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Determination of Marked Components —aloin and aloe-emodin— in Aloe vera before and after hydrolysis

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ABSTRACT

This article proposed an optimal hydrolysis condition and a chromatographic method for the analysis of polyphenol glycosides in aloe extract and its hydrolysates. The aglycones assayed were aloin and aloe-emodin. The linearity ($r > 0.9997$) and validation (< 10%) of the calibration curves were excellent. The amounts of aloin were 76.1 ± 5.9 and 77.7 ± 2.8 μmol/g in decoction and hydrolysate, respectively, while those of aloe-emodin were 8.3 ± 0.5 and 8.1 ± 0.3 μmol/g. The contents of aloin and aloe-emodin in aloe showed no significant difference before and after hydrolysis. This may be due to that aloe sold in Chinese medicine retailer was processed and the glycosides have already been transferred to aglycones. In addition, the components may be decomposed during processing. The method developed here can be applied to determine aglycones and their glycosides in aloe for the quality control.

Key words: aloe, aloe-emodin, aloin, Chinese medicine, hydrolysis, HPLC

INTRODUCTION

Aloe vera, dried leaf gel of *Aloe ferox* Mill (also named Cape aloe; Liliaceae), is an herb and Chinese medicine used to prevent cardiovascular diseases, cancer, neurodegeneration and diabetes[(1)]. Traditionally, it can also be used as laxative[(2)]. Currently, it is extensively used in the cosmetic industry for its anti-inflammatory cure of skin disease[(3,4)], wound and burn healing effects[(5,6)]. Aloe leaves are used to treat asthma, gastrointestinal ulcers, cardiovascular disease, tumors, burns and diabetes[(1)], and it is also used in cosmetics as emollients, moisturizers and cicatrisants[(7)]. It has been reported that aloe exhibited anti-tyrosinase and anti-inflammatory effects[(8-10)]. Aloe vera contains anthraquinones which mainly include aloin (10-glucopyranosyl-1,8-dihydroxy-3-(hydroxymethyl)-9(10*H*)-anthracene; barbaloin), aloe-emodin (Figure 1), chrysophanol and aloinoside A and B[(1,8)]. Aloin, found in nature as a mixture of two diasteromers, is an anthrone glycoside contained in the exudates seeping of freshly cut leaves.

Polyphenols such as flavonoids, anthraquinones, lignan, and aromatic acids were widely distributed in herbs, vegetables and fruits; they were found to exist predominantly as glycosides in nature. It has been reported that polyphenols are associated with many bioactivities including antioxidant activity[(11)], prevention of cardiovascular disease[(12-14)], and anti-tumor activity[(15,16)]. The pharmacokinetics of polyphenols have been clarified in recent years, and it is now known that the glycosides are hydrolyzed to more hydrophobic aglycones by enzymes or enteral bacteria and then absorbed[(17-19)]. After that, polyphenols, such as daidzein, are predominantly excreted as conjugated metabolites (predominantly as sulfates/glucuronides), and only a few of them were present in the parent form[(20)]. In addition, hydrolysis by acid or enterobacter in intestine would significantly elevate the contents of aglycones in decoction[(17-21)].

It is complicated to determine the amount of each

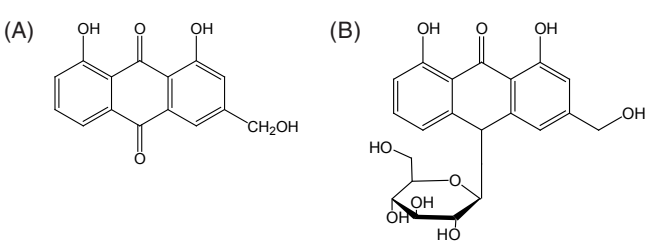

Figure 1. Chemical structures of compounds detected in this study. A: aloin; B: aloe-emodin.

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compound in an herb because there exist many active components in it. However, the specification of Chinese medicine in pharmacopeia, only glycoside or aglycone amount was defined. For example, the contents of baicalin in Scutellariae Radix should not be less than 10% according to the Japanese Pharmacopoeia[22]. Furthermore, according to the Chinese Pharmacopoeia, the content of aloin in *Aloe barbadensis* Miller should be more than 18% and aloin in *Aloe ferox* Miller not less than 6%[23]. It seems not enough to reflect the quality of Chinese medicine.

