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ORIGINAL PAPER

Coumarin contents of tonka *(Dipteryx odorata)* products

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Abstract

Tonka *(Dipteryx odorata)* is a plant native in tropical Africa. The tonka fruits contain a kernel, commercialized as tonka beans. These kernels are known to contain coumarin, a natural flavoring compound widely used in foods and pastries, beverages, and cosmetics. Coumarin is known to be liver toxic and therefore authorities have regulated its contents in foods and alcoholic beverages. The purpose of this study was to quantify the concentration of coumarin in different foods and beverages that contain tonka beans or were prepared thereof. The tonka beans itself or the products thereof were extracted with methanol and these extracts were analyzed using a HPLC-UV/Vis method. We have extracted and measured 20.4 ± 0.4 to 43.4 ± 0.9 mg coumarin per gram in six samples of tonka beans. In sugars aromatized with tonka ($n = 3$) 0.2 ± 0.0 mg/g of coumarin were observed and in two pastes produced from tonka 0.6 ± 0.0 mg/g were determined. A handcrafted 'Tonka Gin' was containing 2.2 ± 0.0 mg/L. Based on the non-observed-adverse-effect level (NOAEL) for hepatotoxicity in animal experiments, the Scientific Panel on Food Additives Flavorings, Processing Aids and Materials in Contact with Food (AFC) established a Tolerable Daily Intake (TDI) of 0.1 mg/kg bw (body weight) coumarin. We suggest to critically evaluate whether the TDI could be exceeded by consuming the products tested containing coumarin.

Keywords Tonka · Coumarin · HPLC-UV/Vis · Risk · *Dipteryx odorata*

Introduction

Coumarin (2 H-Chromen-2-one, Fig. 1) is a colorless crystalline solid with a sweet odor resembling the scent of vanilla and a bitter taste [1]. It was first isolated from tonka bean in 1820 by Alfred Vogel from Munich and was first synthesized in 1868 by William Henry Perkin. Tonka beans, as the first plant from which coumarin was extracted and isolated since they contain 1–3% coumarin, and in rare cases up to 10% [2] have gained interest during the last years as flavoring agent. In extracts from tonka beans besides coumarin, 138 other compounds, e.g. umbelliferone, *o*-coumaric acid, and diterpenoids were identified.

Coumarin has numerous pharmacological activities, which depend on its chemical structure. Some of these pharmacological activities of coumarin include, anti-inflammatory, anticoagulant, antibacterial, antiviral, antifungal, antihypertensive, antitubercular, anticancer, anticonvulsant and neuroprotective [3–5]. On the other hand, coumarin is hepatotoxic. With a tolerable daily intake (TDI) of 0.1 mg/kg bw (body weight), toxicological effects (hepatotoxicity) may occur if the TDI is exceeded [3, 6]. Other adverse effects in humans such as diarrhea or vomiting reported after coumarin exposure were rather scarce and related to high doses [7].

In recent years, cinnamon *(Cinnamomum)* and products thereof were analyzed for its coumarin contents. *Cinnamomum verum (C. zeylanicum)* bark contains only traces of coumarin, whereas barks from all three *cassia* species (*C. cassia*, *C. loureiroi*, *C. burmannii*), especially *C. loureiroi* and *C. burmannii* contain substantial amounts of coumarin (up to 76.1 g/kg). Data on trading numbers indicate that Indonesian *cassia* has replaced the more expensive true cinnamon (*C. verum*, Ceylon cinnamon) in Europe, the United States and Canada [2] a few years ago. German authorities have investigated products produced from cinnamon during the last years [6, 8]. With the scope of a health risk reassessment conducted in June and August 2006, Germany's

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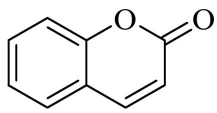

Fig. 1 Structure of coumarin

Table 1 Food in which the presence of the coumarin is restricted [10]

Compound Food	Maximum Level of Coumarin (mg/kg)
Traditional and seasonal bakery	50
Breakfast cereals	20
Fine bakery	15
Desserts	5

Federal Institute for Risk Assessment (BfR) showed concern that can arise for consumers by taking cinnamon-based food supplements. If *cassia* cinnamon is used in the form of powder, there is a possibility for the consumers to exceed the TDI of coumarin [9]. According to Regulation of coumarin in food (EC Nº 1334/2008 of the European parliament and of the Council 16 December 2008 (Annex III)), coumarin must not be added as a substance to the food. In food the maximum level for coumarin has been regulated (see Table 1) [3, 10].

