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Comparison of second-derivative spectrophotometry and HPLC for determination of amygdalin in wild apricot kernels

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ABSTRACT: Amygdalin is a cyanogenic glucoside found in apricot kernels. To facilitate the exploitation and use of wild apricot resources, it is necessary to be able to rapidly and efficiently determine the amygdalin levels within their kernels. To do so, this paper compares two methods, one based on second-derivative UV spectrophotometry and the other on high-performance reversed-phase liquid chromatography (HPLC). The second-derivative spectrophotometry method effectively eliminates background noise and is straightforward to perform while retaining the accuracy of the HPLC method. It is likely to be a useful tool for the large-scale determination of amygdalin concentrations in wild apricot kernels and press cake.

KEYWORDS: cyanogenic glucoside, absorption spectra, chemical analysis, press cake

INTRODUCTION

The dry mature seeds of *Armeniaca sibirica* L., *Armeniaca mandshurica* (Maxim.), and *Armeniaca vulgaris* Lam., which are wild apricot kernels, are usually bitter due to the presence of amygdalin. Amygdalin, the cyanogenic diglucoside D-mandelonitrile-β-D-gentiobioside, is usually present in apricot kernels, bitter almonds, and the seeds of other members of the genus *Prunus* [1,2]. It is potentially dangerous because it can undergo hydrolysis to produce hydrogen cyanide (HCN). The ingestion of apricot kernels can thus cause cyanide poisoning [3].

Various techniques have been used to determine the amygdalin contents of different foods and industrial materials [4]. One of the first such methods developed was the acid hydrolysis method, which is applicable for all cyanogenic glucosides. However, to get accurate results using this method, one must heat the sample in an acidic solution and make measurements at multiple different reaction times in order to estimate the conditions at the start of the reaction by extrapolation. As a result, this method is difficult and less accurate and reproducible than the picrate method. More recently-developed alternatives include the resorcinol method [5] and direct analysis of the seed amygdalin content using high performance liquid chromatography (HPLC). This last method has been applied to the seeds of several apricot cultivars [6,7]. Gas chromatograph/mass spectrometry (GC/MS) and Enzyme immunoassays have also been used for determining the cyanide content of apricot kernels [8,9]. However, most of the current instrument-based methods are very laborious and time-consuming. A newer more effective method is second-derivative spectrophotometry, which has previously been used to determine the amygdalin content of *Prunus mume* Puerarin and wild apricots [10,11]. In this work, we compare the performance of second-derivative spectrophotometry and HPLC in determining the amygdalin content of wild apricot kernels.

MATERIALS AND METHODS

All analyses were performed using methanol as the solvent. D-Amygdalin (D-mandelonitrile β-D-glucosido-6-β-D-glucoside) was purchased from Sigma Chemistry Co. Ltd., Germany.

Wild apricot seeds were purchased in Qingyang City, Gansu Province, China. The seeds were cracked open and the soft kernels were dried to constant weight in an air convection oven at 103 ± 2 °C [12]. The dried kernels were then ground into power using a pestle and mortar and stored at -20 °C until analysis.

The second-derivative spectrum method

Absorption spectra were measured using a Shimadzu recording spectrophotometer, model UV-1700, over a scanning range of 250–275 nm with a high scanning

speed and a derivative wavelength difference of $\Delta\lambda = 2$ nm. Amplitudes were recorded between 266 nm and 268 nm[11,13].

A 0.6 mg/ml amygdalin stock solution in methanol was prepared for use as a standard. A serial dilution of this stock solution in methanol was then prepared to give standard solutions at concentrations of 0, 0.10, 0.15, 0.20, 0.30, and 0.50 mg/ml.

The HPLC method

About 1 ml of filtered apricot kernel extract was filtered through a 0.22 µm micro-filter. The amygdalin content of the solution was determined using a Hitachi Technologies L2000 series liquid chromatograph coupled to a Hitachi L2455 diode array detector. Quantification was performed using a C18 reversed phase column (250 mm × 4.6 mm, 5.0 µm) at 25 °C using a Hitachi L-2300 column oven. Chromatography was performed using a flow rate of 0.8 ml/min, a 15:85 acetonitrile:water mixture as the eluent, 20 µl of sample, and with detection under UV at 214 nm using Hitachi L2455 DAD detector. All analyses were performed in triplicate. Standard curves for the HPLC and spectrophotometric methods were prepared by analysing the stock solution serial dilution using the protocols described above.

Statistical analysis

The reproducibility and stability of the second-derivative spectrophotometry results were analysed for statistical significance using ANOVA (SPSS 16.0). Mean separation was evaluated using Duncan's multiple range test. Differences between the results obtained with HPLC and second-derivative spectrophotometry were analysed using the t-test (SPSS 16.0). Differences were considered statistically significant when $p < 0.05$.

RESULTS

Reproducibility and stability of the second-derivative spectrophotometry results

The second-derivative spectrophotometry calibration curves were linear. Regression analysis yielded the following relationship between concentration (y) and amplitude (x): $y = 5.823\,x + 0.0019$ ($r^2 = 0.9987$). The reproducibility of the analyses was tested for six different sample standing times using five different samples in each case. The intra-day coefficient of variation ranged from 2.4% to 3.5%. The highest amygdalin concentrations were observed for samples left to stand for at least 8 h (Table 1), and the measured amygdalin concentration decreased significantly in samples that had been stored for 5 days or more ($p < 0.05$, Table 2).

Table 1 Reproducibility of measured amygdalin levels from samples ($n = 15$).

