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Investigating the amount of coumarin in cinnamon samples as an indicator of safety and authenticity by liquid chromatography

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Abstract

Cinnamon is one of the world's oldest, important and commonly used spices in food industries. Since cinnamon has different varieties which they have diffident qualities, therefore, development of methods for characterization of different variates and evaluation of quality of cinnamon sample is very important. In this research a simple, fast and sensitive method based on liquid chromatography for determination of coumarin as authenticity and safety index in cinnamon samples has been developed. first, several factors influencing the extraction efficiency of coumarin including composition of extraction solvent and type of sample agitation were studied and optimized. Under the optimal conditions, good linear behavior over the investigated concentration ranges (0.2-500 mg/L) with good correlation of determination, R2> 0.996 were obtained. The relative standard deviations (RSDs) based on six replicate determinations at 100 mg/L level of coumarin was less than 6 percent. In studied samples concentration of coumarin was obtained in the range of 6.1-227.7 mg kg-1. Also, between color and coumarin amount of the samples there is positive significant correlation and samples with dark brown color have higher levels of coumarin.

Keywords Authenticity, Cinnamon, Coumarin, High performance liquid chromatography.

Introduction

Cinnamon is a small evergreen tree in tropical regions that belongs to the Boha family and is native to Sri Lanka and South India. Cinnamon has been known from ancient times as one of the most significant products, which is used in the food and beverage industry, due to its culinary and medicinal properties [1-5]. It is one of the oldest known spices that has wide applications in the food, pharmaceutical and cosmetic [1]. The aroma and flavor of cinnamon is due to the essential aromatic oils that make up 0.5 to 1 percent of its ingredients. The spicy taste and smell of cinnamon is caused by cinnamaldehydes. Cinnamon has many properties such as anti-inflammatory, antioxidant, anti-ulcer, antimicrobial, which also has the potential to reduce blood sugar and lipid, and also has significant medicinal effects in the treatment of type 2 diabetes. In clinical studies, it has been mentioned as an anti-allergen compound [2-5].

Ceylon and Cassia cinnamon are two important and major varieties of cinnamon in the world. Chinese cinnamon

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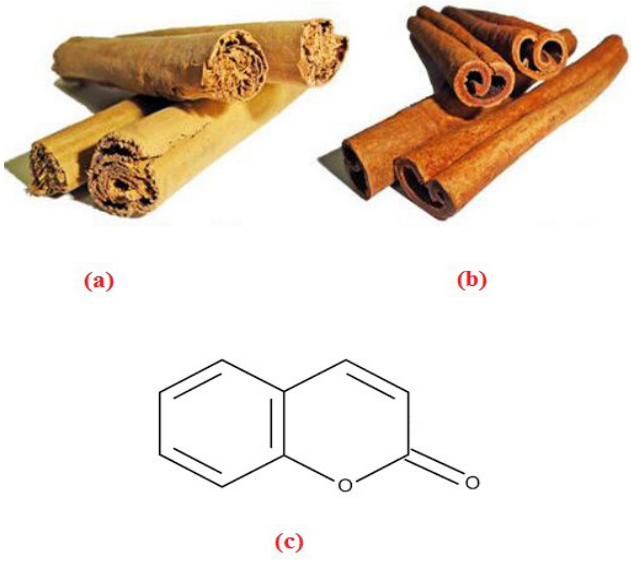

Figure 1. Picture of genuine (ceylon) cinnamon (a), cassia cinnamon (b) and chemical structure of coumarin (c)

