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Determination of coumarin in methanol extract of cinnamon (*Cinnamomum cassia* Blume) using reversed-phase high performance liquid chromatography

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Abstract
Coumarin is a component of natural flavourings including cassia, which is widely used in foods and pastries. The toxicity of coumarin has raised some concerns and food safety authorities have set a maximum limit of 2 mg/kg for foods and beverages in general. An efficient method for routine analysis of coumarin is liquid chromatography with diode array detection. In this work, we determined coumarin levels in methanol extract of cassia cinnamon. Reversed-Phase High Performance Liquid Chromatography (1100-HPLC system), using Phenomenex RP 18e (5 µm) 250 mm column as stationary phase, was selected as the method of analysis. A mixture of acetonitrile: 0.5% (v/v) acetic acid in water (25:75) at flow rate 1 mL/min was used as mobile phase. Detection was done at 278 nm. Using such conditions, retention time for coumarin was 10.4 minutes. The recovery was 99.708%. LOD and LOQ were 0.623 µg/mL and 1.889 µg/mL, respectively. Mean concentration of coumarin in cinnamon bark extract was 916.71 mg/kg.

Keywords: Cinnamon, coumarin, extraction, High-performance liquid chromatography (HPLC)

Introduction
Coumarin is a natural substance occurring in the essential oils of a number of plants used as flavouring ingredients in foods. Coumarin's aroma has been described as sweet, aromatic, creamy vanilla bean odor [1]. Until 1954, when the first toxicological concerns about coumarin were raised, synthetic coumarin was widely used to add flavour, e.g. to the so-called may wines (second-grade white wine flavored with woodruff) [1]. After that, the use of coumarin as a food flavoring was discontinued based on reports of hepatotoxicity prior to the existence of any carcinogenicity and mutagenicity data [2]. According to Lake's estimation, the main source of coumarin in the diet is cinnamon [2]. The cinnamon used domestically and industrially in the preparation of food originates primarily from true cinnamon and from cassia cinnamon (simply referred to as cassia). True cinnamon belongs to the species of *Cinnamomum verum* whereas cassia generally includes the species of Cinnamomum cassia, also known as *Cinnamomum aromaticus*, *Cinnamomum loureiroi* and *Cinnamomum burmannii* Blume [3]. Cassia is cheaper and generally more popular in Europe compared to true cinnamon [4]. True cinnamon and cassia taste different because of a different chemical composition. For example, cassia contains more of the aromatic compound coumarin (1-benzopyran-2-one) compared to true cinnamon [5]. Widespread and increased use of cassia in food products led to evaluate the maximum coumarin limits in cinnamon-flavored food products, thus imposing the necessity of a simple and rapid method of routine analysis. The aim of this study was to develop a simple HPLC method to determine the concentration of coumarin in methanol extract of cinnamon bark.

Materials and methods
Chemicals and standards
Coumarin (≥99%) and glacial acetic acid 99.7% were purchased from Sigma Aldrich (Germany). Acetonitrile (HPLC grade) and Methanol (HPLC grade) were purchased from Fisher Scientific (United Kingdom). Disposable syringe filters with a pore width of 0.2 µm (Chromafil PET-20/25) were purchased from Macherey–Nagel (Germany).

HPLC instrumentation and chromatographic conditions
The HPLC system consisted of an Agilent (Waldbronn, Germany) 1100 HPLC system (binary pump, degasser and autosampler) with diode array detector. LC separation was performed on a reversed phase (Phenomenex, Aschaffenburg, Germany 250×4.6 mm i.d., 5µm, Synergi polar

RP), column thermostatted at 25˚C using mobile phase A (acetonitrile) and mobile phase B (water, 0.5% (v/v) acetic acid) in an isocratic program (25% A: 75%B) with a flow rate of 1mL/min. Injection volume was 20 µL. For quantitative analysis, the wavelength with the highest intensity was used (278 nm) for coumarin. Furthermore, UV/Vis spectrum between 240 and 340 nm was recorded to verify the peak identity of coumarin.

Sample preparation

Barks/Powders [6]

For barks, an adequate amount of plant material was pulverized with a mortar and pestle. Dry plant samples (0.5 g) were weighed and sonicated in 20 mL of methanol 80% for 30 min followed by centrifugation for 10 min at 3000 rpm. The supernatant was transferred to a 25-mL volumetric flask. The procedure was repeated using 5 mL of methanol 80% and respective supernatants were combined. The final volume was adjusted to 25 mL with methanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.2-µm nylon membrane filter. The first 1 mL was discarded and the remaining volume was collected in a LC sample vial. Seven cinnamon bark samples were extracted and tested.

