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ORIGINAL RESEARCH ARTICLE

Development of a liquid fluorometric method for the quantification of Alkylresorcinols in whole wheat

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ABSTRACT

Objective: To assess the quality of whole wheat products, a method for determining alkylresorcinols (ARs)—markers of whole wheat, black wheat, and other cereal products—was developed. **Methods**: The study focused on wheat and black wheat, using factorial design to evaluate the impact of extraction solvents (acetone, alcohol, and ethyl acetate), extraction techniques (ultrasonic extraction for 30 or 60 minutes, and shaking overnight at room temperature), and sample forms (granules or flour) on AR determination. AR detection conditions were optimized by comparing chromatographic settings and fluorescence scanning wavelengths. The method's effectiveness was validated by measuring AR content in various commercially available cereals. **Results**: Considering reagent toxicity and efficiency, the optimal procedure involved ultrasonically extracting comminuted samples with ethanol for 30 minutes, then analyzing with a Waters CORTES-C18 column using an ethanol: acetonitrile (30:70, v:v) mobile phase. Fluorescence detection was performed at an excitation wavelength of 272 nm and an emission wavelength of 296 nm. The method had a linear range of 0.050–10.0 μg/mL with an $R^2 \geq 0.9999$. Precision experiments showed an RSD of less than 5%, and spiked recovery rates ranged from 94.5% to 104%. The total AR content was 47.9–54.3 mg/100 g in commercially available wheat and 53.6–60.9 mg/100 g in black wheat. **Conclusion**: The liquid-fluorescence method established in this study effectively and rapidly separates ARs from wheat and black wheat, offering advantages of simplicity, high sensitivity, and accuracy.

Keywords: alkylresorcinols; whole grain; whole wheat; liquid phase fluorescent chromatography

With more and more evidence supporting the health effects of whole grains, the 2016 edition of the dietary guidelines for Chinese residents advocates that residents increase the intake of whole grains[1], and the number of foods claiming "whole grains" on the market is also gradually increasing. However, so far, the definition of whole wheat food and effective quality evaluation indicators are still lack of sensitive and effective detection technology.

Alkyl resorcinol (1,3-dihydroxy-5-alkylbenzene derivatives, ARS) is a kind of compounds found mainly in wheat in recent years. According to the number of carbon atoms in its saturated alkyl side chain, phenols in wheat bran, such as rye, can be divided into 5-heptadecyl resorcinol (C17:0). 5-hexadecyl resorcinol (C19:0). 5-21alkyl resorcinol (C21:0). 5-23alkyl resorcinol (C23:0). 5-pentadecyl resorcinol (C25:0) and other homologous components. As ARS is rarely found in embryos and endosperm, it has been confirmed to be a

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marker of whole wheat products[2]. Therefore, this study attempts to establish a rapid method by HPLC Fluorescence. Stable ARS determination technology.

1. Materials and methods

1.1. Main instruments

Waters 2695 liquid chromatograph is equipped with fluorescence detector. Empower chromatographic workstation, constant temperature water bath shaker, KQ5200 Ultrasonic instrument, German 5418 Eppendorf centrifuge, nitrogen blowing instrument, METTLER XS205DU and 204 Series electronic analytical balance.

1.2. Reagents

ARs reference materials: C17:0, C19:0, C21:0, C23:0 were purchased from (Sigma, USA), purity >95%; C25:0 (Shanghai Yuanmu, China), purity 73%. After the purity correction, each reference substance containing 1.00 mg ARs was accurately weighed, dissolved in ethanol and made up to 100 mL to make a mixed standard stock solution, which was frozen at −20 ℃. Before use, the stock solution was diluted with ethanol so that the concentration of ARs in the final series of standard application solutions was 0.05–10.00 μg/mL, which was filtered through a 0.45 μm membrane for use.

Reagents such as ethanol, ethyl acetate, acetonitrile, acetone, and isopropanol are all chromatographic grade, and water is first-grade water.

1.3. Establishment of optimal determination conditions

Sample: wheat harvested in 2016. The raw grains of black wheat are provided by COFCO; after mixing, take some parts and crush them with a crusher, and then pack them separately for standby.

Detection conditions: Scan ARS standard application solution within the range of emission wavelength 250~360 nm and excitation wavelength 200~280 nm, and determine the best fluorescence detection conditions according to the intensity of absorption peak.