or C. Cassia is a native of Assam, Vietnam and the eastern Himalayas. In contrast, Ceylon cinnamon with the scientific name C. Zeylancum is native to Ceylon and southwest India [2, 6]. Ceylon cinnamon has a sweet and mild taste, its color is light brown or yellowish brown. Ceylon cinnamon looks like a cigar and its taste is mild and it is grown in India and Sri Lanka and has a low amount of coumarin (Figure 1a). On the other hand, cassia type cinnamon has a spicy and intense taste, a reddish or dark brown color and a hollow tube shape (Figure 1b), which grows in China, Vietnam and Indonesia and has a high amount of coumarin [7]. Due to the high quality and price of Ceylon cinnamon, one of the common frauds in the food industry is the use of cassia cinnamon instead of Ceylon cinnamon. Therefore, appropriate analytical methods are necessary to distinguish these two species from each other. Although the diagnostic test between Ceylon cinnamon and cassia is difficult [8, 9]. But one of the ways of qualitative diagnosis between these two species is to add iodine solution. Iodine solution produces a dark blue color with cassia cinnamon, the intensity the color depends on the amount of cassia, while Ceylon cinnamon does not produce a color [8, 9]. Another important factor to identify these two species is measuring the amount of coumarin in cinnamon samples, because the coumarin content of Ceylon cinnamon is small and about 0.004 percent. On the other hand, cassia cinnamon has high amounts of coumarin up to 1 percent [2-7,10]. Coumarin is a natural product with aromatic and fragrant characteristics, widespread in the entire plant kingdom. It is found in different plant sources such as vegetables, spices, fruits, and medicinal plants including all parts of the plants—fruits, roots, stems and leaves. Coumarin is found in high concentrations in certain types of cinnamon, which is one of the most frequent sources for human exposure to this substance [6]. Coumarins (1,2-benzopyrones or 2H-1-benzopyran-2-ones) represent an important family of naturally occurring benzopyrone compounds, all of which consist of a benzene ring linked to the pyrone ring [1-8] (Figure 1c). After coumarin was synthesized in 1868, it was first put on the market as a flavoring substance [2-10]. However, since coumarin has been found to cause liver toxicity to rats and dogs that were fed with coumarin-containing food, the usage of coumarin as a flavoring substance has become questionable [5-11]. Coumarin has been known as a genotoxic compound in the 1980s and 1990s, and its daily consumption limit by the European Union is 2 mg per kilogram of body weight. Consuming more than the permissible limit of coumarin can lead to liver and kidney damage. In sensitive groups, its low dose can also cause liver enzymes to rise, at the same time, high doses can lead to liver inflammation and jaundice [11-13].

The analysis of coumarin was reviewed by Bogan et al. [15] Early analysis methods included paper chromatography, thin-layer chromatography, colorimetric assays and polarography. Today, high-performance liquid chromatography (HPLC) appears to be the method of choice for coumarin analysis (15). An efficient method for coumarin analysis is the use of high-performance liquid chromatography [7,16-18].

Constanze Sproll et al. used the HPLC system with diode array detector for analysis and safety assessment of coumarin in foods. Mobile phase A (water, 5 mM ammonium acetate buffer, 0.2% (v/v) acetic acid) and mobile phase B (acetonitrile/methanol 1:2 (v/v)) in a gradient program were applied. For quantitative analysis, the wavelength with the highest intensity was used (279.8 nm). Furthermore, UV/Vis spectra between 210 and 400 nm were recorded to verify the peak identity of coumarin and the peak purity. The extraction solvent was methanol, 80% (v/v)) [16].

In a survey in 2013, UPLC-UV/MS was used. All analyses were performed on a Waters Acquity UPLC system (Waters Corp., Milford, MA) that included a binary solvent manager, sampler manager, heated column compartment, photodiode array (PDA) detector, and single quadrupole detector (SQD). The eluent consisted of water with 0.05% formic acid (A) and methanol/acetonitrile (90:10, v/v) with 0.05% formic acid (B). The total run time for an analysis was 7 min. The PDA detection wavelength was 280 nm. Mass spectrometer conditions were optimized to

obtain maximal sensitivity. The source temperature and the desolvation gas temperature were maintained at 150 and 350 °C, respectively [17].
The purpose of this research is to evaluate the status of cinnamon consumed in Iran based on the coumarin index. In this research, high-performance liquid chromatography method was used to separate and measure coumarin. Due to the unknown origin of the varieties available in the Iranian market, the high level of coumarin in the tested cinnamon samples indicates the toxicity of cinnamon and also its inferior variety, which most profit seekers and fraudsters use to increase their profits. Low-value and low-quality varieties are used instead of genuine cinnamon. Therefore, conducting this research, while establishing and validating an efficient method for measuring coumarin in cinnamon samples, provides useful information for evaluating and checking the status of imported cinnamon in the country.