Within the last years various methods were published to analyze coumarin in food which was summarized 2020 by Lončar et al. [3]. These methods were mostly used to successfully quantitate coumarin in cinnamon samples using e.g. planar chromatography GC-MS or HPLC-UV [6, 11, 12].

As coumarin occurs in products flavored with cinnamon, in food supplements, in pharmaceutical preparations, and meanwhile in the more and more popular tonka beans, the aim of our study was to establish an analytical method to determine the amounts of coumarin in tonka beans and tonka containing products on the market for further risk evaluations.

Materials and methods

Coumarin (> 97%), vanillin (> 99%) were purchased from Fluka (Honeywell, USA). 4-Hydroxycoumarine (> 98%) was from Carl Roth (Karlsruhe, Germany), methanol (HPLC grade), acetonitrile (HPLC grade), ammonium acetate, water (HPLC grade), 3,4-dimethoxybenzaldehyde (99%) from Sigma-Aldrich (Taufkirchen, Germany). 3-Ethoxy-4-benzaldehyde (98%) was ordered from Alfa Aesar (Kandel, Germany).

The HPLC system consisted of Agilent Technologies 1200 Series with a quaternary pump (G1311A), a degasser (G1322A), an autosampler (G1329A). The detector is a diode array detector from Agilent (G1315D). HPLC separations were performed with a reversed phase column (Synergi™ 4 µm Polar-RP 80 Å, 250 × 4.6 mm, Phenomenex, Aschaffenburg, Germany) equipped with a SecurityGuard™ cartridge for Polar-RP HPLC columns (Phenomenex, Aschaffenburg, Germany) tempered at 40 ºC. The mobile phase is composed of three different solvents: A) a buffer consisting of ammonium acetate (5mM acidified with 0.2% acetic acid, B) ACN and C) methanol. The elution gradient used was as follows: between 0 and 3.2 min held with 70% A, 20% B, 10% C; from 3.2 to 22.2 min increased from 70% A to 20% A, 20% B to 53.3% B, 10% C to 26.7% C. Then from 22.2 to 24.2 min again 70% A, 20% B, 10% C held for 10.8 min and re-equilibrated with 70% A, 20% B, 10%C. The flow rate was 1 mL/min, the detection wavelength was 280 nm, and the injection volume was 10 µL at room temperature. Additionally, UV/Vis spectra between 280.8 and 360.5 nm were recorded to verify the identity of the coumarin peak.

Different samples of tonka beans ($n = 6$) and tonka bean containing foods were purchased: Tonka bean pastes ($n = 2$) that contain 2% of tonka bean, vanilla sugars with tonka ($n = 2$), organic coconut tonka sugar ($n = 1$), tonka extract ($n = 1$), tonka cacao ($n = 1$), ice cream with tonka ($n = 1$), almond tonka milk chocolate ($n = 1$) and tonka gin (handcrafted, 47% Vol; $n = 1$), respectively. All products were purchased in retail stores or via internet. For the extraction of the tonka beans, 3 g were ground in a mortar to a fine powder [6]. Three aliquots of one gram were extracted with 50 mL of methanol: water (80:20 *V: V*; extraction solution) for 2 h, stirring with a magnetic bar. Other tonka containing foods were weighted (1–10 g, depending on the matrix and expected coumarin content) and extracted with extraction solution for 2 h with stirring at room temperature. For the vanilla sugar and coconut sugar samples that contained tonka bean, aliquots of 5 g each of those powders were extracted with methanol-water as reported above.

