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Determination of coumarin in seasonal bakery products using QuEChERS and GC-MS

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Cinnamon is a traditional herbal drug, but more importantly, it is used as a flavor compound in the production of foodstuff. Due to the content of significant concentrations of coumarin in Cassia cinnamon, effective control of the coumarin content in seasonal bakery products like ginger bread and cinnamon biscuits is urgently needed. Here we present a novel, fast and fully validated protocol for the determination of coumarin in marketed bakery products using the QuEChERS sample preparation technique in combination with GC-MS analysis. Ten grams of homogenized sample was mixed with 20 mL acetonitrile/water (1:1) and 5 g magnesium sulfate/sodium chloride mixture (4:1). The organic phase was cleaned by dSPE with 25 mg magnesium sulfate/PSA (5:1). The LOD was 0.15 µg/mL and the LOQ 0.50 µg/mL. We detected a mean coumarin content of 19.5 µg/kg in 9 out of 14 seasonal food products (ranging from 1.45 to 39.4 mg/kg). No coumarin was detected in five cinnamon containing products. With this investigation we demonstrate that the QuEChERS sample preparation, previously applied mainly to the analysis of pesticides in vegetables, is also suitable for other complex matrices.

1. Introduction

Coumarin is a natural component of various cinnamon species, which is regulated by the European Food Safety Authority (EFSA) because of its possible adverse health effects (EFSA 2008). High levels of coumarin exposure have shown non-genotoxic carcinogenic effects in animal experiments and although it has not been observed yet in human population or clinical studies, precautions must be taken (Carlton et al. 1996, National Toxicology Program 1993). Still, because of the non-genotoxic nature, carcinogenic effects will only manifest when a certain concentration level is surpassed, and up to this point consumption can be allowed. However, in terms of setting a limit, the other *in vivo* observable effect of coumarin is of even greater importance. The compound's hepatotoxic effect has a much lower threshold at which adverse health effects emerge. Therefore, the tolerable daily intake (TDI) is influenced mostly by the hepatogenic properties of coumarin. For example, elevated liver enzyme levels (e.g. aminotransferases) which are indicative of hepatotoxity, are observed after the daily intake of 400 mg coumarin (Loprinzi et al. 1999), and another study relates elevated liver enzyme levels to the intake of 100 mg coumarin daily (Cox et al. 1989). Interestingly, these investigations suggest that there is a minor population sub-group (single digit percent), which is especially prone to coumarin's hepatotoxity, while the rest of the population can endure higher levels of coumarin intake without suffering from adverse health effects. Taking all these factors into consideration, the EFSA has established a TDI of 0.1 mg/kg bodyweight as well as coumarin content limits for different food product categories. Breakfast cereals are allowed to contain 20 mg/kg of coumarin, fine bakery ware 15 mg/kg, and desserts 5 mg/kg. The highest limit of up to 50 mg/kg is set for seasonal and traditional bakery ware. Because of this, one can easily reach the TDI in christmas time, when a lot of seasonal bakery containing cinnamon is consumed. Therefore, it is reasonable to regularly check the amount of coumarin in seasonal bakery products.

Sproll et al. (2008) used HPLC-UV to determine the coumarin content in cinnamon star cookies after extraction in a methanol-/water mixture. They determined a mean coumarin content of 25 mg/kg (up to 88 mg/kg) in 40 out of 47 cookies. Later the year Raters & Matissek (2008) used LC-MS to lower the limit of quantification (LOQ), and Carrez I (potassium ferrocyanide solution)/Carrez II (zinc sulfate solution) precipitation to improve sample clean-up. After analyzing 307 samples, a mean coumarin content of 22.1 mg/kg (up to 103.0 mg/kg) was detected in cinnamon star cookies and 5.3 mg/kg (up to 33.2 mg/kg) in gingerbread. Ballin et al. (2014) used a methanol/water extraction step and HPLC-UV to detect mean levels of 19.5 mg/kg (up to 35.0 mg/kg) coumarin in 18 traditional/seasonal bakery products. The downside of these reported methods are the multiple centrifugation, precipitation and filtration steps, which have to be performed prior to HPLC analysis.