Chromatographic conditions: Use altlantis T3 chromatographic column (4.6 mm × 250 mm, 5 μm). waters cortes-C18 column (4.6 mm × 150 mm, 2.7 μm) with cortes-C18 (3.9 mm × 5 mm, 2.7 μm) protective column, under the conditions of column temperature of (38 ± 5) ℃, sample injection volume of 20 μL and flow rate of 1 mL/min, ARS mixed standard machine measurement is carried out; by comparing different mobile phase systems (a: isopropanol acetonitrile, b: ethanol acetonitrile, c: methanol acetonitrile). Flow matching ratio. The effect of elution method (isocratic or gradient) on the separation of five ARS components. Peak time stability. Detector response value, etc., to determine the appropriate chromatographic conditions.

Extraction conditions: Adopt the factorial design of two factors and three levels to explore the extraction solution (ethanol, Acetone, Ethyl acetate). Extraction method (ultrasonic extraction 30, 60 min or room temperature oscillation overnight) on the determination results of ARS of rye flour, the best extraction conditions were established by variance analysis.

Sample pretreatment: In order to explore the influence of sample state on ARS extraction effect, wheat grains/flour before and after crushing are used respectively. The ARS of black wheat grain/powder was determined, and the necessity of crushing before sample extraction was judged by comparing the difference of results.

1.4. Methodological evaluation

According to the methodology. Price principle, establish:

Standard curve: Take a series of standards and apply them to the liquid machine for determination. Take the concentration as the abscissa and the peak area as the ordinate to establish a linear regression equation ($y = ax + b$).

Precision and accuracy: wheat flour. Black wheat flour was used as samples for 6 times of parallel determination, and the background level $x \pm s$ was obtained, and the relative standard deviation (RSD) was calculated; take another sample, use a medium level and conduct a standard addition recovery experiment at a low concentration, then calculate the recovery rate.

Detection limit and quantitative limit: take blank matrix for spiked experiment, and the signal-to-noise ratio (s/n) is 3 respectively. At 10 o'clock, the detection limit and quantitative limit were determined for the concentration of the tested compound.

1.5. Determination of ARS of commercial whole wheat products

After the method was established, some commercially available whole wheat flours were collected, including whole wheat flour (Chinese fine fiber, medium and high gluten), wheat flour (Chinese fine fiber, medium and high gluten), whole triticale flour (high gluten), triticale flour (high gluten); In addition, other grain samples such as highland barley, oats, glutinous rice (commonly known as chicken feet valley), quinoa and other grains were collected, and the ARs double-sample parallel determination was carried out after crushing and mixing, and the data were expressed as $\bar{x} \pm s$.

1.6. Data processing

Excel 2013 is used to input and edit the data, and SAS9.4 software is used for statistical analysis.

2. Results

2.1. Establishment of the best determination conditions

The mobile phase of the chromatographic column is ethanol acetonitrile (volume ratio 3:7). ARS can be effectively separated within 7 min, and the baseline is stable.

Fluorescence chromatographic scanning determined that ARS was at the emission wavelength of 296 nm. There is a maximum absorption peak at the excitation wavelength of 272 nm. The study of chromatographic conditions shows that ARS can be effectively separated by different chromatographic columns and mobile phase systems. It can be seen from **Figure 1** that the peak time of A or B mobile phase system is short and the peak area response value is high. Considering the chromatographic resolution comprehensively, peak retention time, the consistency between the mobile phase and the sample extract and the toxicity of the reagent were finally determined as CORTES-C18.