Stock solutions of coumarin, vanillin, 4-hydroxycoumarin, and ethylvanillin were prepared (13.1, 12.3, 12.3, and 10.9 mg solubilized in 10 mL of methanol/water (80/20%), respectively. And 251.5 mg of the internal standard, 3,4-dimethoxybenzaldehyde, was dissolved in 10 mL methanol/water (80/20%). All stock solutions were diluted 1:10 and measured by HPLC-UV/Vis to identify the characteristic retention time of each compound. The retention times were as follows: coumarin (12.7 min), vanillin (8.6. min), 4-hydroxycoumarin (9.6 min), and ethylvanillin (11.1 min) and 12.1 min for the internal standard 3,4-dimethoxybenzaldehyde, respectively. To validate the HPLC-UV/Vis method, standard solutions containing coumarin, 4-hydroxycoumarin, vanillin, and ethylvanillin with concentrations between 1 and 100 µg/mL (seven concentrations) were prepared containing the same amount of 3,4-dimethoxybenzaldehyde

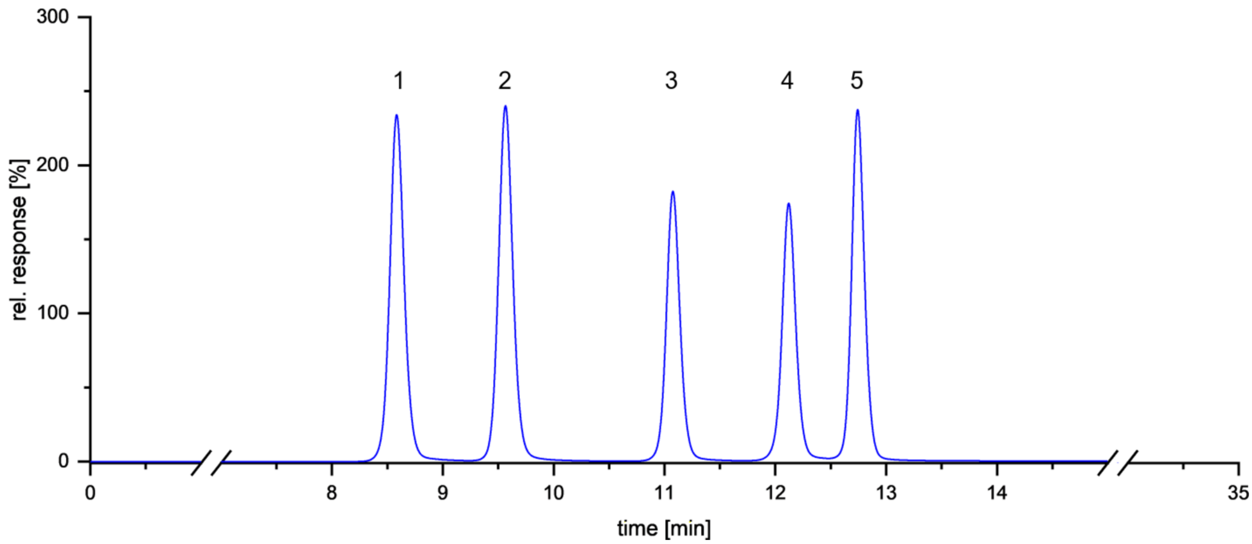

Fig. 2 HPLC-UV/Vis chromatogram of a standard solution containing 50 µg/mL of each analyte at a wavelength of 280 nm. 1 = vanillin t = 8.6 min, 2 = 4-hydroxycoumarin t = 9.6 min, 3 = ethylvanillin t = 11.1 min, 4 = 3,4-dimethoxybenzaldehyde (IS) t = 12.1 min and 5 = coumarin t = 12.7 min

(50 µg/mL) as an internal standard. The calibration curve linearity was evaluated between 1 and 100 µg/mL. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from regression line residual standard deviation using real matrices [6]. A good linear correlation ($r^2 = 0.9996$) was achieved.

For HPLC-UV/Vis analyses, the samples were diluted 1:10. All the samples contained the internal standard 3,4dimethoxybenzaldehyde in a concentration of 50 µg/mL. All solid samples were filtered with 0.45 µm disposable filters (Carl Roth, Karlsruhe, Germany). The gin sample was diluted: 900µL of undiluted gin was pipetted into a HPLC vial, followed by the addition of 100 µL of diluted standard.