Preparation of Standard Solution

Stock standard solution was prepared by dissolving 10 mg of coumarin standard in methanol 80% and adjusting the final volume to 100 ml (0.1 mg/ml).

Validation of Analytical Method [According to ICH Guidelines] [7]

Linearity

Standard solution was pipetted 0.2, 1, 2, 5, and 7mL, respectively, and adjusted with methanol 80% to 10 mL. The obtained solutions were 2, 10, 20, 50, and 70 µg/mL, respectively. The solutions were injected into the chromatograph injection gate. Linearity is determined based on the correlation coefficient (r) in the linear regression (y = bx + a) of the relation between the peak area and concentration [8].

Accuracy

Accuracy is measured by determining the recovery (%) [9]. Standard solution 2 µg/mL was used and six replicates were prepared. Recovery is calculated by the formula:

$$\text{Recovery (\%)} = \frac{\text{measured concentration}}{\text{actual concentration}} \times 100\%$$

Precision

Precision is measured by determining three concentrations of the analyte with three times replication or minimum six times replication at 100% concentration of the analyte [7]. Standard solution 2 µg/mL was used and coefficient of variance (CV) was calculated.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were obtained based on the standard deviation and slope using the following formulas [7]:

$$LOQ = \frac{10 \times SD}{b}$$

$$LOD = \frac{3.3 \times SD}{b}$$

SD: standard deviation, b: slope of the linear regression

Results and Discussion

Coumarin was identified at a retention time of 10.4 min. (Fig.1). In all samples, the UV profiles of coumarin were consistent with literature data [10] showing a global maximum at around 278 nm. (Fig.2)

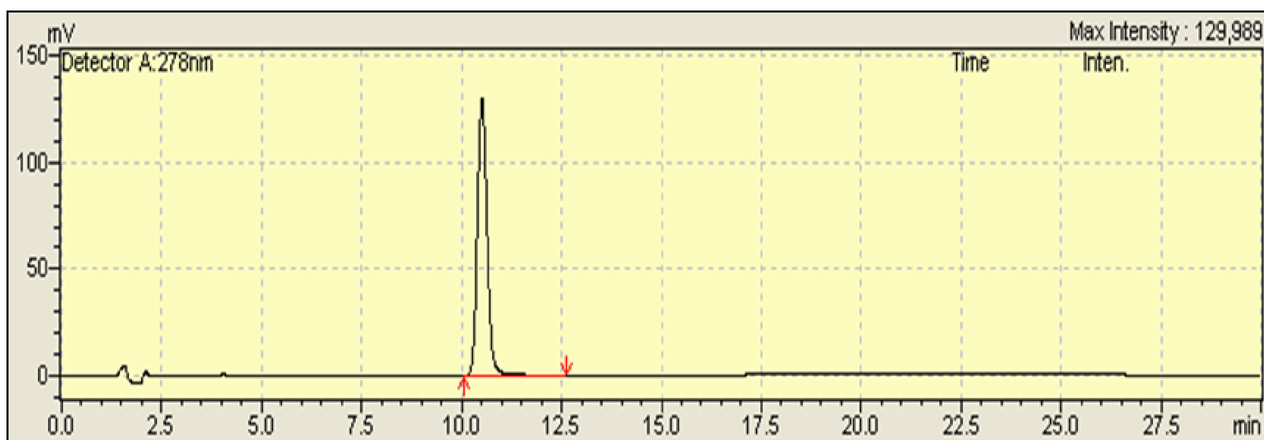

Fig 1: A chromatogram of coumarin standard at a concentration of 0.1 µg/mL and obtained at a wavelength of 278.1 nm.