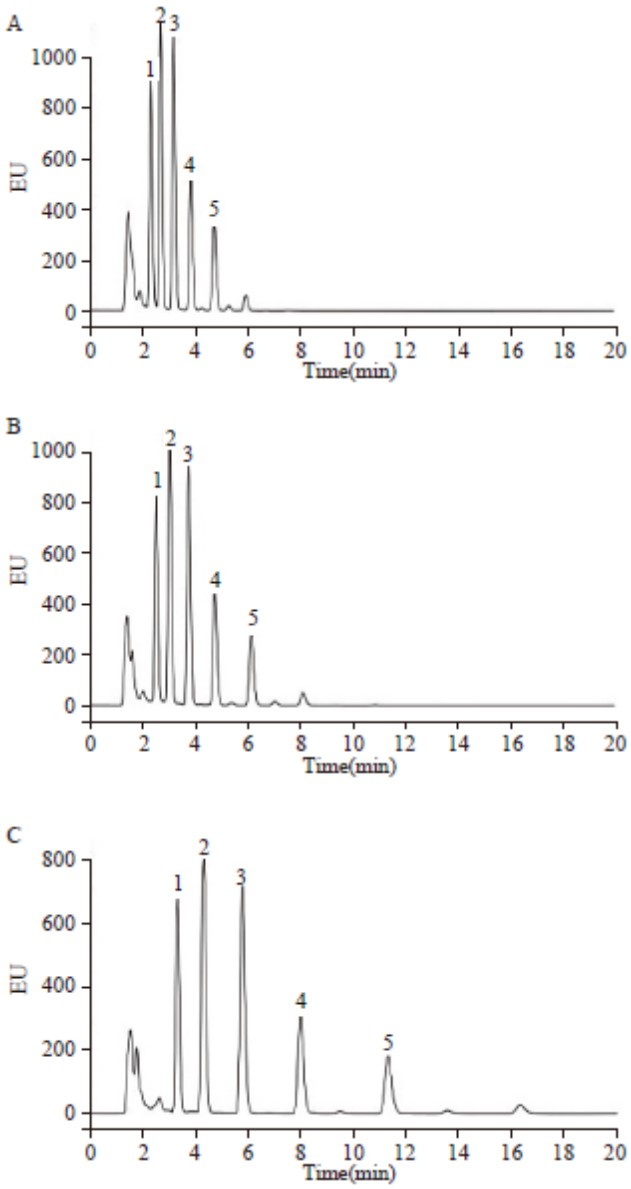

Figure 1. Peaks of ars separated by different mobile phases; **(A)** isopropyl alcohol-acetonitrile; **(B)** ethanol-acetonitrile mobile; **(C)** methanol-acetonitrile mobile.

2.2. Establishment of the best sample extraction conditions

Table 1 lists the determination results of ARS of rye flour under different extraction conditions. The analysis of variance and Bonferroni method showed that the extraction effect of acetone was the worst ($P < 0.05$), while there was no significant statistical difference in the content and total amount of ARS components when extracted with ethanol or ethyl acetate, whether ultrasonic or room temperature oscillation overnight ($P > 0.05$). Considering the efficiency and toxicity of reagents, ethanol ultrasound for 30 min was finally selected as the extraction condition.

Table 1. Comparison of extraction conditions for ars determination in black wheat flour (n = 3, mg/100 g).

Extraction methods	**Ars**	**Extraction regents**		
		Ethanol	**Acetone**	**Ethylacetate**
Ultrasound for 30 min	C17:0	5.29 ± 0.05	5.72 ± 0.25	5.91 ± 0.56
	C19:0	16.6 ± 0.1	15.2 ± 0.2	15.8 ± 0.3
	C21:0	24.1 ± 0.1	21.8 ± 0.4	22.7 ± 0.5
	C23:0	4.92 ± 0.04	4.43 ± 0.10	4.62 ± 0.10
	C25:0	2.62 ± 0.05	2.31 ± 0.04	2.45 ± 0.06
	Total	53.6 ± 0.3	49.4 ± 0.8	51.4 ± 0.4
Ultrasound for 60 min	C17:0	5.19 ± 0.03	5.35 ± 0.05	5.78 ± 0.23
	C19:0	16.3 ± 0.1	14.8 ± 0.5	16.0 ± 0.2
	C21:0	23.8 ± 0.1	21.4 ± 0.7	22.9 ± 0.2
	C23:0	4.89 ± 0.04	4.38 ± 0.12	4.63 ± 0.05
	C25:0	2.56 ± 0.02	2.30 ± 0.06	2.52 ± 0.07
	Total	52.7 ± 0.3	48.22 ± 1.25	51.8 ± 0.4
Shaking for 24 h	C17:0	5.44 ± 0.09	5.83 ± 0.38	6.54 ± 0.38
	C19:0	16.2 ± 0.1	13.9 ± 0.1	15.6 ± 1.1
	C21:0	23.5 ± 0.0	19.7 ± 0.1	22.3 ± 1.6
	C23:0	4.77 ± 0.02	3.99 ± 0.03	4.54 ± 0.32
	C25:0	2.53 ± 0.06	2.18 ± 0.11	2.38 ± 0.13
	Total	52.4 ± 0.1	45.7 ± 0.5	51.4 ± 3.4

For wheat grain/flour. The comparative analysis of the determination results of black wheat grain/flour suggests that the pretreatment and crushing of samples before extraction can significantly improve the extraction efficiency of ARS (P < 0.05, **Table 2**).