For method validation, tonka bean samples prepared by standard addition containing coumarin concentrations of 20, 40, 80 and 200 µg/mL were analyzed. With these samples measurements were performed and recoveries of 107, 108, 102 and 100% achieved. The LOD was 0.3 µg/mL which means that with this method we are able to identify coumarin concentrations as low as 0.15 mg/g. The LOQ was 1.5 µg/mL. The LOQ and LOD were defined as signal to noise ratio equal to 10 and 3, respectively.

Results

Many reliable methods are available to determine coumarin in food samples [6, 11, 12]. We have used HPLC-DAD and optimal chromatographic conditions were achieved with a mobile phase that composed of ammonium acetate and acetic acid buffer, methanol, and acetonitrile. With this mixture, perfectly separated peaks were obtained for all the compounds of the standard solutions (see Fig. 2). The retention time for coumarin was 12.7 min.

Table 2 Coumarin concentration [mg/g] in different tonka samples analyzed ($n = 3$) by HPLC-UV/Vis. The results are shown as mean ± standard deviation of three independent analyses of the samples

Samples	Amount of Coumarin [mg/g]
Tonka Bean 1	35.5 ± 0.5
Tonka Bean 2	20.4 ± 0.4
Tonka Bean 3	42.8 ± 1.4
Tonka Bean 4	29.1 ± 0.5
Tonka Bean 5	32.5 ± 1.7
Tonka Bean 6	43.4 ± 0.9
Vanilla Sugar 1	0.2 ± 0.0
Vanilla Sugar 2	0.2 ± 0.0
Tonka Sugar 1	0.8 ± 0.0
Tonka Paste 1	0.6 ± 0.0
Tonka Paste 2	0.6 ± 0.0
Tonka extract	3.0 ± 0.1
Tonka Cocoa	0.8 ± 0.0
Coconut Sugar	0.2 ± 0.0
Tonka Gin (handcrafted)	2.2 ± 0.0 mg/L
Ice cream with tonka	36.3 ± 0.0
Almond tonka milk chocolate	0.2 ± 0.0

In the samples under study, different amounts of coumarin were analyzed in triplicate (see Table 2). Highest amounts were observed for tonka beans ($n = 6$) with 20.4–43.4 mg/g. In the three (vanilla) sugar samples aromatized with tonka, 0.2 ± 0.0 mg/g were determined. A paste produced from tonka (containing 2% tonka bean) revealed 0.6 ± 0.0 mg/g. The tonka paste was sold in a 50 g tube. Therefore, the total amount in the whole tube was calculated to be 30 mg coumarin. A handcrafted commercially available 'Tonka Gin' contained 2.2 ± 0.0 mg/L. The tonka extract contained

3.0 ± 0.1 mg/g coumarin. 36.3 ± 0.0 mg/g coumarin was detected in an ice cream with tonka. An almond tonka milk chocolate contained 0.2 ± 0.0 mg/g coumarin. Additionally, a tonka cacao was analyzed and 0.8 ± 0.0 mg/g coumarin was detected.

As expected, using the HPLC/UV-Vis method it was possible to determine vanillin peaks in sugar samples containing both, coumarin and vanillin. In those samples 0.2 ± 0.0 mg/g vanillin were measured (see Table 3). The maximum vanillin intake resulting from the consumption of orthosilicic acid-vanillin complex (OSA-VC) would be less than 0.3 mg/kg body weight (bw) per day, corresponding to less than 5% of the acceptable daily intake (ADI) value for vanillin of 10 mg/kg bw per day established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002 [13, 14]. For the vanillated sugar samples, a coumarin concentration of 0.2 mg/g was obtained, corresponding to 1.6 mg of coumarin per ready-to-use sachet (total weight of 8 g).

Another sample that was analyzed was a coconut sugar that contained tonka bean. Here, a coumarin concentration of 0.2 ± 0.0 mg/g was found, and this concentration in the entire product, which has a total weight of 10 g, corresponds to a total amount of 2.0 mg coumarin per bag.

