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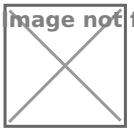

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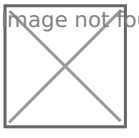

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Coumarin in grounded cinnamon and teas containing cinnamon - extraction and determination by HPLC method

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ABSTRACT

Cinnamon, a commonly used spice as well as a part of many commodities, such as breakfast cereals, teas and bakery products, represents an important part of the diet. Cinnamon, among many bioactive components, contains an important active compound coumarin. Numerous studies have shown that coumarin has a beneficial health effect on the body in optimal consumption, while increased long-term consumption can lead to adverse health effects. In this paper, the amount of coumarin in ten different products found on the Croatian market, including ground spices and teas, was analyzed. In addition, different extraction parameters were analyzed in order to find the best method of extraction of coumarin from cinnamon and products containing cinnamon.

Introduction

Cinnamon has become one of the most widely used spices in the world. In addition, cinnamon is used for therapeutic purposes in traditional Asian medicine due to various pharmacological properties, such as antioxidant, anti-inflammatory, anticancer, antifungal, and antidiabetic activity. Other research shows that cinnamon consumption protects the pancreas, stimulates the digestive system, prevents urinary tract inflammation, reduces the risk of heart disease, and protects the nervous system (Dvorackova et al., 2015).

Two forms of cinnamon are often be found on the market, Ceylon and Cassia cinnamon namely. Ceylon or "real" cinnamon is obtained by drying the inner bark of the tree *Cinnamomum verum* J. S. Presl, which is sometimes reffered to as *Cinnamomum zeylanicum*. This species belongs to the *Lauraceae* family and is grown in Sri Lanka, Madagascar, and the Seychelles. Another form of cinnamon is called cassia cinnamon or more simply, cassia. This form of cinnamon is obtained from other types of wood, the most important of which are Chinese cassia (*Cinnamomum cassia Blume*, syn. *Cinnamomum aromaticum*) and Indonesian cassia (*Cinnamomum burmannii*), by drying the inner bark of the tree (Woehrlin et al., 2010). These two forms of cinnamon are very difficult to distinguish according to their appearance in ground form. However, in the form of sticks, it is possible to distinguish between cassia sticks, which are relatively hard and consist of a thick layer of bark, and Ceylon cinnamon sticks, which consist of several thin layers of bark (Woehrlin et al., 2010). These two types of cinnamon also differ in price, *i.e.* cassia is cheaper than Ceylon cinnamon and has replaced the more expensive Ceylon cinnamon in various products in Europe, Canada, and the US (Wang et al., 2013). Apart from the appearance and price, the most important difference lies in the concentration of coumarin where cassia cinnamon has a much higher concentration of coumarin than Ceylon cinnamon. Data in the literature indicate that coumarin concentrations in Ceylon cinnamon vary from a point below the limit of detection to 190 mg/kg. In cassia, on the other hand, these values range from 700 to 12000 mg/kg (Woehrlin et al., 2010).

Pharmacologically important compounds that justify its popularity are for sure compounds