It is important to establish a simple, rapid and accurate quantitative method for the quality control of Chinese medicine. This study was aimed to develope a quantitative method for absorbable glycosides of the herb. In this study, an easy, economical, and efficient hydrolysis condition and HPLC-UV/VIS method were developed for anthraquinone-rich herbs or formulation. Aloin and aloe-emodin, the major components in *Aloe Ferox* Miller[1-4,24], were determined by HPLC with a UV-VIS detector before and after acid hydrolysis.

MATERIALS AND METHODS

I. *Materials*

Aloe ferox was a kind gift sponsored by a Chinese medicine company. The exudate of Aloe leaves were collected and concentrated by boiling and then cooling[25]. On cooling, a solid amorphous extract is formed. Aloe-emodin, aloin and 6,7-dimethoxycoumarin (6,7-DMC; as internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile, methyl alcohol and ethyl acetate were from J. T. Baker, Inc. (Phillipsburg, NJ, USA). Ortho-phosphoric acid was from Riedel-deHaën AG (Seelze, Germany). All the solvents were of HPLC grade and Milli-Q plus water (Millipore, Bedford, MA, USA) was used throughout this study.

II. *Sample Preparation*

The decoction of aloe was prepared according to the standard decoction method[26]. Briefly, 20 g of the aloe crude drugs were weighed and pulverized, and then soaked with 400 mL water. The mixtures were then boiled to 200 mL. The decoctions were then concentrated to 0.4 g/mL by vacuum-drying at 45°C and aliquots were stored at -20°C until used.

III. *Investigation of Hydrolysis Conditions*

(I) *Hydrolysis Time*

Equal volume of HCl (1.2 N) and aloe decoction were mixed, and the mixtures were heated in a water bath at 80°C for 0.5, 1, 2, 4, and 6 h, respectively. The mixture was extracted by an equal volume of ethyl acetate and centrifuged at 4,000 ×*g* for 10 min. The supernatant was collected and dried under nitrogen gas. The residue was dissolved in methanol and spiked with the internal standard for HPLC analysis.

(II) *Concentration of HCl*

Five hundred microliters of 1.2 N and 2.4 N HCl were added into 500 μL of decoction, respectively, and the mixtures were heated in a water bath (80°C) for 1 h. The mixtures were extracted with ethyl acetate, and the process described above was repeated.

(III) *Hydrolysis Temperature*

Equal volume of HCl (1.2 N) and aloe decoction were mixed, and the mixtures were heated in a water bath at 80°C or 100°C for 1 h. The mixtures were extracted with ethyl acetate, and the process described above was repeated.

(IV) *The Effect of Light*

Five hundred microliters of HCl (1.2 N) was added into 500 μL of decoction. The test tubes containing the mixture were either wrapped with aluminum foil to protect them from light or left unwrapped. The mixtures were heated and extracted as described above.

IV. *Instrumentation and HPLC/UV-VIS Analysis*

The HPLC/UV-VIS system consisted of a Shimadzu HPLC system equipped with a pump (LC-10AT vp, Shimadzu, Japan), an automatic injector (SPD-10AF, Shimadzu, Japan), a UV-VIS detector (SPD-10A vp, Shimadzu, Japan) and a degasser (ERC-3415, Japan). Separations were effected over an Apollo C18 reversed-phase column (5 μm, 4.6 × 250 mm, Alltech Associates, Inc. USA) maintained at ambient temperature. The mobile phase consisted of acetonitrile (A) and 0.1% aqueous phosphoric acid (B) with a gradient elution of 24% A at 0-12 min, 24-50% A at 12-22 min, 50-24% A at 22-40 min and 24% A at 40-50 min. The flow rate was 1.0 mL/min and the separation was monitored by absorbance at 254 nm. The injection volume was 20 μL.

V. *Method Validation*

(I) *Resolution*

The selectivity of the method was determined by standards and samples analysis. The peaks of aloe-emodin and aloin were identified by comparing the retention times, UV spectra and addition test with those of the standards. The resolution of two peaks was determined by following equation:

$$Rs = [2(t_{RB}-t_{RA})] / (W_A+W_B)$$

Where t_{RA} and t_{RB} mean the retention times of peak A and B, and W_A and W_B mean the widths of peak A and B, respectively.

