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Analytical Methods

HPLC analysis and safety assessment of coumarin in foods

Constanze Sproll, Winfried Ruge, Claudia Andlauer, Rolf Godelmann, Dirk W. Lachenmeier *

Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weißenburger Str. 3, D-76187 Karlsruhe, Germany

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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, and a maximum level of 10 mg/l for alcoholic beverages. An efficient method for routine analysis of coumarin is liquid chromatography with diode array detection. The optimal sample preparation for foods containing cinnamon was investigated and found to be cold extraction of 15 g sample with 50 mL of methanol (80%, v/v) for 30 min using magnetic stirring.

In the foods under investigation, appreciable amounts of coumarin were found in bakery products and breakfast cereals (mean 9 mg/kg) with the highest concentrations up to 88 mg/kg in certain cookies flavoured with cinnamon. Other foods such as liqueurs, vodka, mulled wine, and milk products did not have coumarin concentrations above the maximum level.

The safety assessment of coumarin containing foods, in the context of governmental food controls, is complicated as a toxicological basis for the maximum limits appears to be missing. The limits were derived at a time when a genotoxic mechanism was assumed. However, this has since been disproven in more recent studies. Our exposure data on coumarin in bakery products show that there is still a need for a continued regulation of coumarin in foods. A toxicological re-evaluation of coumarin with the aim to derive scientifically founded maximum limits should be conducted with priority.

Keywords: Cassia; Cinnamon; Coumarin; Flavourings; High-performance liquid chromatography (HPLC)

1. Introduction

Coumarin is a natural substance occurring in the essential oils of a number of plants used as flavouring ingredients in foods. The occurrence of coumarin was reported in the following plant materials: *Anthoxanthum odoratum* (sweet vernal grass), *Asperula odorata* (sweet woodruff), *Cinnamomum aromaticum* (cassia bark), *Dipterix odorata* (tonka bean), *Eupatorium triplinerve* (white snakeroot), *Hierochloe odorata* (holy grass), *Melilotus coerulea* (sweet trefoil), *M. officinalis* (common melilot), *Melittis melissophyllum* (bastard balm), *Primula elatior* (oxlip) and *Trilisa odoratissima* (deer tongue) (MAFF, 1995). Coumarin's aroma has been described as sweet, aromatic, a creamy vanilla bean odour with nut-like tones that are heavy, but not sharp or brilliant (Clark, 1995). 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 maywines (second-grade white wine flavoured with woodruff) (Clark, 1995). After that, the use of coumarin as a food flavouring was discontinued based on reports of hepatotoxicity prior to the existence of any carcinogenicity and mutagenicity data (Lake, 1999). According to Lake's estimation, the main source of coumarin in the diet is cinnamon (Lake, 1999). A major source in alcoholic beverages is *H. odorata,* which is used to flavour a special kind of vodka, the so-called subrowka produced mainly in eastern Europe (Nykänen, 1984).

Coumarin was first suspected to have genotoxic and carcinogenic effects in the 1980s (AFC, 2004). On this basis,

* Corresponding author. Tel.: +49 721 926 5434; fax: +49 721 926 5539.
E-mail address: Lachenmeier@web.de (D.W. Lachenmeier).

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the Codex alimentarius provided general requirements for natural flavourings that included specific maximum levels for coumarin in the final product ready for consumption (Codex alimentarius, 1985). For foods and beverages in general, the maximum level is 2 mg/kg, with the exception of special caramels and alcoholic beverages for which the maximum level is 10 mg/kg. It must be noted that coumarin must not be added as such to food and beverages. It may only be contributed through the use of natural flavourings provided that the maximum levels in the final product ready for consumption are not exceeded. The Codex alimentarius maximum levels were subsequently introduced into European law in 1988 (European Council, 1988).

More recent evidence has suggested that coumarin is not a genotoxic agent (Lake, 1999). The International Agency for Research on Cancer (IARC) has classified coumarin as belonging to group 3 (''not classifiable as to its carcinogenicity in humans''). No epidemiological data relevant to the carcinogenicity of coumarin were available and there was only limited evidence in experimental animals for the carcinogenicity of coumarin (IARC, 2000).

Based on the non-observed-adverse-effect level (NOAEL) for hepatotoxicity in animal experiments, the Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food (AFC) established a Tolerable Daily Intake (TDI) of 0.1 mg/kg bw (AFC, 2004). The German Federal Institute for Risk Assessment (BfR) derived the same numerical value for a TDI under consideration of human data available from the pharmaceutical use of coumarin (Abraham, 2007).