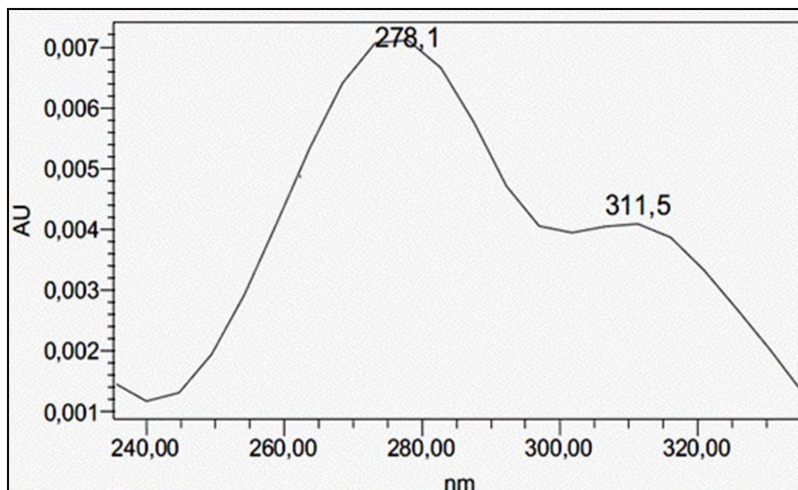

Fig 2: The UV profile (240-340 nm) obtained from coumarin eluted at 10.4 min.

Linearity

The method gave linear response for coumarin (Fig. 3) within the concentrations 0.2, 1, 2, 5, and 7 µg/mL with r^2 = 0.999.

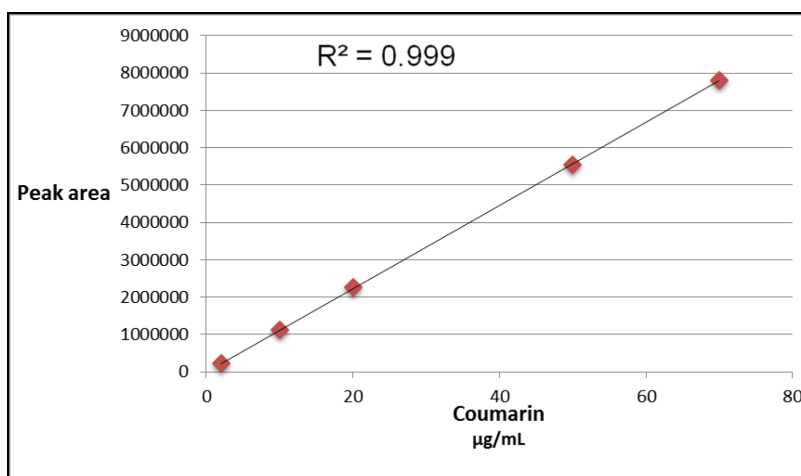

Fig 3: Linear response of peak area against the concentration of coumarin (ug/mL).

Accuracy and Precision

Determination of accuracy and precision was obtained from the calculation of six-time-measurements at concentration 2 µg/mL. The measurement results are shown in Table 1. Instrument response (peak area) entered into the linear regression equation which has been previously calculated, so that the measured concentration was obtained. Then the recovery and coefficient of variance were calculated. The values show that the method used for coumarin quantification presents high accuracy and precision.

Table 1: Accuracy and precision of coumarin

Actual concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (%)	SD	CV (%)
2 µg/mL	1.977	98.85%	0.02865	1.44%
	1.991	99.55%		
	1.985	99.25%		
	1.973	98.65%		
	1.988	99.4%		
	2.051	102.55%		
Mean	1.994	99.708%		

LOD and LOQ were calculated with the formula previously described

$$LOQ = \frac{10 \times SD}{b} = \frac{10 \times 21037}{111354} = 1.889\ \mu g/ml$$

$$LOD = \frac{3.3 \times SD}{b} = \frac{3.3 \times 21037}{111354} = 0.623\ \mu g/ml$$

LOD and LOQ indicate coumarin concentration limit that can still be detected and quantized using this method.

Determination of Coumarin in Cinnamon Bark Extract

Determination of coumarin concentration in the extracts was achieved by entering the instrument's response (peak area) to the linear regression equation. coumarin concentration from seven measurements are shown in Table 2. Average concentration of coumarin contained in cinnamon bark extract is 916.71 mg/Kg

Table 2: Determination of coumarin in cinnamon bark extracts

Sample number	peak area	Coumarin concentration mg/Kg
1	548726	97
2	1015464	448.4
3	2684729	1185.5
4	593607	262.12
5	6897080	1218.4
6	737033	325.6
7	6519210	2880
Mean	2713692.714	916.71

Conclusion

Our HPLC method can be used to determine the concentration of coumarin in cinnamon-bark extract using mobile phase A (acetonitrile) and mobile phase B (water, 0.5% (v/v) acetic acid) in an isocratic program (25% A: 75%B) with a flow rate of 1mL/min. Detection wavelength was 278 nm. This method fulfilled validation criteria and presents simple and rapid measurements of coumarin levels.

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