In order to establish a new, fast and reliable analytical method for the determination of coumarin content in food products, we chose the QuEChERS (quick, easy, cheap, efficient, rough, and safe) procedure (Anastassiades et al. 2003) for sample preparation. The main feature of QuEChERS is the combination of single phase extraction and liquid-liquid phase partitioning, followed by a dispersive solid-phase extraction (dSPE) for further sample clean-up, thus providing a fast and efficient sample preparation. Although this method was initially established for multi-residue analysis of pesticides in foodstuff, it has become popular in other analytical fields, too. For example, Müller et al. (2011) used QuEChERS for the analysis of nicotine in dried mushrooms, and Plössl et al. (2006) analyzed drugs in plasma. Altogether, the QuEChERS method has proven its ability to facilitate sample preparation starting from diverse complex matrices.

Here we report a QuEChERS based approach to determine coumarin in seasonal bakery ware, and its application for the quality control of cinnamon spiced food samples.

2. Investigations and results

2.1. Method development

Fourteen seasonal bakery products with cinnamon (eleven gingerbreads, two cinnamon stars and one almond biscuit) were purchased from local supermarkets. For the QuEChERS procedure a mixture of acetonitrile/water (1:1) was chosen for the extraction step. This mixture was successfully used in many QuEChERS approaches, including the original work (Anastassiades et al. 2003; Posyniak et al. 2005; Fagerquist et al. 2005), and it also resembles closely the well-established methanol/water or ethanol/water mixtures used for coumarin extraction in previous work (De Jager et al. 2007; Sproll et al. 2008; Wang et al. 2013; Ballin et al. 2014). 3’,4’-(Methylenedioxy)acetophenone (Fig. 1) was chosen as internal standard, because it was already used successfully for the determination of coumarin in the past (De Jager et al. 2007; Stanfill et al. 1999; Lisko et al. 2015).

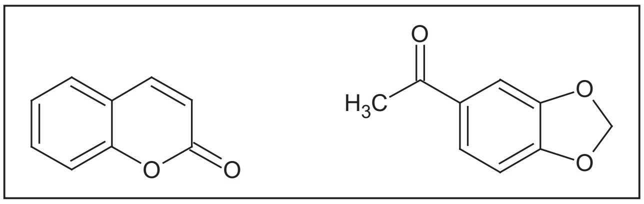

Fig. 1: Structures of coumarin (left) and internal standard 3‘,4‘-(methylenedioxy)acetophenone (right).

2.2. Method validation

The analytical method was validated by evaluating linearity, precision, and accuracy. The upper level of calibration was set at 50 µg/mL, which is equivalent to a sample containing the highest allowed concentration of coumarin in seasonal food (50 mg/kg), and the lowest level was set at the LOQ to allow for a maximum of sensitivity. Standard solutions of coumarin in acetonitrile were prepared for each calibration level (0 µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, and 50 µg/mL). Internal standard (3’,4’-(methylenedioxy)acetophenone) was added to a concentration of 10 µg/mL and three samples of each concentration were analyzed. Linear correlation between concentration and response of the detector was confirmed after visual inspection of the linear regression line and calculation of the correlation coefficient ($R^2 = 0.9993$). The limit of detection (LOD) and limit of quantification (LOQ) was calculated according to the EURACHEM guideline. The standard deviation of the lowest calibration standard was multiplied by 3.3 to determine the LOD (0.15 µg/mL equals 0.15 mg/kg) and multiplied by 10 to gain the LOQ (0.50 µg/mL equals 0.50 mg/kg). The calculated LOQ was verified by injecting this concentration (0.50 µg/mL) six-fold. The resulting relative standard deviation (RSD) was 6.0 %.