(II) *Calibration Curves and Linearity*

For the determination of anthraquinones in aloe, aloe-emodin and aloin were individually dissolved in methanol and diluted in series to 6.25-200 μg/mL for aloin, and 62.5-200 μg/mL for aloe-emodin as standard solutions. 6,7-DMC (50.00 μg/mL) was spiked into each standard solution as the internal standard. The calibration curves were constructed by plotting the peak area ratios of each standard to the internal standard versus the concentration of each standard. The linear regressions and correlative coefficients were determined based on the calibration curves.

(III) *Precision and Accuracy*

The variability of inter-day and intra-day measurements were used to evaluate the reproducibility and repeatability of this HPLC method. The intra-day assays were performed by measuring three replicates on the same day and the inter-day assays on three consecutive days. The real concentrations were derived from standard curves and used to calculate the standard deviation (SD) and coefficient of variation (CV), which were used as indexes for the precision and relative error for accuracy, respectively.

(IV) *Sensitivity*

Lower limit of quantification (LLOQ) represents the lowest concentration of analyst in a sample that can be determined with acceptable precision (CV < 15%) and accuracy (relative error within ± 15%), whereas limit of detection (LOD) represents the lowest concentration of analyst in a sample that can be detected with a signal-to-noise ratio (S/N) > 3.

(V) *Recovery*

Three concentrations of the calibration standard were individually spiked into the decoction or hydrolysate, and assayed by HPLC-UV/VIS method. The recoveries were determined by the percentage of calculated concentration out of theoretical concentration.

VI. *Statistics*

Each experiment was performed in triplicate and all data are presented as mean ± SD.

RESULTS AND DISCUSSION

HPLC chromatograms of aloin, aloe-emodin, and decoction of aloe vera and hydrolysate of aloe vera were shown in Figures 2A-C, respectively. Aloin and aloe-emodin of aloe in decoctions as well as the internal standard were well resolved within 40 min by gradient elution. The retention time of aloin, 6,7-DMC and aloe-emodin were 17.0, 19.2 and 34.6 min, respectively (Figures 2). The resolutions (Rs) of aloin were 1.12 and 1.13 in decoction and hydrolysate, respectively. Rs of aloe-emodin in both decoction and hydrolysate were over 1.5. Rs is not only a parameter for the separation of two peaks, but also the efficiency of the column. The results indicated the analysis condition is optimized. The linear regressions and concentration ranges of the standard curves for aloe were shown in Table 1. The calibration curves were linear over the concentration range of 12.5 to 200 μg/mL for aloin and 6.25 to 200 μg/mL for aloe-emodin. Regression equation and correlation coefficients (r) were not less than 0.9997 indicating a response of good linearity for this method. S/N's of 3 and 10 were considered as LOD and LLOQ, respectively. The LOD was 0.5 μg/mL for aloin and 0.2 μg/mL for aloe-emodin; the LLOQ was 1.3 μg/mL for aloin and 0.5 μg/mL for aloe-emodin. These results indicated a good sensivity exhibited by the proposed method.

The intra-day and inter-day analytical precision and accuracy of these standard compounds are shown in Table 2. Validation of this method indicated that all coefficients of