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collectively called coumarins. These compounds, in small quantities, show a favorable pharmacological effect on the organism, while in excessive concentration they have a hepatotoxic effect in several species and a carcinogenic effect in rodents. Coumarins are a family of compounds that contain a 1,2-benzopyrone ring, often found in a large number of fruits, vegetables, herbs and spices. For most coumarins, no harmful effect on the human body has been shown for digestion through edible plants (Wang et al., 2013). Coumarin (1,2-benzopyrone) itself, is a secondary metabolite and was first found in the seeds of tonka (*Dipteryx odorata*) in 1822, while its laboratory synthesis was firstly performed in 1868 (Ehlers et al., 1995; Woehrlin et al., 2010). Since then, it has been used as a flavor enhancer as it has a sweet and vanilla-like aroma (Solaiman and Al-Zehouri, 2017). With the advancement of analysis, in 1950, the hepatotoxic effect of coumarin on laboratory animals was noticed, which led to the ban on its use. Its carcinogenic properties were determined and in 2004 the European Food Safety Authority (EFSA) determined non-covalent binding of coumarin to DNA, which indicated the fact that its carcinogenicity was not associated with DNA coil disruption (Woehrlin et al., 2010). Accordingly, EFSA sets an acceptable daily intake (TDI) for animals of 0.1 mg/kg body weight based on the maximum dose that does not cause harm (NOAEL). In 2008, EFSA established the same TDI for humans (EFSA 2008), despite further research showing different effects of coumarin on primate metabolism and the metabolism of other animals. Unlike rodents, the primary metabolic pathways of primates do not develop hepatotoxic metabolites. Furthermore, it has been shown that most people are not sensitive to coumarin in the same way as rodents that have been used for research related to the toxicity of this compound. Moreover, coumarins are beginning to be used for therapeutic purposes, but due to its potential hepatotoxicity in some patients, certain states prohibit its use in this manner (Wang et al., 2013). Due to the increasing use of cassia cinnamon in food products, research in Germany has shown that coumarin concentrations in some foods exceed the acceptable daily intake (TDI) limits as cassia contains more coumarin than Ceylon cinnamon. On this occasion, the European Parliament and the Council of Europe in 2008 set limits on coumarins in cinnamon-containing foods. They are 50 mg/kg for traditional or seasonal bakery products, 15 mg/kg for other bakery products, 20 mg/kg for breakfast cereals and 5 mg/kg for desserts (Wang et al., 2013). Although cinnamon, as a spice, is the largest source of coumarin in food, the legal limit for coumarin in cinnamon still does not exist. In general, there is currently no legal limit to any natural ingredient found in herbs or spices (BfR, 2012).

Previous research has provided insight into the structure of coumarin, its pharmacological properties as well as the positive and negative impact on the human body. In addition, a difference was observed in the coumarin concentration of the two types of cinnamon, Ceylon cinnamon, and cassia where Ceylon cinnamon contains less coumarin than cassia. Scientists around the world have noted an increase in cassia in cinnamon containing foods, resulting in an increase in coumarin intake in the human body. This research aimed to establish the most appropriate extraction method and determine the concentration of coumarin in some foods containing cinnamon on the Croatian market.

Materials and methods

Chemicals

The standard compound coumarin (≥ 99%) (Sigma Chemical Co., St. Louis, MO, USA) was used as a standard for making the calibration curve. All solvents were of analytical grade and purchased from J.T. Baker (Avantor, Phillipsburg, NJ, USA).

Cinnamon and cinnamon products

In this research, coumarin was extracted from ten various food products, with the emphasis on the cinnamon as a spice or in the form of tea and juice. Products were purchased in 2019 from the Croatian market, mostly from larger supermarkets. The list of products and types of packaging can be seen in Table 1.

Extraction

Two types of extraction techniques were used to determine the optimal extraction conditions for the model sample (C2), ultrasonic-assisted extraction (UAE) and extraction by heating and stirring on a magnetic stirrer using two solvents, methanol and ethanol at 30 °C and 50 °C for 30 and 60 min, respectively. The weight of grounded samples was 500 mg and the volume of the solvent used was 1 mL. Each extraction was performed in two parallels. After determining the optimal conditions, the other food samples were extracted with methanol for 10 min at 30 °C and 50 °C. After extraction, the samples were centrifuged for 5 min at 5000 rpm and then decanted. The supernatant was diluted with methanol, filtered

through a PTFE 0.22 µm filter, and subjected to HPLC analysis.

Table 1. List of products, manufacturers and types of packaging.

Sample mark	Product	Type of packaging
C1	Ground cinnamon	Glass packaging
C2	Ground cinnamon	Glass packaging
C3	Ground cinnamon	Plastic bag
C4	Ground cinnamon	Glass packaging
C5	Ground cinnamon	Plastic bag
C6	Ground cinnamon	Plastic bag
C7	Tea with orange, cinnamon and clove	Paper box
C8	Apple and cinnamon tea	Paper box
C9	Apple and cinnamon tea	Paper box
C10	Cinnamon and apple juice	Plastic bottle