Table 2. Detected ars contents in whole grain granule or flour before and after milling (n = 6, mg/100 g).

Sample	**Particle**	**Ars**					
		C17:0	**C19:0**	**C21:0**	**C23:0**	**C25:0**	**Total**
Wheat	Granule	0.99 ± 0.08	4.12 ± 0.32	5.36 ± 0.44	1.37 ± 0.11	1.08 ± 0.08	12.9 ± 1.0
	Flour	3.32 ± 0.11	14.0 ± 0.6	19.0 ± 0.6	4.87 ± 0.16	3.76 ± 0.10	45.0 ± 1.4
Black wheat	Granule	1.40 ± 0.12	4.97 ± 0.36	6.13 ± 0.38	1.51 ± 0.10	1.09 ± 0.06	15.1 ± 1.0
	Flour	5.73 ± 0.20	19.8 ± 0.7	24.9 ± 0.8	5.43 ± 0.16	3.17 ± 0.08	59.1 ± 2.0

Therefore, the processing process before the sample is put on the machine is: weigh 2G of the sample and put it in a 50 mL volumetric flask, fix the volume of ethanol to the scale line, shake it fully, ultrasonic for 30 min, take 2 mL and put it in a centrifuge tube, centrifuge it at a speed of 10,000 r/min for 5 min, take 1 mL of the supernatant, filter it through a 0.45 m membrane, and then use the liquid-phase fluorescence method for determination.

2.3. Methodological evaluation

The linear equation of ARS standard curve shows that there is a good linear relationship between ARS concentration and peak area within a certain concentration range (R^2 > 0.9999); the detection limit and quantitation limit are at ppm level (**Table 3**).

The precision experiment shows that the RSD of 6 parallel determinations of wheat flour and rye flour is 2.41%–3.46%, indicating that the method has good repeatability (**Table 4**). The results of the three-level spiked recovery experiment showed that the recovery rates of the five components were 94.5% to 104%, indicating that the method had high accuracy (**Table 5**), which was suitable for the qualitative and quantitative determination of ARS in whole wheat.

Table 3. Linear regression equation, determination coefficient, LOD and LOQ of ars.

Ars	Linear equation	Determination coefficient R^2	Linearity range (kg/mL)	LOD (ng/mL)	LOQ (ng/mL)
C17:0	Y = 9.77e + 006*X* + 2.90e + 005	0.9999	0.0630–6.30	2.50	7.50
C19:0	Y = 9.98e + 006*X* + 1.17e + 005	0.9999	0.0960–9.60	3.80	11.4
C21:0	Y = 1.00e + 007*X* + 6.66e + 003	0.9999	0.0970–9.70	3.90	11.7
C23:0	Y = 1.00e + 007*X* + 6.54e + 004	0.9999	0.0470–4.70	1.90	5.70
C25:0	Y = 6.70e + 006*X* + 2.26e + 004	0.9999	0.0510–5.11	5.10	15.3

Table 4. Precision of the ars determination by parallel tests (n = 6).

Analytes	Detection values mg/100 g						$\bar{x} \pm s$	RSD (%)
	1	2	3	4	5	6		
Wheat flour								
C17:0	3.38	3.37	3.40	3.11	3.33	3.36	3.32 ± 0.11	3.28
C19:0	14.3	14.3	14.2	13.1	14.4	14.1	14.1 ± 0.5	3.26
C21:0	19.2	19.3	19.1	17.8	19.4	19.0	19.0 ± 0.6	3.06
C23:0	4.89	4.94	4.89	4.55	4.99	4.92	4.87 ± 0.16	3.24
C25:0	3.80	3.79	3.79	3.55	3.84	3.78	3.76 ± 0.10	2.78
Black wheat flour								
C17:0	5.94	5.39	5.77	5.71	5.90	5.64	5.73 ± 0.20	3.46
C19:0	20.6	18.7	19.9	19.9	20.3	19.7	19.8 ± 0.7	3.37
C21:0	25.8	23.5	25.1	25.1	25.5	24.6	24.9 ± 0.8	3.29
C23:0	5.59	5.15	5.48	5.48	5.52	5.34	5.43 ± 0.16	2.89
C25:0	3.25	3.04	3.20	3.21	3.22	3.13	3.17 ± 0.08	2.41

Table 5. Recovery of ars in wheat flour (n = 6).