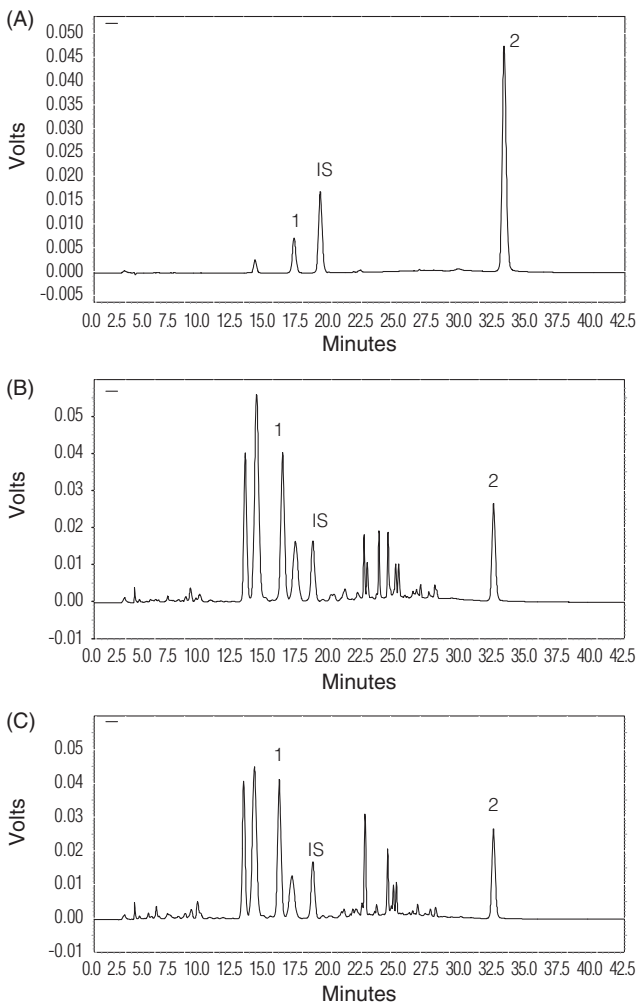

Figure 2. HPLC chromatograms of aloe decoction and its hydrolysate.
A: standard; B: aloe decoction; C: aloe hydrolysate.
1: aloin, 2: aloe-emodin, IS: 6,7-DMC.

Table 1. The regression equations, concentration ranges and correlation coefficients of constituents in aloe

Constituents	Regression equations	Conc. Range (μg/mL)	Correlation coefficient (*r*)	LOD (μg/mL)	LLOQ (μg/mL)
Aloin	y = 0.0066 x - 0.0150	12.50 - 200	0.9997	0.5	1.3
Aloe-emodin	y = 0.0608 x + 0.0185	6.25 - 200	0.9999	0.2	0.5

Table 2. Intra-day and inter-day analytical precision and accuracy of the constituents in aloe

Constituents	Conc. (μg/mL)	Intra-day		Inter-day	
		Precision	Accuracy	Precision	Accuracy
		Mean ± S.D. (C.V.%)	(%)	Mean ± S.D. (C.V.%)	(%)
Aloin	200	201.3 ± 0.3 (0.1)	0.6	201.3 ± 0.5 (0.2)	0.7
	100	97.6 ± 0.6 (0.6)	-2.4	97.5 ± 1.0 (1.0)	-2.5
	50.0	48.6 ± 0.5 (1.0)	-2.8	48.9 ± 0.4 (0.9)	-2.3
	25.0	26.3 ± 0.2 (0.1)	5.2	26.1 ± 0.4 (1.6)	4.4
	12.5	13.0 ± 0.5 (3.8)	4.0	13.3 ± 0.3 (2.3)	6.4
Aloe-emodin	200	200.7 ± 0.3 (0.1)	0.4	200.7 ± 0.8 (0.4)	0.4
	100	98.9 ± 1.2 (1.2)	-1.1	98.5 ± 1.5 (1.5)	-1.5
	50.0	49.3 ± 0.3 (0.7)	-1.4	50.0 ± 1.1 (2.2)	-0.1
	25.0	25.3 ± 0.1 (0.3)	1.1	25.1 ± 0.3 (1.1)	0.5
	12.5	12.9 ± 0.4 (2.9)	3.1	12.8 ± 0.5 (3.8)	2.2
	6.25	6.5 ± 0.3 (4.6)	4.0	6.4 ± 0.4 (6.3)	2.4

variation for intra-day and inter-day analysis were less than 5%, and the relative errors were below 10%; the variations of the relative errors became more significant while the concentrations of standards decreased. The recoveries are shown in Table 3. The recoveries were 97.9-101.7% for aloin and 94.3-110.4% for aloe-emodin in aloe decoction, and 90.6-103.2% for aloin and 96.3-105.7% for aloe-emodin in hydrolysate.