Human exposure to coumarin from the diet has been calculated to be around 0.02 mg/kg/day (AFC, 2004; Lake, 1999). The theoretical maximum daily intake (TAMDI) of coumarin via food was estimated to be 4.085 mg/day (0.07 mg/kg bw/day) (AFC, 2004).

The analysis of coumarin was reviewed by Bogan et al. (1997). 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 (Adam & Postel, 1992; Archer, 1988; Bettero & Benassi, 1983; Bourgaud, Poutaraud, & Guckert, 1994; de Jager, Perfetti, & Diachenko, 2007; Ehlers, Pfister, Bork, & Toffel-Nadolny, 1995; Ehlers, Platte, Bork, Gerard, & Quirin, 1997; He et al., 2005; Huang & Sheu, 2000; Jürgens, 1981; Martino, Ramaiola, Urbano, Bracco, & Collina, 2006; Sagara et al., 1987; Thompson & Hoffmann, 1988; vande Casteele, Geiger, & van Sumere, 1983; Villeneuve, Abravanel, Moutounet, & Alibert, 1982; Walters, Lake, & Cottrell, 1980). For the analysis of coumarin in alcoholic beverages an official HPLC method is available from the International Organization of the Flavour Industry (Grundschober, 1997). For the general application to flavoured foods including bakery products an isotope dilution liquid chromatography/tandem mass spectrometry (LC–MS/MS) method was recently proposed by Raters and Matissek (2007), which was shown to be very sensitive and selective but requires relatively expensive instrumentation.

The aim of this study was to develop a more simple, rapid and economic HPLC method with diode array detection for the analysis of coumarin that would be suitable for all kinds of coumarin containing foods. A survey of a large number of samples was conducted to provide current data for exposure estimation and risk assessment of coumarin in food.

2. Materials and Methods

2.1. Samples

A total of 120 samples submitted to the CVUA Karlsruhe were analyzed for coumarin. The samplings were conducted by local authorities between September 2006 and January 2007, either directly from the manufacturers and importers or from the retail trade.

2.2. Reagents and materials

Coumarin (>99%), methanol (HPLC grade), ethanol (HPLC grade), acetonitrile (HPLC grade), chloroform, ammonium acetate, and water (HPLC grade) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Disposable syringe filters with a pore width of 0.2 μm (Chromafil PET-20/25) were from Macherey–Nagel (Düren, Germany). For the tempering of the sample the heating circulator bath DC10-W26 (Haake, Karlsruhe, Germany) was used.

2.3. High-performance liquid chromatography

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 × 2 mm i.d., 4 μm, Synergi polar RP) column thermostatted at 40 °C using 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 with a flow of 0.2 mL/min: 0–2 min: 70% A; 2–20 min: 70–20% A; 20–22 min: 20–70% A; 22–30 min: 70% A. 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.

2.4. Sample preparation

For solid foods, a 15 g portion of the sample was placed into a 50 mL wide-necked volumetric flask. The flask was filled with extraction solvent (methanol, 80% (v/v)) just below the calibration mark, a magnetic stir bar was added,

and the flask was immediately sealed with a plug. The flask was agitated for 30 min using a magnetic stirrer at room temperature. After removing the magnetic stir bar, the flask was temperated at 20 °C and filled up to the calibration mark with extraction solvent. In case of very high coumarin contents (e.g. in cinnamon flavourings), a lower sample weight was chosen (1 g) and the sample extract was diluted with methanol to reach the linear range of the HPLC method (approx. 1:10). For liquid foods (i.e. alcoholic beverages) no sample preparation was necessary. The beverages can be directly injected into the HPLC system without dilution. Products with a high water content (e.g. milk products, yoghurt) were diluted with pure methanol to gain a methanol–water mixture in the range of the optimized setting of 80% and a portion of only 10 g was used. All samples were filtered through disposable syringe filters before 5 μL of the extract was injected into the HPLC system.

2.5. Optimization studies

In a preliminary test, the extraction of coumarin with methanol (80%), ethanol (80%), acetonitrile and chloroform was studied. A sample of cinnamon star cookies was homogenized and extracted using the four solvents for 10 min under stirring at room temperature ($n = 4$). To systematically study the extraction, statistically designed experiments were used. A first experiment was used to find the significant influences with a minimum amount of experiments. This was done with a D-optimal screening design (Box, Hunter, & Hunter, 2005; Montgomery, 2005). The D-optimal algorithm was used because it chooses an ideal subset of all possible combinations and significantly reduces the number of required experiments compared to standard design types. In a second experiment, the two variables with a significant influence on the extraction were studied using response surface methodology with a central composite design. The experiments, parameters and chosen ranges of variables are shown in Table 1. To additionally study the effect of agitation, 46 samples of different bakery products were analyzed using magnetic stirring as well as ultrasonication.