2. Material and methods

2.1 instruments

The high-performance liquid chromatography (HPLC) was KNAUER, Euro Chrom model, made in Germany, was equipped with a K-1001 four-channel pump, a UV-Vis detector, and a K-2600 degasser. The separations were done in a C-18 chromatography column (250mm x 4.6mm, 5µm). The injection volume was 20 µl and the column temperature was ambient temperature. Also, the flow rate of the mobile phase was 1 ml/min. The detection wavelength was 280 nm. The pH of the solutions was adjusted with a pH meter, model WTW Inolab (made in Germany). An ultrasonic water bath of 40 kHz with a power of 0.138 kW (Techno-Gas, Italy) was used to extract coumarin from cinnamon samples and also to degas the mobile phase. Deionized water was prepared by a water purification device manufactured by Aqua Max-Ultra Youngling Company (Seoul, South Korea). A centrifuge manufactured by Poya Electric Company of Iran was used to centrifuge the samples.

2.2 Chemicals and standards

All the chemicals used were of analytical grade. Coumarin standard was obtained from Sigma-Aldrich (Germany). Ethanol, methanol, acetonitrile, sodium hydroxide and ammonium acetate were purchased from Merck, Germany. The base standard solution (1000 mg/l) of coumarin was prepared by weighing 50 mg of the coumarin standard and making it up to volume with 50 ml of methanol. Working standard solutions were prepared by suitable dilution of the base solution with distilled water. The mobile phase for liquid chromatography was prepared by mixing 2 volumes of acetonitrile and 1 volume of ammonium acetate buffer.

2.3 Sample preparation

20 cinnamon samples (11 stick and 9 ground cinnamon samples) were obtained from market in Tehran. In sampling, we tried to get imported samples as much as possible from different countries and brands. Next, the stick samples were ground into powder. Then 1 gram of each sample was carefully weighed and transferred to Erlen 100 ml. Then 25 ml of extraction solution (methanol: water with a ratio of 1:1) was added to Erlenmeyer flask. In order to extract coumarin, Erlen was stirred for 30 minutes in a shaker at a speed of 150 rpm at ambient temperature or placed in an ultrasonic bath for 5 minutes. Then each sample was filtered using filter paper and the filtered solution was injected into the liquid chromatography to determine the amount of coumarin.

2.4 Statistic analysis

In this research, the results were analyzed based on a completely random design with SPSS 20 software. To compare the averages, Duncan's multiple range test was used at the 5 percent level. Excel 2010 software was used to draw the graphs. 20 cinnamon samples (11 stick and 9 ground cinnamon samples) were tested.

3. Results and discussion

3.1 Optimization of extraction parameters

There are many techniques in the literature described for the extraction of coumarins from food [19]. A systematic study of coumarin extraction carried out by Bourgaud et al., [20] showed that the extraction with ethyl acetate, diethyl ether and chloroform was not adequate and applicable in the extraction of coumarin, unlike extraction using polar solvents such as methanol, ethanol and water. Therefore, methanol and ethanol were most commonly used for extraction of coumarin in cinnamon and cinnamon containing food samples. In addition, the extraction time did not affect the amount of coumarin extracted, and thus the amount of coumarin did not differ significantly in the extract obtained by using Soxhlet extraction with methanol for 48 h from that obtained by stirring on a magnetic stirrer for 30 min at room temperature.
It has been shown that for the detection and quantification of coumarin in different products, it is sufficient to carry out direct extraction with 80% methanol with the necessary homogenization of food. Using the method developed in the work by Sproll et al. [16] matrix interferences are low and emulsions are not formed, even with products containing a lot of fat. For this reason, according to Sproll et al. [16] extraction using 80% methanol was under consideration in Germany for inclusion in official meth-

ods for the preparation of samples for the determination of coumarin in food. The same was shown in the work of Rahim et al. [21], whereby solid food was extracted with methanol using ultrasound for a period of 10 min to enhance extraction. Extraction by ultrasound for 10 min was sufficient, since extending the extraction time does not increase the extraction efficiency.

First, the effect of two factors, the type and stirring time of the solution, as well as the composition of the extraction solvent, were investigated. Ultrasonication of the sample solution can accelerate coumarin extraction. Therefore, efficiency of shaking and ultrasocation methods were compared. To minimize extraction time, different times were tested for ultrasonication. The obtained results showed that, extraction efficiency for 5 min ultrasonication is comparable with 30 min shaking.

The obtained results (Figure 2) showed that the type of ultrasonic stirring or shaker has no significant effect on the amount of coumarin extraction ($p<0.05$).

This can be due to the proper contact surface between the sample and the extraction solvent because the samples were in powder form.