Ars	Background value (Hg/100 g)	Added value (Hg)	Detected value after Addition (pg)	Average spike recovery ± standard deviation (%)
C17:0	3.32	9.45	16.5 ± 0.6	104 ± 6
		18.9	26.0 ± 0.7	101 ± 4
		37.8	44.5 ± 0.4	99.6 ± 1.0
C19:0	14.0	14.4	42.4 ± 1.0	97.0 ± 7.1
		28.8	58.6 ± 1.1	103 ± 4
		57.6	86.7 ± 1.3	100 ± 2
C21:0	19.0	14.6	52.1 ± 1.4	94.5 ± 9.0
		29.1	69.3 ± 1.5	104 ± 5
		58.2	97.3 ± 1.7	100 ± 3
C23:0	4.87	7.05	16.8 ± 0.4	97.9 ± 5.3
		14.1	24.4 ± 0.4	102 ± 3
		28.2	38.3 ± 0.4	100 ± 3
C25:0	3.76	7.67	15.2 ± 0.3	98.6 ± 3.3
		15.3	23.3 ± 0.2	101 ± 1
		30.7	38.7 ± 0.5	101 ± 2

2.4. Determination results of ARS in some grains

After the ARS determination method was established, the ARS values in some commercial cereals were determined in this study (**Table 6**).

In general, the total amount of ARS in wheat flour is 47.9~54.3 mg/100 g, and that in black wheat flour is 53.6~60.9 mg/100 g. C19:0 in ARS component. C21:0 content is high; in comparison, the ARS content of the samples with the word "whole grain" in the name is slightly higher than that of the samples without the word "whole grain", indicating that the products will be affected by raw materials and processing methods.

Table 6. Results of ars content in commercial wheat and black wheat samples (mg/100 g).

Species	Ars					Total
	C17:0	C19:0	C21:0	C23:0	C25:0	
Wheat flour						
With med-high gluten	3.09 ± 0.02	17.8 ± 0.1	19.1 ± 0.0	4.68 ± 0.01	3.20 ± 0.04	47.9 ± 0.0
With med-high gluten, from whole granules	3.39 ± 0.03	17.3 ± 0.0	24.8 ± 0.0	5.49 ± 0.14	3.23 ± 0.01	54.2 ± 0.0
Chinese style, fine	3.14 ± 0.04	15.7 ± 0.0	22.6 ± 0.0	5.28 ± 0.01	2.92 ± 0.02	49.7 ± 0.0
Chinese style, fine, from whole granules	3.48 ± 0.08	17.3 ± 0.2	24.8 ± 0.2	5.59 ± 0.04	3.24 ± 0.04	54.3 ± 0.3
Black wheat flour						
With gluten	3.59 ± 0.02	18.2 ± 0.1	23.9 ± 0.0	5.08 ± 0.00	2.81 ± 0.01	53.6 ± 0.1
With gluten, from whole granules	3.95 ± 0.03	20.1 ± 0.0	27.6 ± 0.1	5.96 ± 0.01	3.27 ± 0.03	60.9 ± 0.2
Barley flour, highland	0.167 ± 0.005	1.37 ± 0.06	2.23 ± 0.111	1.28 ± 0.02	5.86 ± 0.26	10.9 ± 0.4
Oatmeal, organic	Tr*	Tr	Tr	Tr	Tr	Tr
Eleusine coracana	Tr	Tr	Tr	Tr	Tr	Tr

*Tr means data lower than LOD.

In addition, the content of ARS in other cereals is about 50% in highland barley); oats. AR is not detected in Xizi; although the ARS chromatographic response of quinoa is high, the component types are complex, so they are not measured (**Figure 2 and 3**).

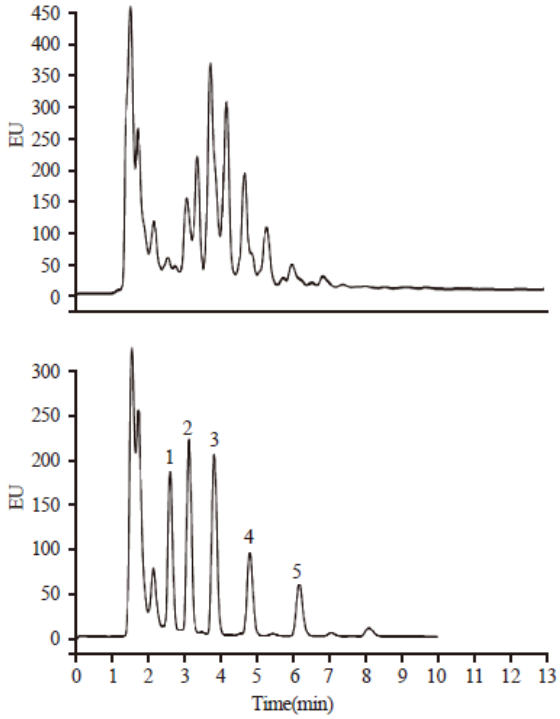

Figure 3. Determination of ars in quinoa and ars standards by liquid-fluorescence chromatograms.
1: ARC17:0; 2: C19:0; 3: C21:0; 4: C23:0; 5: C25:0.