2.6. Validation studies

To validate the method, commercial samples were extracted and analyzed for several times using the optimized procedure given above. To determine accuracy, samples were spiked with different coumarin concentrations. The calibration curve linearity was evaluated between 0.5 and 40 μg/mL. The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated from the regression line residual standard deviation using real matrices (DIN 32 645, 1994; Meier & Zünd, 2000).

2.7. Statistics

The experimental designs and calculations were done using the Software Package Design Expert V6 (Stat-Ease Inc., Minneapolis, Minnesota, USA). The experiments were evaluated using Analysis of Variance (ANOVA) to find the variables' significance and their interactions in the models. The models were checked for consistency by looking at the lack of fit and possible outliers.

3. Results and discussion

3.1. Optimisation of coumarin extraction

The only systematic study of the extraction of coumarin from plant material was conducted by Bourgaud et al. (1994). Ethyl acetate, diethyl ether and chloroform all extracted coumarin poorly. Extraction with polar solvents (water, ethanol, methanol) was shown to be the most efficient. Indeed, Soxhlet extraction with boiling methanol for 48 h gave results not significantly different from extraction with methanol at room temperature under stirring for 30 min.

In a preliminary test, we compared the extraction of coumarin with methanol (80%), ethanol (80%), acetonitrile and chloroform from bakery products (Fig. 1). In general, Bourgaud's results for the extraction of coumarin can be confirmed for foodstuffs. The best solvent appears to be methanol, which was used in the following, more detailed optimisation experiments.

First, a screening experiment was conducted to check the influence of agitation type (ultrasonication or stirring), solvent concentration (methanol, 60%, 80%, 100%), sample weight (5 g, 10 g) and extraction time (10 min, 30 min). The model was significant ($p < 0.0001$, $R = 0.87$). The agitation type had a significant influence ($p = 0.0091$): the extraction yield was significantly higher using magnetic stirring than

Table 1
Variables and ranges used in the experimental designs for extraction optimization

Design type	Number of experiments	Variables	Name	Levels/range
Factorial screening experiment				
D-Optimal, 2-factor interaction	25	*A*	Agitation	Ultrasonication, magnetic stirring
		B	Methanol concentration (%)	60, 80, 100
		C	Sample weight (g)	5, 10
		D	Extraction time (min)	10, 30
Response surface experiment				
Central composite, quadratic	21	*A*	Methanol concentration (%)	50–100
		B	Sample weight (g)	5–15

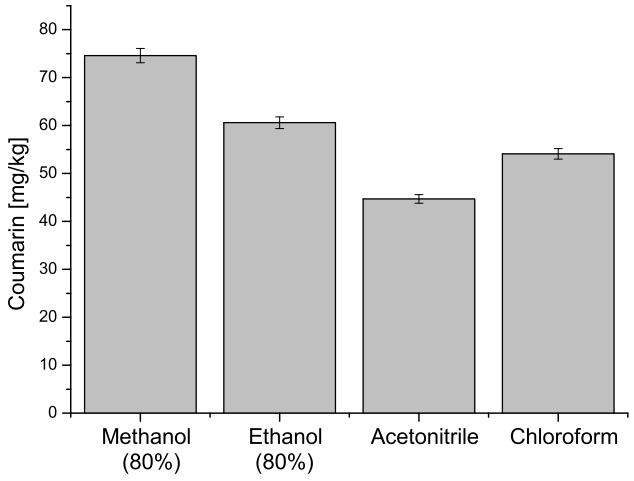

Fig. 1. Comparison between different solvents for extraction of coumarin from a bakery product matrix.

with ultrasonication. This result was verified by a series of 46 samples analysed using both agitation methods. On average, the yield with magnetic stirring was 3% higher than with ultrasonication ($p < 0.0001$). Magnetic stirring was, therefore, used in all further experiments.

The extraction time in the time segment of 10–30 min had no significant influence on the extraction yield ($p = 0.8185$). Methanol concentration ($p < 0.0001$) and sample weight ($p < 0.0001$) showed the largest and highly significant influences on the extraction of coumarin. Therefore, these two parameters were studied in more detail in the second response surface design using the central composite algorithm.