Selectivity was achieved by using characteristic ions for the analyte (*m/z* 146) and the internal standard (*m/z* 149). The molecule peak *m/z* 146 (base peak) was chosen for coumarin and the molecule peak *m/z* 149 for 3’,4’-(methylenedioxy)acetophenone (base peak). When injecting only the analyte, no peak was detected at *m/z* 149. *Vice versa*, when injecting only the internal standard, no peak could be observed at *m/z* 146.

Precision was determined by calculating RSD of calibration standards at 10 µg/mL, 30 µg/mL, and at 50 µg/mL (n = 6). The mean RSD was 5.3% (ranging from 5.1 - 6.8%).

Matrix effects were determined by comparing the signal of coumarin in acetonitrile in comparison to spiked blank matrix (each 10 µg/mL, n = 3). The peak areas were almost identical (2.8 % deviation), which indicates that the matrix has no relevant effect on the quantification. A recovery experiment was used to determine the loss of analyte during the sample preparation. For that a standard solution was compared to real blank sample matrix, both spiked with coumarin to a concentration of 10 µg/mL relating to the acetonitrile volume. This time the spiked matrix sample was

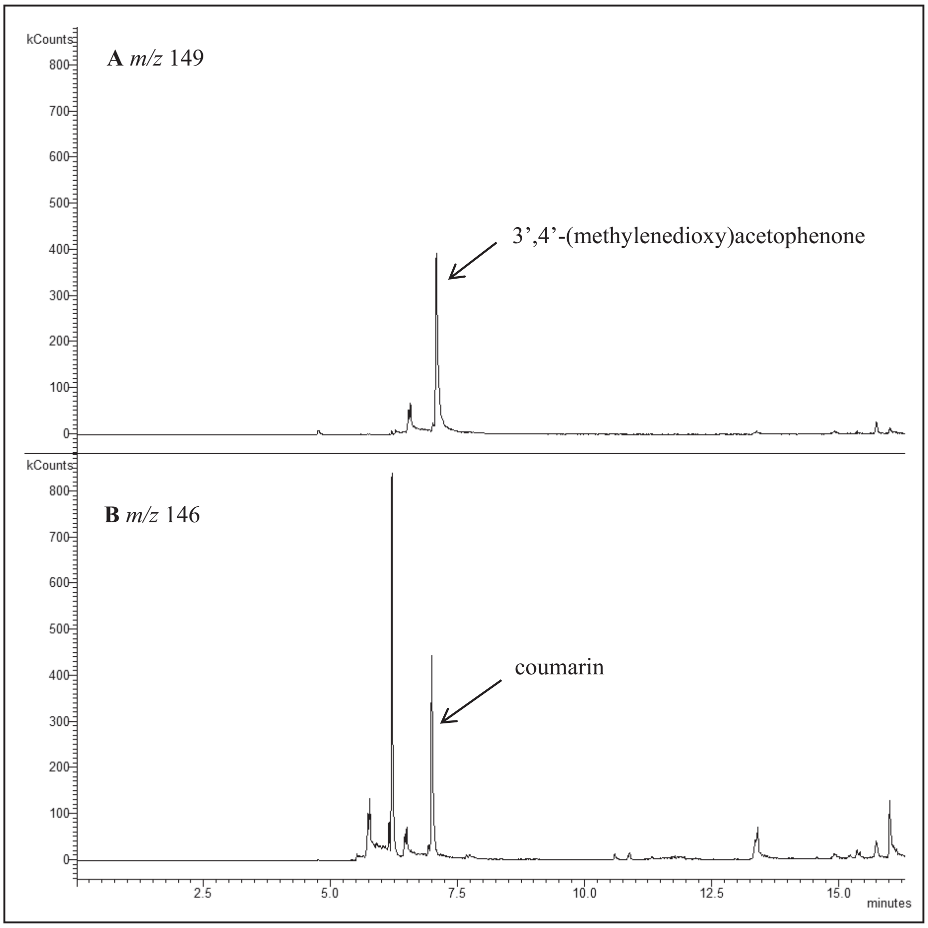

Fig. 2: Selected ion chromatograms of bakery product **3**; **A** internal standard 3’,4’-(methylenedioxy)acetophenone (10 µg/mL, *m/z* 149) at 6.99 min, **B** analyte coumarin (*m/z* 146) at 7.08 min.