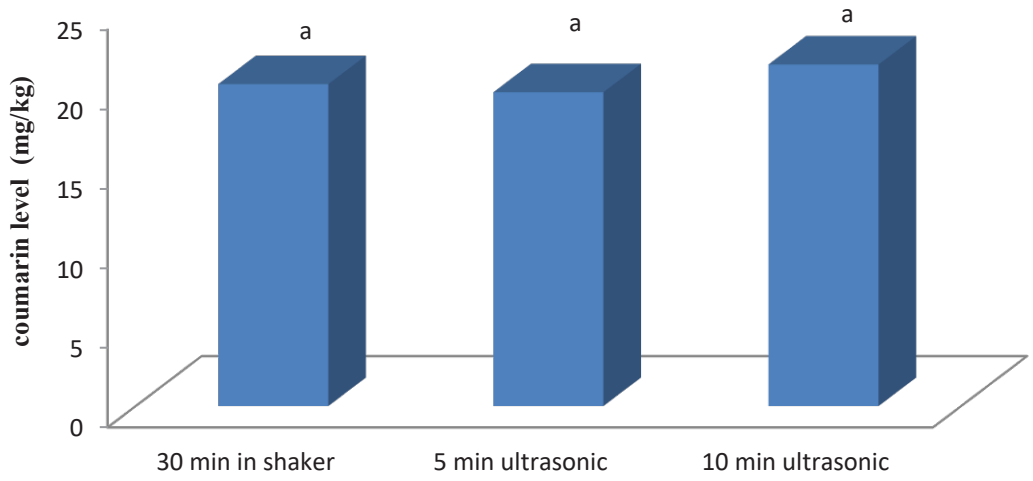

Figure 2. Effect of different time of ultrasonication and shaker on the amount of coumarin of Indian cinnamon stick code number 3

In order to check the best extraction solvent combination, different proportions of methanol and water were checked (Figure 3). The results showed that the ratio of methanol to water 50:50 has the best extraction efficiency. The results of comparing the averages showed that the ratio of 50:50 has a significant difference with other ratios of methanol and water ($p\geq0.05$). Coumarin is a polar and hydrophilic compound, so it is predictable that better extraction efficiency can be achieved with polar solvents. Comparing the amount of coumarin extraction in different modes shows that high ratios of water or high ratios of methanol both have lower extraction efficiency than the 50:50 mode.

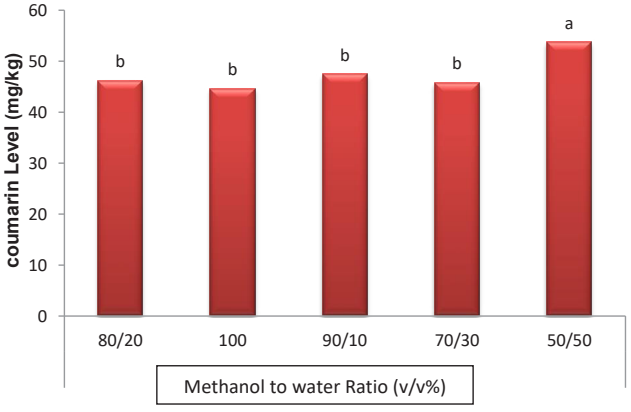

Figure 3. Effect of methanol to water ratio on the amount of coumarin

3.2 Validation of the test method

In Table 1, the validation parameters of the method under optimal conditions, including limit of detection (LOD), limit of quantification (LOQ), percentage of relative standard deviation (RSD%), linear range and correlation coefficient of the calibration chart are given. The proposed method was linear in the range of 0.2-500 mg/L and the correlation coefficient of the linearity method was R2 ≥ 0.99 (Figure 4). The relative standard deviation percentage of the method was obtained by performing 6 repeated tests in one day and in several consecutive days using the standard solution of coumarin with a concentration of 100 mg/L, 2.6 and 5.9, respectively. Also, the detection limit and quantitative limit of the method were 0.1 and 0.2, respectively.