The second experiment also had statistical significance ($p < 0.0001$, $R = 0.81$). The results show that the methanol concentration ($p = 0.0137$) and the sample weight ($p < 0.0001$) both have a significant influence on the extraction. Furthermore, there appears to be a significant interaction between both parameters ($p = 0.0209$) and a quadratic influence of the solvent concentration ($p = 0.0015$). The response surface contour plot is shown in Fig. 2. An optimal range with above 90% extraction yield is around 80% of methanol and above 10 g of sample weight.

By default, we used the following optimised setting for the extraction of coumarin in our survey of foods: methanol 80%, 15 g sample weight, magnetic stirring, and 10 min extraction time.

The present study shows that coumarin content can be determined easily by this direct extraction with 80% methanol with no sample pretreatment (besides homogenization of the foods). The optimised extraction method provides extracts with low matrix interferences and does not form emulsions even if used for fat-containing foodstuffs. Subsequent measurement with HPLC requires no further sample clean-up at all. In spite of this, no problem with HPLC column degradation was detected. One and the same column was used for all optimization experiments and the food survey described in this paper. Even in very large samples series (i.e. 70 samples measured over 2 days), the calibrations showed to be very stable ($R = 0.9999$ between calibrations at the beginning and end of the series).

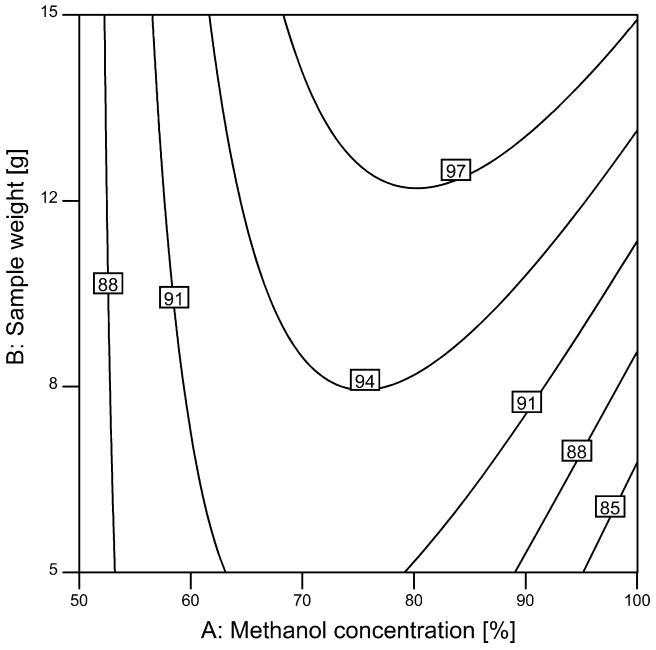

Fig. 2. Contour plot showing the optimization results for the extraction of coumarin using a central composite design (contours show extraction yield in %, conditions are detailed in Table 1).

The chosen conditions for sample preparation and chromatographic separation are very robust, and the experimental design shows that slight deviations (e.g., of extraction time) have no influence on the accuracy of the results.

The validation results (Table 2) demonstrate satisfactory precisions in ranges between 0.60% and 3.25%, and accura-

Table 2
Method validation results for the determination of coumarin in different cinnamon flavoured products

Product	Coumarin level (mg/kg)	Precision (RSD) (%) ($n = 5$)	Accuracy (mean bias) (%) ($n = 5$)
Mulled wine #1	4	0.96	4.31
Mulled wine #2	10	1.00	−1.01
Mulled wine #3	20	1.70	0.20
Mulled wine #4	40	1.42	1.09
Yoghurt #1	2	2.66	−10.01
Yoghurt #2	4	1.10	0.70
Yoghurt #3	4	1.11	0.70
Drinking yoghurt	4	1.43	0.50
Quark cheese #1	4	0.60	−0.10
Quark cheese #2	4	0.65	−0.04
Rice Pudding #1	4	0.88	0.02
Rice Pudding #2	4	0.58	2.25
Cinnamon Star Cookies #1	93	2.40	Not determined
Cinnamon Star Cookies #2	51	1.95	Not determined
Cinnamon Star Cookies #3	88	3.25	Not determined

cies between 0.02% and 10.01%, independent from the tested food groups. The validation data also prove that the conditions optimised for the extraction of bakery products are transferable to other matrices such as milk products. Coumarin exhibited an excellent linearity in the range between 0.1 mg/l and 40 mg/l with a regression coefficient greater than 0.9999. The limits of detection and quantitation were 0.1 mg/l and 0.3 mg/l, respectively.