Chemical Characterization of the Extracts

There is no official method for the determination of coumarin in cinnamon and cinnamon products, but several methods have been described in the literature that applies different determination techniques as well as different modifications of the same technique. The high-pressure liquid chromatographic (HPLC) method was chosen for the determination, with modifications of the method developed by Solaiman and Al-Zehouri (2017). The analysis was performed on a semi-preparative high-pressure liquid chromatography (HPLC) device manufactured by Agilent, 1260 Infinity II series. The HPLC system used for the analysis consisted of a quaternary pump (G7111A), a column chamber (G7116A), a photodiode array detector (G7115A), an autosampler (G7157A), and a fraction collector (G1364E). The system was operated using a computer program Prep LC Offline (Agilent, Santa Clara, California, SAD). A Cosmosil 5C18-MS-II (Nacalai Tesque) column (250x4.6 mm), filled with 5 µm particles, was used to separate the components. The separation was achieved by gradient elution at a flow rate of 1mL/min, and with a 1% solution of acetic acid in water (A) and methanol (B) were used as the mobile phase. The gradient was set as follows: 0–5 min: 80% A; 5–15 min: 80–40% A; 15–35 min: 40–20% A; 35–40 min: 20-80% A. The equilibration time to starting conditions was 10 minutes.

Coumarin identification was performed based on the retention time and comparison of the absorption spectrum of coumarin in samples of cinnamon and cinnamon products with the spectrum of standard. Quantification was made based on external calibration.

A standard coumarin stock solution was prepared in methanol in concentration of 1 mg/mL. Based on the expected ranges of coumarin concentrations in the samples, a series of 6 solutions of different concentrations were made; 20; 30; 50; 100; 150 µg/mL in methanol. Based on the expected ranges of coumarin concentrations in the samples, a series of 6 solutions of different concentrations were made; 20; 30; 50; 100; 150 µg/mL. The retention time of coumarin on this method was 18.085 min. The separation was performed in a column heated to 25 °C, and coumarin detection was performed at a wavelength of 300 nm, with spectrum recording in the wavelength range from 190 nm to 400 nm. 20 µL of the sample was injected into the device. Sample analysis was performed in duplicate, and two injections were performed from each prepared solution. The results of the content in the analyzed samples were expressed in mg/kg.

Results

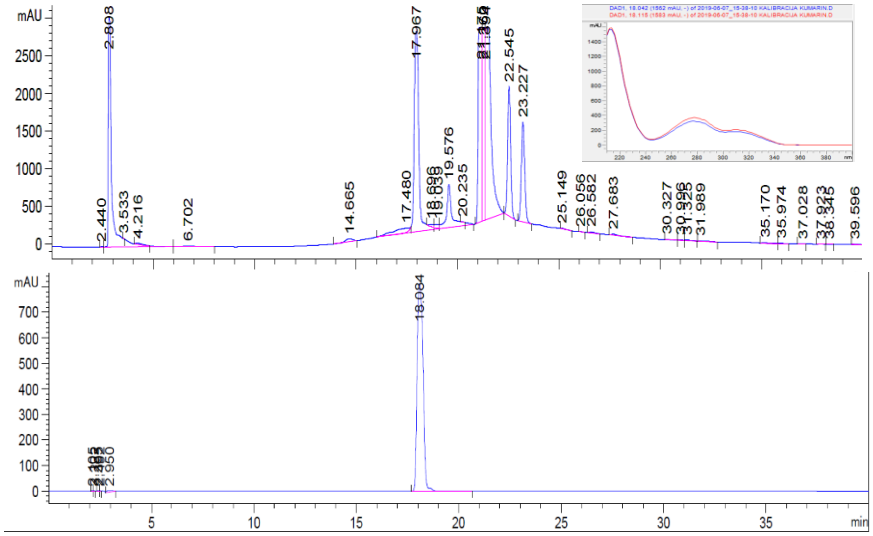

Fig. 1. Comparison of the absorption spectra and chromatograms of coumarin standards and coumarin components in the sample C2 with column retention time of 18.0804 min

Table 2. Coumarin content in sample C2 obtained by extractions of different duration (10, 30 and 60 min) in different solvents (methanol and ethanol) at different temperatures (30 °C and 50 °C).