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Table 1. Figures of merit of the proposed method

Repeatability percentage		LOQ (mg/L)	LOD (mg/L)	correlation coefficient	Linear range (mg/L)
In several days (number=6)	In 1 day (number=6)				
5.9	2.6	0.2	0.1	0.996	0.2-500

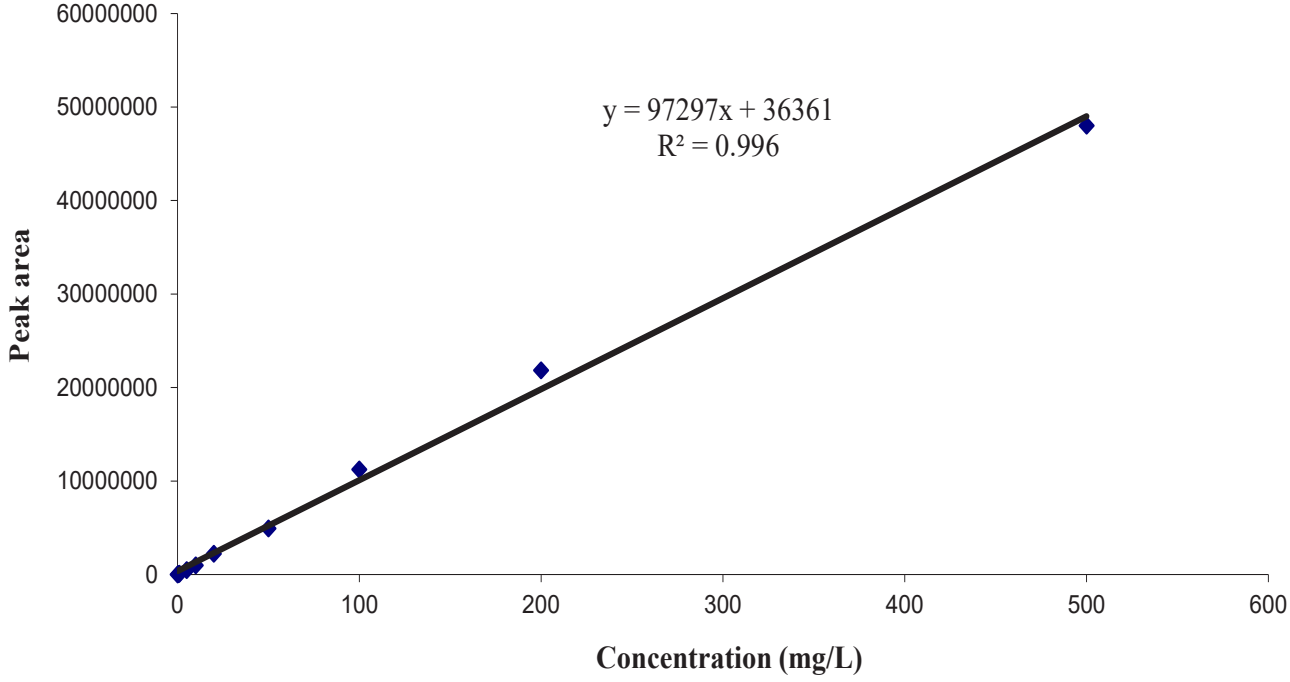

Figure 4. Calibration curve of coumarin

3.3 Analysis of real samples

In order to evaluate the efficiency of the proposed method, the samples of stick and cinnamon powder collected under optimal conditions were extracted and measured with 2 repetitions. The obtained concentrations of coumarin in different samples are given in Table 2. Also, the accuracy of the method was evaluated by calculating the recovery percentage for spiked samples with a concentration of 100 mg/L (Table 3). Figure 5 shows the chromatograms of code 2 cinnamon stick sample before and after the spike.

$$\%\text{Recovery} = \frac{(C_{found} - C_{initial})}{C_{added}} \times 100$$

C_{found}: The obtained (measured) concentration for the spiked sample
$C_{initial}$: The initial concentration obtained for the pre-spike sample
C_{added}: concentration added to the sample

According to the results reported in Table 2 in the sticks samples, the highest amount of coumarin (222.7 mg/kg) belongs to the code 9 cinnamon sticks sample (Chinese cinnamon) and the lowest amount of coumarin (6.1 mg/kg) belongs to the cinnamon stick was code 1 (Indian

cinnamon). The results show that cinnamons of Chinese origin have high coumarin content and Indian cinnamons have low coumarin content, which is an expected result. Also, among the samples of cinnamon powder, the highest amount of coumarin (141.4 mg/kg) belongs to treatment code 5 and the lowest amount of coumarin (0.21 mg/kg) belongs to code 4.