Table 1: Overview of analyzed confectionaries sorted by price range

#	Confectionary	Weight per piece [g]	Price per kg[a]	Content of coumarin [mg/kg ± SD] (n = 3)	Content of coumarin [mg/piece ± SD] (n = 3)	Max. pieces 9 kg child[b]	Max. pieces 70 kg adult[b]
1	Gingerbread	28.6	€	n.d.	n.d.	-	-
2	Gingerbread	28.6	€	7.78 ± 0.46	0.22 ± 0.02	4.0	26.9
3	Gingerbread	50.0	€	20.5 ± 3.13	1.02± 0.20	0.9	5.9
4	Gingerbread	50.0	€	17.9 ± 1.23	0.89 ± 0.07	1.0	6.7
5	Gingerbread	28.6	€	n.d.	n.d.	-	-
6	Almond biscuit	20.0	€	1.45 ± 0.09	0.03 ± 0.002	32.1	214
7	Gingerbread	28.6	€€	10.4 ± 0.40	0.30 ± 0.01	3.0	20.2
8	Gingerbread	60.0	€€	26.6 ± 1.00	1.60 ± 0.07	0.6	3.8
9	Cinnamon stars	5.8	€€	n.d.	n.d.	-	-
10	Gingerbread	33.3	€€€	n.d.	n.d.	-	-
11	Gingerbread	55.0	€€€	27.3 ± 0.73	1.50 ± 0.05	0.6	4.0
12	Gingerbread	68.8	€€€	23.9 ± 0.46	1.64 ± 0.03	0.5	3.7
13	Cinnamon stars	5.6	€€€	39.4 ± 1.02	0.22 ± 0.01	4.1	27.4
14	Gingerbread	50.0	€€€€	n.d.	n.d.	-	-

[a] € 0-10 Euro per kg; €€ 10-20 Euro per kg; €€€ 20-30 Euro per kg; €€€€ from 30 Euro per kg; [b] calculated with TDI (tolerable daily intake) of 0.1 mg coumarin/kg bodyweight according to EFSA (European Food Safety Authority); n.d. not detected

processed as described prior to analysis and results were quantified. The recovery of coumarin was calculated as 92.7 % (n = 3).

2.3. Results

We found a mean coumarin content of 19.5 µg/kg in 9 out of the 14 seasonal food products, with values ranging from 1.45 (sample **6**) to 39.4 mg/kg (sample **13**). No coumarin was detected in five food products, although cinnamon addition was declared on the packing (Table 1). A sample chromatogram is given in Fig. 2.

3. Discussion

We successfully implemented the QuEChERS approach to develop a method of determining coumarin in seasonal bakery food products. The method has been shown to be precise, accurate and suitable for the intended concentration range for this complex matrix.

Coumarin concentrations up to 39.4 mg/kg (sample **13**) were measured, and in five of the 14 bakery products no coumarin was detected (samples **1**, **5**, **9**, **10**, **14**). The most plausible explanation for these differences is that two types of cinnamon are on the market and are legally specified as "cinnamon" in the list of ingredients. Ceylon cinnamon (from *Cinnamomum verum*) contains only traces of coumarin, whereas the cheaper Cassia cinnamon (from *Cinnamomum cassia*) contains 2650 mg/kg – 7017 mg/kg coumarin (Blahová et al. 2012). Alternatively, the amount of Cassia cinnamon spice used in the particular recipe was low enough for coumarin to fall under the limit of detection (0.15 mg/kg).