Time (min)	Solvent	Temperature (°C)	Coumarin (mg/kg)
10	MeOH	30	182.46±1.15
10	EtOH	30	10.90±0.25
30	MeOH	30	171.04±0.91
30	EtOH	30	71.66±0.15
60	MeOH	30	169.15±0.15
60	EtOH	30	69.22±1.50
10	MeOH	50	157.33±0.41
10	EtOH	50	13.79±0.05
30	MeOH	50	138.62±0.16
30	EtOH	50	27.39±0.03
60	MeOH	50	131.34±0.15
60	EtOH	50	46.13±0.53

Table 3. Display of coumarin content in different samples depending on the type of extraction and temperature. Extractions were performed by stirring or ultrasound (UAE) at 30 °C or 50 °C for 10 min in methanol as solvent in two samples.

Sample	Extraction type	Temperature (°C)	Coumarin (mg/kg)
C1	Stirring	30	179.91±4.92
	UAE	30	184.54±2.61
	Stirring	50	173.31±2.28
	UAE	50	200.58±5.18
C2	Stirring	30	179.06±5.18
	UAE	30	158.80±1.21
	Stirring	50	145.35±0.18
	UAE	50	138.34±0.39
C3	Stirring	30	10.58±0.04
	UAE	30	9.13±0.01
	Stirring	50	17.81±0.08
	UAE	50	7.41±0.06
C4	Stirring	30	176.76±0.48
	UAE	30	154.47±0.02
	Stirring	50	167.89±5.81
	UAE	50	38.85±0.00
C5	Stirring	30	159.76±0.00
	UAE	30	148.43±11.33
	Stirring	50	150.55±0.67
	UAE	50	133.83±2.84
C6	Stirring	30	182.30±0.02
	UAE	30	183.75±0.32
	Stirring	50	167.26±2.72
	UAE	50	153.19±0.60
C7	Stirring	30	7.98±0.01
	UAE	30	10.80±1.21
	Stirring	50	3.15±0.02
	UAE	50	14.07±0.02
C8	Stirring	30	41.07±0.00
	UAE	30	51.60±0.20
	Stirring	50	39.84±0.06
	UAE	50	58.46±0.13
C9	Stirring	30	21.86±0.14
	UAE	30	20.02±0.07
	Stirring	50	11.28±1.33
	UAE	50	20.79±0.10

Discussion

HPLC

After the chromatographic conditions were adjusted to the applied column and device, the linearity of the method was estimated in the range of 10 to 150 μg/mL, which is a range of the expected coumarin concentrations in the products. As shown in Figure 1, the method was linear in the concentration range with the correlation coefficient of 0.996. In addition to comparing the retention time of coumarin standards with the retention time of the components in the sample, the absorption spectra of the standards and components in the sample were also compared (Figure 1). Based on this, we can confirm that the component with a retention time of 18.085 minutes is the desired component, coumarin.

Influence of extraction parameters on coumarin yield

The extraction efficiency is influenced by a number of extraction parameters such as extraction time and temperature, as well as the solvent. In this study, the influence of solvent, temperature and extraction time of two extraction techniques was examined to determine the optimal conditions for further extractions of coumarin in food. As can be seen from the results, all of the mentioned parameters influenced the coumarin content in the obtained extracts. The influence of each parameter is discussed as described below.

Extraction method

Two very common and simple extraction techniques were used in this research, mixing on a magnetic stirrer and ultrasonically assisted extraction (UAE). A comparison of these two extraction techniques was performed, since their mechanisms are completely different. From the results listed in Table 3., it can be seen that a higher coumarin content was achieved in the extracts obtained by mixing and heating, compared to the UAE. In general, and according to the literature, extraction performed by mixing using a magnetic stirrer is 7% more efficient than ultrasonically assisted extraction. The results agree with the results of Sproll et al. (2008) who also showed a 4% higher mixing efficiency compared to ultrasound-assisted extraction possible due to better mixing of the sample and the solvent especially at lower temperatures, whereby a better mass yield occurs.

Temperature

The results show that extraction at 30 °C is more efficient than extraction at 50 °C (Table 1). In contrast, Bourgaud et al. (1994) in their work with the plant *Psoruleu cinerea* showed a higher level of coumarin in extraction with methanol at boiling temperature compared to extraction at room temperature. In addition, Medeiros-Neves et al. (2015) in a study of coumarins from *Pterocaulon balansae* point out the most favorable extraction at 60 °C. Wang et al. (2013) in their work extracted coumarin from ground cinnamon at room temperature. These results could indicate the optimality of coumarin extraction from cinnamon at room temperatures and the optimality of coumarin extraction from other plant material at elevated temperatures. The difference in coumarin content in cinnamon at 30 °C and 50 °C is possibly caused by the degradation of coumarin at higher temperatures (Lapornik et al., 2005). Looking at Table 3 and Figure 9, it can be concluded that coumarin content is higher during extraction at 30 °C in ground cinnamon, while in teas extraction at 50 °C results in higher coumarin levels.