Table 2. Coumarin concentration in cinnamon stick and powder samples

color of the cinnamon sample	coumarin concentration[a] (mg/kg)	sample code	cinnamon sample type
Light brown	6.1 ± 0.4	stick 1	stick
Very dark brown	91.9 ± 0.05	stick 2	
Medium brown	17.4 ± 0.8	stick 3	
Medium brown	15.9 ± 0.5	stick 4	
Medium brown	15.8 ± 0.4	stick 5	
Light brown	16.1 ± 0.8	stick 6	
Medium brown	30.3 ± 0.9	stick 7	
Light brown	23.6 ± 0.7	stick 8	
Very dark brown	222.7 ± 10.7	stick 9	
Very dark brown	189.9 ± 8.3	stick 10	
Very dark brown	197.7 ± 6.7	stick 11	
Medium brown	87.8 ± 1.9	powder 1	**powder**
Light brown	75.9 ± 2.4	powder 2	
Light brown	59.1 ± 1.7	powder 3	
Light brown	21.0 ± 0.5	powder 4	
Very dark brown	141.4 ± 4.8	powder 5	
Light brown	54.1 ± 0.9	powder 6	
Medium brown	76.8 ± 3.1	powder 7	
Medium brown	78.5 ± 2.3	powder 8	
Light brown	47.8 ± 1.1	powder 9	

a: Average of three repeated measurements

Investigations of the coumarin content in commercially available ground cinnamon samples are reported to vary from 5 to 7670 mg/kg [7, 14-16]. However, coumarin contents below 100 mg/kg were considered to originate from Cinnamomum verum [18]. Therefore, some of the tested sample from Iran market (75% of the samples) are originate from Cinnamomum verum.

Table 3. Results of percentage recovery of coumarin measurement in cinnamon samples

recovery (percent)	measured value (mg kg^{-1})	added amount of coumarin (mg kg^{-1})	sample name
—	6.1	—	stick code 1
93.8	99.5	100	
—	91.9	—	stick code 2
97.0	186.2	100	
—	87.8	—	powder code 1
95.7	179.8	100	
—	21.0	—	powder code 4
92.6	112.1	100	