The validation results indicate that the developed HPLC–DAD procedure is equally suitable as the LC–MS/MS procedure of Raters and Matissek (2007) to determine coumarin in foods in the interesting range around the limit of 2 mg/kg. The advantages of HPLC–DAD over LC–MS/MS are its lower cost for instrumentation and operation. The optimized sample preparation is also simpler than that of Raters and Matissek (2007) as it requires no Carrez clarification and there is no need for standardization using isotope dilution. A recent interlaboratory trial has shown that our HPLC–DAD procedure with external standardization excellently performs against LC–MS/MS with deuterium labelled coumarin as internal standard. The optimized sample extraction using 80% of methanol is currently considered for inclusion in the draft of an official German standard method for the determination of coumarin in foods.

3.2. Coumarin levels in flavourings

The analysis results of coumarin in different types of *Cinnamomum* species are shown in Table 3. According to German regulations the botanical species is mostly not labelled on food packages. The German word "Zimt" includes cinnamon and cassia. Cinnamon is recognised as the dried inner bark of cultivated varieties of *C. zeylanicum* Blume principally from Sri Lanka, but also from India, Madagascar and the Seychelles. The most common form, however, is cassia or cassia cinnamon. Cassia is derived from different sources. Chinese cassia (*C. aromaticum* Nees syn. *C. cassia* Nees ex. Blume), is grown commercially in China and Vietnam. Indonesian cassia, also called Padang cassia, is mainly exported to the USA (ISO, 1997b; Ravindran, Nirmal Babu, & Shylaia, 2004). Vietnam cassia or Saigon cassia was in earlier literature identified as *C. loureirii* Nees, but according to Ravindran et al. (2004) Vietnam cassia should be nothing else than Chinese cassia. Separate ISO specifications exist for cassia (ISO, 1997a) and cinnamon (ISO, 1997b). Cinnamon of the Sri Lankan Type has a characteristically different flavour to cassia. Our results confirm that cassia contains significantly higher levels of coumarin than cinnamon. The main source of the ground products labelled as "Zimt" appears to be cassia, not only because of the high contents of coumarin but also because of its typical and strong flavour and its lightly sweet taste characteristics (Jayatilaka, Poole, Poole, & Chichila, 1995). Cassia is also the variety that is predominantly found in retail trade as well as in pastry shops (Weber, 2007).

Flavouring a food with 0.1% (w/w) of cassia containing 3000 mg/kg of coumarin would lead to a concentration above the limit of 2 mg/kg in the food. As there are no maximum levels for coumarin in flavourings, even the products with unusually high coumarin concentrations up to 8790 mg/kg cannot be rejected by the food safety authorities. Therefore, because of the different flavouring characteristics, including the differences in coumarin content, a change in food policy that demands the different species, cinnamon and cassia, be labelled with their specific names appears to be required.

3.3. Coumarin levels in foods

In the foods under investigation, appreciable amounts of coumarin were only found in cereals and bakery products (Table 3). The highest coumarin contents were found in cinnamon star cookies, which are one of Germany's most popular Christmas cookies. In fact, 85% of all cookie samples were above the Codex alimentarius maximum level of 2 mg/kg; the mean concentration was 25 mg/kg and a max-

Table 3
Coumarin content in different flavours and flavoured foods

Product group	No. of samples	Coumarin-positive (%)	Samples above maximum level (%)	Coumarin (mg/kg)		
				Mean	Range	Median
True cinnamon (Sri Lanka/Ceylon)	5	0	–[a]	nd	–	–
Cassia Cinnamon	5	100	–[a]	3612	2880–4820	3330
Cinnamon (category not labelled)	20	85	–[a]	2419	nd-8790	2895
Cinnamon star cookies	47	85	85	25	nd-88	22
Other bakery products and breakfast cereals	13	46	46	9	nd-32	-
Cinnamon-flavoured Liqueur	2	0	0	nd	–	–
Vodka flavoured with sweet grass	7	71	0	4	nd-8	5
Mulled wine	16	0	0	nd	–	–
Milk products (yoghurt, quark cheese, rice pudding)	5	60	0	0.7	nd-2	0.5

[a] The Codex alimentarius maximum levels apply only to flavoured foods but not the flavourings themselves.

imum concentration of 88 mg/kg was detected. The majority of the cookies were obviously made with cassia. In other bakery foods including breakfast cereals, the coumarin incidence was lower with 46% above the 2 mg/kg level and a lower mean concentration of 9 mg/kg.