The contents of the two anthraquinones in decoctions of various hydrolysis conditions were shown in Figure 3. As Figure 3A showed, the contents of aloin were not significantly different before and after hydrolysis and the aloe-emodin content decreased as the heating was extended. In addition, the contents of anthraquinones decreased when the hydrochloric acid concentration and heating temperature were elevated (Figure 3B and 3C). Furthermore, light did not significantly influence the contents of both components (Figure 3D). We speculate that various anthrone-glycoside derivatives would be cleaved into their aglycones, and the results after hydrolysis of the same components in various preparations could be different.

Table 4 showed the contents of constituents of aloe before or after acid hydrolysis. The amounts of aloin (76.1 ± 5.9 to 77.7 ± 2.8 μmol/g) and aloe-emodin (8.3 ± 0.5 to 8.1 ± 0.3 μmol/g) in the aloe decoction did not increase significantly after acid hydrolysis. Since fresh aloe leaves contain more than 90% water[1,27]; heating and condense is necessary for storage. Generally, aloe purchased from the retailer was processed by heating. Aloin and aloe-emodin are present largely as aglycones in aloe, and the contents were similar to those of aloe hydrolysate, which might be due to the cleavage of the sugar moiety by heat during processing[23]. In our previous study, emodin and physcion in crude *Polygoni multiflori* radix increased after hydrolysis, while that in processed *Polygoni multiflor*i radix were not[24]. The results of these two studies were consistent. The active

Table 3. Recoveries (%) of constituents from aloe decoction and hydrolysate

	Constituents	Conc. Spiked (μg/mL)	Recoveries (decoction) (%)
			Mean ± S.D.
Decoction	Aloin	100.0	101.7 ± 5.6
		50.0	101.0 ± 3.2
		25.0	97.9 ± 1.4
	Aloe-emodin	100.0	94.3 ± 0.8
		50.0	100.5 ± 3.5
		25.0	101.4 ± 3.5
Hydrolysate	Aloin	200.0	105.7 ± 6.3
		100.0	97.6 ± 3.4
		40.0	96.3 ± 5.4
	Aloe-emodin	125.0	103.3 ± 7.6
		62.5	90.6 ± 4.8
		31.5	91.8 ± 3.3