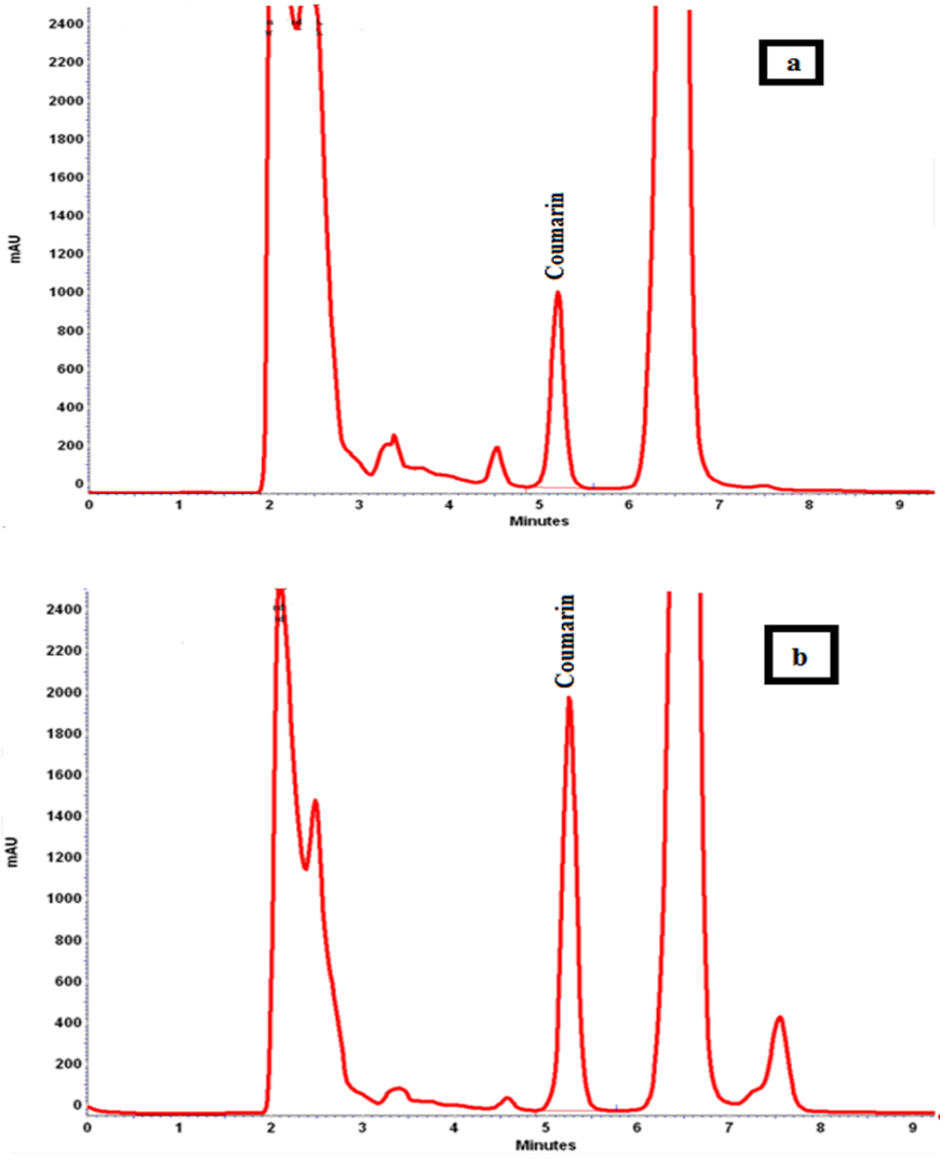

Figure 5. Chromatogram of code 2 cinnamon stick sample before (a) and after adding (b) the control amount (100 mg Kg^{-1})

3.4 Investigation the relationship between color and coumarin content of cinnamon samples

The relationship between the color of cinnamon samples and their coumarin content was investigated (Table 2). The color of the cinnamons included light brown, medium brown, and very dark brown, and each color was assigned a score of 1 to 3, respectively. Then the Pearson correlation between the amount of coumarin and the color of the cinnamon sample was checked. The results of correlation analysis between the amount of coumarin and cinnamon color in stick samples showed that there is a significant positive correlation with correlation coefficient R2=0.66. This means that the darker the brown cinnamon sample, the higher the coumarin content. On the other hand, samples with light brown color have less coumarin. Also, the results of the correlation analysis between the amount of coumarin and the color of cinnamon in the powder samples showed that there is a significant positive correlation with a correlation coefficient of R2=0.77. This means that the darker the brown cinnamon sample, the higher the coumarin content. On the other hand, samples with light brown color have less coumarin.

The results obtained in this research are in accordance with the results of the research conducted by Blahova and Svobodova [7] in evaluating the coumarin level of cinnamon powder samples available in the Czech market. An independent sample of Vroom type cinnamon from a farm in Sri Lanka was used for testing. The results of 60 cinnamon samples analyzed by them showed that all the samples obtained from Czech retail markets were from cassia cinnamon and had a high coumarin content between 2650 and 7017 mg/kg, while the coumarin content of the sample obtained from Sri Lanka (control sample or genuine cinnamon) had less coumarin content than the detection limit of the method. They also found a significant relationship between the color of cinnamon and its coumarin content, so that darker cinnamon samples had higher coumarin content.

In research conducted in 2008 by Sproll et al. [15] for the analysis of coumarin with high performance liquid chromatography, the use of polar solvents such as methanol and ethanol mixed with water showed a very high efficiency compared to non-polar solvents such as ethyl acetate and chloroform, which are consistent with the results obtained in this research. They also stated that extraction efficiency increases significantly in the presence of ultrasound.

3.5 Conclusion

In general, it is possible to express the efficiency of high-performance liquid chromatography (HPLC) in measuring the amount of coumarin in different types of stick and cinnamon powder. The results showed that the amount of coumarin varies in different types of cinnamon sticks and cinnamon powder. The highest amount of coumarin among all treatments (cinnamon stick and powder) belonged to Chinese cinnamon stick and the lowest amount of coumarin belonged to Indian cinnamon stick. Also, the results show a significant positive relationship between the color of the cinnamon sample and its coumarin content, and it was found that cinnamon samples with a darker brown color have a higher amount of coumarin. 25% of the tested samples (dark brown samples) had coumarin contents above 100 mg/kg were considered to originate from Cinnamomum verum [18]. Finally, it can be concluded that the coumarin index is a very suitable parameter for evaluating the authenticity, quality and safety of cinnamon samples offered in the market.

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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