Standing time (h)	Assay value (dry base, %)	Coefficient of variation (%)
0	$5.01 \pm 0.18^{d*}$	3.5
2	5.18 ± 0.16^{c}	3.1
4	5.14 ± 0.14^{c}	2.8
6	5.25 ± 0.17^{bc}	3.2
8	5.33 ± 0.16^{ab}	3.0
10	5.38 ± 0.13^{a}	2.4

[*] Entries in this column that are followed by the same letter do not differ significantly from one-another ($p < 0.05$).

Extraction recoveries

The ratio of the assay value to the spiked amount for standard was calculated as $(A-B)/C$, where A is the amount detected, B is the amount of sample without standard, and C is the spiked amount of the standard. The recovery of amygdalin was 99.08% (Table 3).

Differences between the results obtained using HPLC and second-derivative spectrophotometry

The HPLC calibration curve was linear. Regression analysis yielded the following equation describing the relationship between peak area (y) and concentration (x): $y = 280\,225\,x + 1806.5$ ($r^2 = 0.9991$).

The paired-samples t-test showed that the amygdalin concentrations measured using second-derivative spectrophotometry were higher than those determined using HPLC ($p = 0.000$). However, the paired samples correlation for the two methods was 0.975 ($p = 0.000$), indicating that their results are strongly correlated. Two samples were found to contain no amygdalin by both HPLC and second-derivative spectrophotometry.

DISCUSSION

HPLC is generally considered to be more practically convenient than either gas chromatography or capillary electrophoresis[14]. It has been used to analyse amygdalin levels in the flowers of *Eriobotrya japonica*, apricot kernels, almonds, and other species[6,15,16].

Derivative spectrophotometry is a useful tool in chemical analysis because it can separate overlapping signals and eliminate background. This makes it possible to quantify analyses without initial separation or purification. It has therefore found applications in the pharmaceutical industry, clinical medicine, and

Table 2 Stability of amygdalin in samples ($n = 10$).

Standing time (h)	1-day	2-day	3-day	4-day	5-day
0	$5.06 \pm 0.17^{a*}$	4.98 ± 0.14^{ab}	4.97 ± 0.14^{ab}	5.00 ± 0.19^{a}	4.84 ± 0.13^{b}
2	5.28 ± 0.08^{a}	5.12 ± 0.16^{bc}	5.10 ± 0.11^{bc}	5.18 ± 0.13^{ab}	5.02 ± 0.01^{c}
4	5.20 ± 0.12^{a}	5.14 ± 0.17^{a}	5.10 ± 0.14^{a}	5.12 ± 0.16^{a}	4.97 ± 0.11^{b}
6	5.29 ± 0.17^{a}	5.26 ± 0.20^{a}	5.21 ± 0.19^{ab}	5.29 ± 0.20^{a}	5.08 ± 0.18^{b}
8	5.37 ± 0.15^{a}	5.39 ± 0.22^{a}	5.33 ± 0.16^{ab}	5.36 ± 0.12^{a}	5.20 ± 0.17^{b}
10	5.43 ± 0.12^{a}	5.40 ± 0.17^{ab}	5.37 ± 0.06^{ab}	5.45 ± 0.14^{a}	5.29 ± 0.12^{b}

[*] Entries between the columns that are followed by the same letter do not differ significantly.

Table 3 Testing the recovery of amygdalin ($n = 5$).

Sample amount (mg)	Spiked amount (mg)	Assay value (mg)	Recovery rate (%)
8.07 ± 0.10	0.98	9.04 ± 0.10	99.08

Table 4 Differences in the results obtained with HPLC and second-derivative spectrophotometry ($n = 16$).

HPLC (mg/ml)	Second-derivative spectrophotometry (mg/ml)	
0.18 ± 0.09	0.21 ± 0.10	
Paired difference	**t-value**	**Sig. (2-tailed)**
0.028 ± 0.023	4.918	0.000

biochemistry, among other fields[10]. Because it is simple to perform, can be done rapidly, and gives accurate results in the determination of amygdalin, it may be an attractive tool for routine use in quality control laboratories that can be used by relatively unskilled workers. The methanol used in this work is a volatile and toxic solvent. To minimize the amount of methanol used, we tested an alternative extraction process using a Soxhlet apparatus[6]. While this reduced the volume of methanol used in the extraction, it increased the amount of time required for sample preparation, and so the ultrasonic method was preferred. While amygdalin is readily dissolved in methanol, it could also potentially be extracted into water containing 0.1% citric acid under reflux, which may be a more attractive option[14]. It should be noted that there are both bitter and sweet wild apricot kernels. We found that sweet kernels contained no amygdalin, in keeping with a report on sweet apricots cultivars form Mallorca (Spain)[17]. The amygdalin concentrations measured using second-derivative spectrophotometry were generally somewhat higher than those determined using HPLC. However, for samples containing between 0.217 mg/ml and 0.284 mg/ml, there was a strong correlation between the concentrations measured using the two methods, making it possible to estimate the HPLC value from the spectrophotometric data.

Second-derivative spectrophotometry thus appears to be a powerful and generally useful tool for measuring the amygdalin contents of wild apricot kernels. Moreover, it should also be suitable for determining the amygdalin content of the press cake. To avoid contaminating the press cake with toxic methanol, one could initially extract amygdalin using edible ethanol, then dry the resulting extract in a vacuum oven and re-dissolve it in methanol to obtain a sample suitable for analysis by second-derivative spectrophotometry.

The second-derivative spectrophotometry method is likely to be a useful and attractive tool for routine use in quality control laboratories for the large-scale determination of amygdalin concentrations in wild apricot kernels and press cake.

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