Samples **1**, **5**, and **10** were sold under different labels, but were produced at the same production site. All three of them had no detectable coumarin, indicating that the same recipe or at least the same source material, i.e. Ceylon cinnamon, was used. Two other products (samples **8**, **12**) that were produced in another production site, also had similar coumarin contents (23.9 and 26.6 mg/kg). These coincidences are plausible since manufacturers often produce the same bakery product for different retailers, and quite often these products are sold at different prices.

In summary, none of the 14 seasonal bakery products surpassed the allowed limit of 50 mg/kg, but detected coumarin contents still remain high in a couple of products (up to 39.4 mg/kg). Since the daily input limit is 0.1 mg/kg bodyweight (EFSA 2008), one can reach this limit quite fast. When consuming product **12** an average adult (70 kg) would reach the daily intake limit by consumption of about three and a half pieces, for a child it is one half piece of this product. While a temporary overdose up to three times the TDI over a period of 1-2 weeks is still considered safe (EFSA 2008), a moderate consumption of seasonal bakery products containing cinnamon as a flavoring is nevertheless recommended.

4. Experimental

4.1. Reagents

Coumarin (≥ 99 %) and 3',4'-(methylenedioxy)acetophenone (98 %) were purchased from Sigma Aldrich (Schnelldorf, Germany). Magnesium sulfate (*p. a.*), sodium chloride (*p. a.*), and acetonitrile (HPLC grade) were purchased from VWR (Darmstadt, Germany). PSA sorbent material was obtained from Agilent Technologies (Waldbronn, Germany). Water was taken from the institute's own deionizing apparatus and distilled before use.

4.2. Sample preparation

Each product was blended for 60 s with a food processor and 10 g was weighed in 50 mL falcon tubes (n = 3). After the addition of 10 mL water, 10 mL acetonitrile, and internal standard (to a final concentration of 10 µg/mL in acetonitrile) the mixture was shaken vigorously for 1 min. Next, 5 g of a magnesium sulfate/sodium chloride mixture (4:1) were added to initiate the phase partitioning. After centrifugation (2,500 g, 5 min at room temperature) 1.5 mL of the organic phase was transferred to a 2 mL microcentrifuge tube containing 25 mg of a magnesium sulfate/PSA mixture (5:1). The resulting suspension was shaken manually for 1 min and then centrifuged (10,000 g, 5 min). Then 1 mL of the supernatant was transferred into a autosampler vial and injected into the GC-MS. Signals were quantified using a calibration curve calculated from two sets of calibration standards (0, 10, 20, 30, 40, 50 µg/mL) that were run before and after the sample batch. Each product was analyzed in triplicate and results were stated in mg/kg.

4.3. GC-MS Method

Coumarin and the internal standard 3',4'-(methylenedioxy)acetophenone were analyzed with a Varian 3800 gas chromatograph coupled with a Saturn 2200 ion trap from Varian (Darmstadt, Germany). Injection mode was "splitless" (injection volume: 1 µL) at a temperature of 270 °C and a flow rate of 1.4 mL/min carrier gas helium 5.0. For separation an Agilent VF-5ms capillary column 30 m × 0.25 mm × 0.25 µm + 10 m EZ-Guard column (Waldbronn, Germany) was used with the following oven program: starting at 50 °C for 2 min, then ramping up to 150 °C at 25 °C/min, ramping up to 240 °C at 10 °C/min and finally up to 310 °C at 50 °C/min where the final temperature was held for 1 min. The total run time was 16.1 min. The temperatures were 200 °C for the ion trap, 50 °C for the manifold, and 270 °C for the transfer line. Data was collected in scan mode (*m/z* 50 –250) and following selected ions for peak integration following selected ions were used: *m/z* 146 for coumarin, *m/z* 149 for 3',4'-(methylenedioxy)acetophenone.

Conflicts of interest: Non declared.

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