Solvent

The results (Table 3) clearly show the higher efficiency of methanol as a solvent for the extraction of coumarin from cinnamon compared to ethanol. This result agrees with the research of Bourgaud et al. (1994) and Sproll et al. (2008) in which the higher efficiency of methanol was also confirmed. For further research, it would be useful to use methanol of different concentrations to see the most optimal one. Also, it would be good to test other samples with different solvents, *i.e.* to expand the research to a larger number of samples, as well as the solvents.

Extraction time

The extraction time using methanol of 10 min showed the highest content of coumarin (Table 3) in the obtained extracts, and by increasing the extraction time, the coumarin content decreased. Although Bourgaud et al. (1994), as well as Sproll et al. (2008), emphasize that the extraction time does not affect the coumarin concentration, they do not say that increasing the extraction duration decreases the coumarin concentration. The probable reason for this is the degradation of coumarin (Lapornik et al., 2005). It is interesting to note that degradation accelerates over a longer extraction duration at a temperature of 50 °C (Figure 8). In contrast, increasing the duration of extraction with ethanol as the solvent increases the coumarin levels. In support of this are the results of research by Dvorackova et al. (2015) who showed that increasing the extraction time of coumarin from

cinnamon in ethanol as a solvent increases the level of extracted coumarin.

Coumarin content in the samples

Considering that the concentration of coumarin in Ceylon cinnamon extends up to 190 mg/kg, and since the concentrations of the analyzed samples do not exceed this level, it is very likely that the samples contain Ceylon cinnamon. From Table 3, it can be seen, as expected, a higher amount of cinnamon in ground cinnamon compared to teas. It is important to note that coumarin was not found in sample C10 (cinnamon apple juice) or was below the detection level. Sample of ground cinnamon C1 contains the most coumarin (184.58 mg/kg), followed by C6 (171.62 mg/kg), while sample C3 contains the least coumarin (11.23 mg/kg). In the study conducted by Woehrlin et al. (2010) it is shown that the coumarin content in Ceylon cinnamon on the German market, which varies from 65 to 185 mg/kg is in line with the results of this study.

As already mentioned, teas contain much less coumarin than pure spices, which was to be expected since cinnamon is not their only ingredient. Ballin and Sørensen (2014) in their study point out coumarin levels that range from 0 to 12 mg/kg in cinnamon-containing teas. In comparison, the results of this study stand out with significantly higher concentrations of coumarin in cinnamon-containing teas ranging from 9 to 48 mg/kg. The reason for this could be the use of cassia instead of Ceylon cinnamon in teas or the use of a much larger amount of cinnamon. Likewise, coumarin is more biologically available to the body in the form of a fluid matrix such as tea (Abraham et al., 2011).

Conclusions

The most effective method of extraction of coumarin from ground cinnamon is with methanol as a solvent at a temperature of 30 °C for 10 min using stirring on a magnetic stirrer. The most effective method of extraction of coumarin from teas is with methanol as a solvent at a temperature of 50 °C using stirring on a magnetic stirrer. Teas have a lower coumarin content than ground cinnamon due to the lower content of cinnamon in teas. The coumarin content in ground cinnamon varies from 11 to 185 mg/kg which corresponds to the coumarin content in Ceylon cinnamon. The content of coumarin in teas varies from 9 to 48 mg/kg tea in dry form, which is significantly higher compared to the research of Ballin and Sørensen (2014) and indicates the use of a larger amount of cinnamon or the use of cassia cinnamon. No coumarin was found in the apple juice Amarena cinnamon (C10) or it is below the level of detection, which indicates a small content of cinnamon in the juice.

Author Contributions: The research design, funding and the final proof writing was performed by Maja Molnar. Investigation and method validation was performed by Martina Jakovljević Kovač and Lovro Mihajlović. Data validation and draft writing was performed by Martina Jakovljević Kovač.

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