3. Discussion

3.1. Progressiveness and operability of the method

At present, the published determination techniques of ARS mainly include gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS); spectrophotometry is recommended in the whole wheat flour industry standard issued by the national grain administration of China[3]. Before carrying out this study, according to the GC-MS method[4] published by Landberg et al., an attempt was made to use thermo gas chromatography in series with a triple quadrupole mass spectrometer to determine ar. The results showed that although GC-MS method can realize the separation of several ARS components, it failed to reproduce the good precision of the experiment under the conditions of this experimental study, which may be related to the need for derivatization of ARS during sample processing, which is easy to introduce impurities or interference peaks, or cause sample loss, the reproducibility and stability of the results are affected.

The establishment of this method was put forward on the basis of consulting the relevant determination methods of phenolic compounds in water[5]. Because phenolic substances (including ARS) are organic compounds with strong polarity and have fluorescence, HPLC Fluorescence method was tried to establish. Ars is easily soluble in organic solvents such as methanol, ethanol, acetone, ethyl acetate, hexane, propanol, dichloromethane and chloroform. Different extraction conditions are compared. Chromatographic conditions. Fluorescence spectrum scanning, and finally established the best experimental conditions.

As ARS only exists in the outer layer of grain, but not in endosperm and germ rich in starch, Ross et al.[6] believe that more impurities will be introduced into the grinding process, so ARS can be extracted directly

from whole grain in theory, but in order to ensure the extraction efficiency, it is recommended to use a long-term direct extraction method (24 h~48 h). In order to avoid the oxidation of ARS caused by long-time extraction, factorial design was used to explore the reagent. The influence of the extraction method on the results was compared with the 24 h oscillation method, which proved that the ethanol ultrasonic extraction method established by this method has high extraction efficiency, and the sample crushing before extraction is very necessary.

3.2. ARS characteristics of different wheat crops

Research shows that ARS is wheat. The main symbolic components of Triticum aestivum L. The Academy of Sciences of the State Food Administration[7] has used spectrophotometry to determine the total amount of ARS in 36 wheat samples of different varieties in China, ranging from 438 to 1348 μg/g. The total amount of ARS in wheat measured in this study is 47.9~54.3 mg/100 g, the content level is basically the same, and the distribution range is narrow. On the one hand, it is related to the small number of samples measured in this study, on the other hand, it is related to the high specificity of this method.

Observing the ARS spectra and content levels of different wheat crops, it can be found that the ratio of wheat and rye is C19:0. C21:0 is the main method. This method can not only detect 5 components, but also quantitatively determine C15:0 if the flow matching ratio is adjusted and ethanol acetonitrile gradient elution is carried out (volume ratio 0~4 min 2:98, 4~15 min 30:70, flow rate 1 mL/min). In particular, black wheat samples can separate 6 homologous components. Although the total amount of ARS in highland barley is less than 1/4 of that of whole wheat, the C25:0 ratio is high, which can be used as the characteristic peak of ARS components.

Quinoa is not a cereal grain in terms of plant classification. Its grain does not have a typical caryopsis structure, but because it is rich in starch, it is also classified as a food crop like buckwheat, and is one of the sources of whole grain. This study shows that quinoa contains ARS components with high response values, but different from wheat ARS spectra, there are double peaks or unclear baseline separation between peaks, indicating that quinoa AR is not only high in content but also complex in components. Ross et al.[8] recently published research also showed that 17 commercial quinoa species contain linear ARS with 17~26 odd or even carbon atoms, as well as branched ARS (BCAR) and methyl ARS (MAR). As a marker of quinoa, the analytical technology of ARS needs to be further studied.

To sum up, this study established a rapid, efficient, and sensitive specific HPLC fluorescence method suitable for the determination of ARS in wheat crops and their products..

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Conflict of interest

The authors declare no conflict of interest.

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