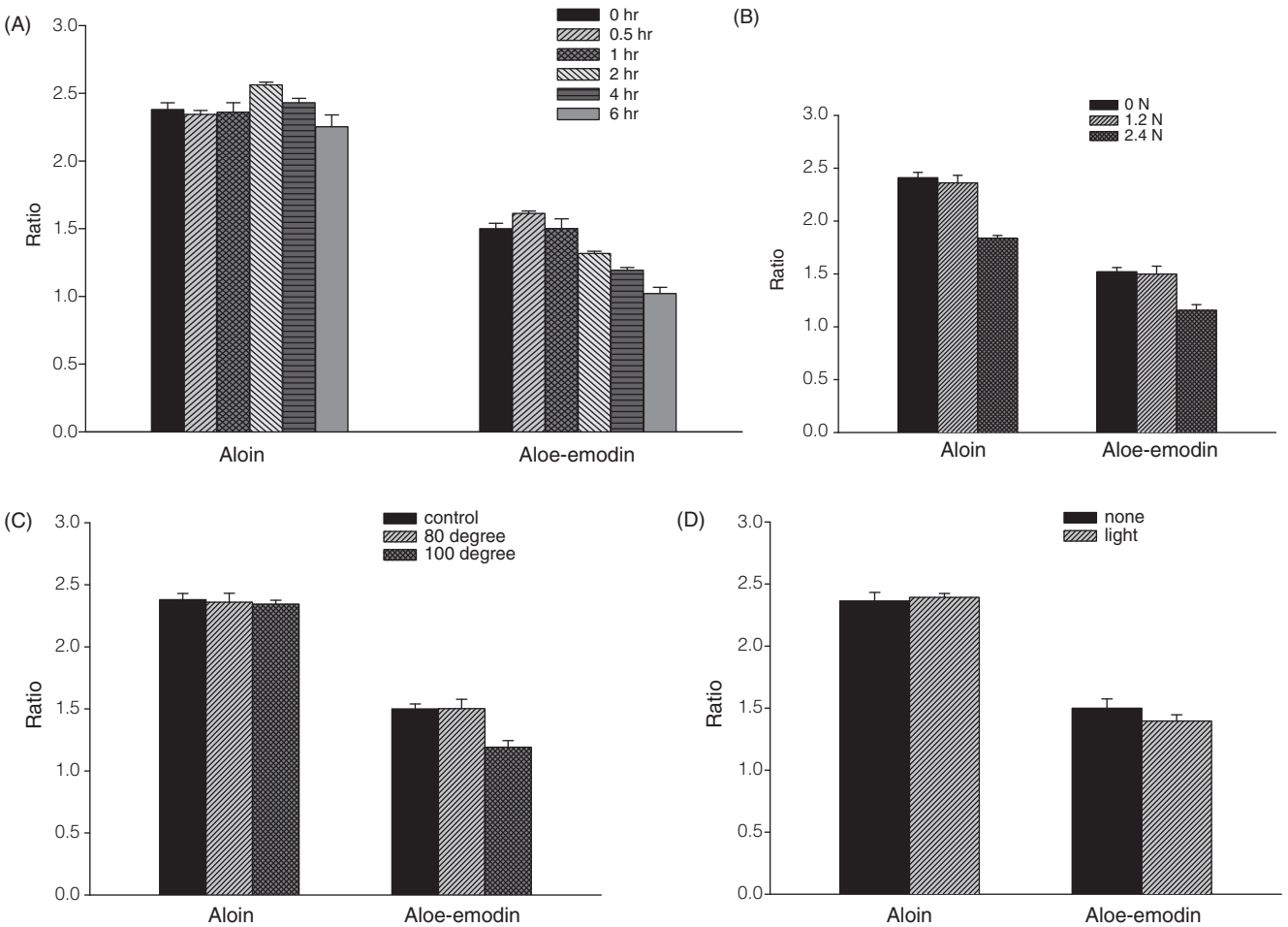

Figure 3. Histograms of aloe-emodin and aloin contents in aloe decoction after acid hydrolysis.
A: the time effect on acid hydrolysis; B: the influence of hydrochloric acid concentration on acid hydrolysis; C: the temperature effect on acid hydrolysis and D: the influence of light on constituents.

Table 4. Comparison of contents (μmoL) of polyphenols in aloe decoction and its hydrolysate

Constituents	Decoction (μmoL/g)	After acid hydrolysis (μmoL/g)	Difference (%)
Aloin	76.1 ± 5.9	77.7 ± 2.8	2.1
Aloe-emodin	8.3 ± 0.6	8.1 ± 0.3	-2.2

components in Chinese medicine is predominant in glycoside form, but the glycosides will hydrolyzed to the corresponding aglycones after acid or bacteria hydrolysis[28]. Some reports suggested that processing may cause irreversible modification of polysaccharides and the active ingredients contents in aloe products became very little[29,30]. It may also explain why the contents of aloin and aloe-emodin were not significantly different between decoction and hydrolysate.

Hence many polyphenol or anthraquinone standards are not commercially available, only one glycoside or aglycone was assessed quantitatively in Chinese herbs in Pharmacopeia[22,23]. For most crude Chinese herbs, polyophenols and anthraquinones exist mainly in glycosides, especially in *O*-glycosides, and the amount of aglycones increases noticeably after processing involving hydrolysis by acid or enzymes, or hydrolysis by gastric acid. Thus, in some case, the bioavailability of some aglycones in Chinese medicine may be over 100% due to the deglycosylation from glycosides other than quantified ones in the gastrointestinal tract. Most of them are susceptible to absorption in more hydrophobic forms[31,32]. Therefore, it is helpful to measure the total contents of glycosides and their corresponding aglycones in order to understand the fate of these polyphenols in body systems. In our previous study, we have reported that the C-glycosides, such as puerarin, are neither cleaved by acid nor by enterobacteria[20]. In the previous study, *Polygoni multiflori* radix and rhubarb decoction were incubated with acid to cleave the sugar moiety from anthraquinones glycosides[21]. In addition, fermentation of Puerariae radix by *Bifidobacterium breve* would cleave the glycosides to aglycones[33]. The effect of enzymes is more specific, while that of acid is rapid and stronger. Acid hydrolysis would be a suitable method for the quantitation of absorbable anthraquinones. For processed herbs such as aloe, the amount of aglycones could be measured directly before hydrolysis[21].

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