Table 3 Vanillin concentrations [mg/g] in vanilla sugar samples. The results are shown as mean ± standard deviation of three independent analyses of the samples

Samples	Amount of Vanillin [mg/g]
Vanilla Sugar 1	0.2 ± 0.0
Vanilla Sugar 2	0.2 ± 0.0

Discussion

Paying attention to the limit for coumarin allowed for alcoholic beverages, it is known that the maximum is 10 mg/L [6]. In the beverage analyzed in this investigation, a concentration of 2.2 mg/L of coumarin could be determined. This concentration is within the allowed limit which means that it does not violate the limits of the EU regulation (s. Table 1) [3, 4, 10]. According to our findings 1 gram of tonka bean contains 20.4–43.4 mg of coumarin. Data are reported for coumarin in different other foods such as cinnamon cookies, gingerbread (Lebkuchen), as well as food supplements and, pharmaceutical products [6, 8].

With all this it is important to know that a child can exceed the TDI by simply consuming staple food such as bakery products and breakfast cereals aromatized with tonka or cinnamon. On the internet lots of different recipes containing tonka bean are available. E.g. a Panna Cotta prepared with one bean of tonka in 500 mL cream. This means, that one bean weighing 1 g will add about 20 to 40 g coumarin to 500 mL resulting in four portions. At the end, about 5 to 10 mg per portion will be ingested exceeding the TDI. Here it must be mentioned, that the TDI is established for a daily consumption, whereas most people consume the abovementioned aromatized foods not on a daily base e.g. only on Christmas time.

A child weighing approximately 15 kg might exceed the limit of 0.1 mg/kg/day (1.5 mg coumarin) easily by consuming coumarin rich foods such as with tonka and cinnamon aromatized products. Whereas adults weighing approximately 60 kg and more, they could consume higher amounts (maximum of 6 mg coumarin for a person with 60 kg). According to research, a child weighing approximately 15 kg could only consume 3 to 4 cookies (of 5 g each) containing coumarin without exceeding the limit, while an adult weighing around 60 kg could consume up to 10 cookies [6]. It could be estimated that the ingestion of one portion Panna Cotta containing about 20 mg coumarin, would be 1.3 mg/kg bw for a child and 0.3 mg/kg bw for an adult weighing 60 kg exceeding the TDI easily. Additionally, adverse effects reported for coumarin intake such as vomiting or diarrhea were only occurring by consumption of high doses [7]. It should be noted that people could be exposed to slightly higher amounts of coumarin, when they consume products aromatized with cinnamon and/or tonka and additionally being exposed to coumarin via aromatized by body care products or parfums added to the skin.

Conclusion

HPLC/UV-Vis is an effective technique for the rapid and accurate detection of coumarin in tonka bean and foods containing it. The sample preparation procedure is simple, directly extracting the sample with methanol and stirring. With the established method, we could reveal the highest coumarin amounts in tonka beans ($n = 6$) with contents of 29.1 ± 0.5–43.4 ± 0.9 mg/g and in one ice cream sample with tonka with 36.3 mg/g coumarin. In two vanilla sugars flavored with tonka, we found 0.2 mg/g and the amounts in one tonka sugar, two tonka pastes, and one tonka extract were 0.8 mg/g and 0.6 mg/g, respectively. Furthermore, we determined coumarin amounts of 0.2 mg/g for a coconut sugar, 0.8 mg/g for a tonka cocoa, and 2.2 mg/L for a handcrafted tonka gin.

Without knowing any facts about coumarin, it is very easy to exceed the TDI or have adverse effects due to it. For this reason, it is crucial that its existence be made known both in natural and processed products. The fact that maximum levels of coumarin as a food additive is regulated (see Table 1) was reasonable, given the importance of protecting the public from any potentially harmful substances. A reliable and unequivocal assay for coumarin is needed to monitor the sometimes high levels of coumarin in foods on the market [13] as the TDI can be easily exceeded.

As a possible measure against the excessive consumption of coumarin, maximum consumption levels could be indicated on foods containing coumarin or foods used for seasoning (e.g. tonka beans).

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Data availability Data is provided within the manuscript.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval This article does not contain any studies with human or animal subjects.

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