Cinnamon or cassia flavoured liqueurs and mulled wines did not contain coumarin levels above the detection limit and in other alcoholic beverages, coumarin was only found in flavoured vodkas. However, none of the vodka samples had concentrations above the limit of 10 mg/kg. This may derive from the fact that coumarin levels in alcoholic beverages have been under scrutiny for a long time since Bandion determined coumarin levels higher than 10 mg/kg in two Polish vodkas in 1974 (Bandion, 1974).

A relatively low incidence of coumarin was also found in cinnamon or cassia flavoured milk products, none of which had a concentration above the 2 mg/kg level.

3.4. Exposure estimation and risk analysis of coumarin in foods

Generally, foods with flavouring concentrations above the maximum levels are not marketable. However, the findings of relatively high coumarin levels in traditional bakery products led to the question of whether a re-evaluation of the maximum levels is necessary.

The Codex alimentarius maximum levels were derived in a time when the carcinogenic mechanism of coumarin was unknown. Therefore, a genotoxic mechanism was assumed, so that even the lowest amounts of coumarin would have been able to cause tumours. Since then, an extensive body of research has focused on understanding the aetiology of tumours derived from coumarin. The data support the conclusion that coumarin is not DNA-reactive (Felter, Vassallo, Carlton, & Daston, 2006). Moreover, there appears to be a non-linear dose-response relationship for coumarin-induced toxicity and carcinogenicity, with tumour formation being observed only at high doses which are also associated with hepatic and pulmonary toxicity (Lake, 1999).

As a scientific foundation for the Codex alimentarius maximum levels appears to be missing, the European Union (EU) is discussing to completely delete the regulations about coumarin in the current proposal for a revision of the regulation on flavourings and certain food ingredients with flavouring properties for use in and on foods (European Parliament, 2006).

To date, the most useful guideline for the evaluation of coumarin in foods appears to be the previously mentioned TDI value of 0.1 mg/kg bw.

Table 4 shows the amounts of different foods required for children or adults to reach this TDI value. As can be seen, the amounts of milk products required are much higher than the usual daily intake of such products. The same is true for coumarin containing vodka, which would be toxic due to ethanol before even nearing the TDI of coumarin. For example, a 60 kg adult would need to consume a whole bottle (750 ml) of the sample with the highest concentration (8 mg/kg) to exceed the TDI. If at all, this is only likely for persons with alcohol dependence. Interactions with the liver toxic and carcinogenic effects of ethanol should also be considered in the evaluation of alcoholic beverages (Lachenmeier, 2007). More problematic than vodkas or other legal alcoholic beverages may be so-called surrogate alcohols (Lachenmeier, Rehm, & Gmel, 2007). There are indications that cosmetic products likely to be consumed as surrogate alcohol (e.g. aftershaves) may contain higher levels of coumarin, because there is no limit for coumarin in cosmetics (Abraham, 2007).

Table 4
Estimation of food amounts required to exhaust the TDI of coumarin

Product group	Amount of food required to reach the TDI of 0.1 mg/kg b.w. (worst-case scenario calculated with the maximum concentration determined for each food group as given in Table 3)	
	15-kg child	60-kg adult
Cinnamon star cookies (g)	17	68
Other bakery products and breakfast cereals (g)	47	188
Vodka flavoured with sweet grass (g)	–	750
Milk products (yoghurt, quark cheese, rice pudding) (g)	750	3000

The highest relevance appears to be the coumarin contents in bakery products. A child could reach the TDI by consuming approximately 3–4 cinnamon star cookies (typical weight of cookie: 5 g), whilst an adult would need approx. 10 cookies. The TDI could also be reached by consuming a 75-g portion of breakfast cereals for a child or three portions for an adult. However, these calculations were made using the maximum concentrations found in our study.

To conclude, the present study shows that TDI values could possibly be reached simply by consuming staple foods such as bakery products and breakfast cereals. This clearly shows there is a need to regulate coumarin. Furthermore, from a public health standpoint, toxicological re-evaluation of coumarin by the food safety authorities is recommended with the aim to derive scientifically founded maximum limits. We completely concur with the position of the BfR (Abraham, 2007) that there should be a continued regulation of coumarin in foods, and that the EU should not completely abolish their coumarin limit.

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