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HPLC method for the determination of coumarin and quassine in foods and beverages

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1. SAFETY

This method involves the use of several hazardous chemicals and procedures likely to produce an increased risk to the operator. A COSHH assessment should be written to identify the risks associated with this method.

1.1. General precautions

1.1.1. Protective clothing including laboratory coat (buttoned), safety spectacles and gloves should be worn at all times.

1.1.2.1 Samples should be regarded as a biological hazard. Direct contact with skin is best avoided and proper attention to hygiene must be maintained.

1.2. COSHH

UNDER THE CONTROL OF SUBSTANCES HAZARDOUS TO HEALTH (COSHH) REGULATIONS THIS METHOD HAS BEEN ASSESSED FOR ANY RISK TO HEALTH TO OPERATORS THROUGH ITS USE. A FULL ASSESSMENT HAS BEEN COMPLETED AND IS KEPT ON FILE (x). THIS ASSESSMENT IS ACCESSIBLE TO STAFF AND BEFORE STARTING THIS METHOD ANYONE USING THIS PROCEDURE MUST HAVE A COPY OF THE COSHH ASSESSMENT.

Particular attention is drawn to the following toxic chemical. Contact by inhalation, skin absorption or ingestion must be avoided. Work in a fume cupboard if necessary.

Chemical X	Toxic, T+

1.3. First Aid

Any injury must be reported, in the first instance, to a qualified First Aider and recorded. The First Aider will decide on further action. All accidents, incidents and near misses should be reported to the Health and Safety team.

2. INTRODUCTION

Coumarin (IUPAC 2-chromenone, CAS No. 91-64-5, $C_9H_6O_2$ = 146.1; Figure 1),

Coumarin is a naturally occurring compound present in a wide variety of microorganisms and higher plants. Coumarin was first isolated from Tonka beans, and is found at high levels in some essential oils, particularly cassia leaf oil (up to 83,300 mg /kg) cinnamon leaf oil (40,600 mg/kg), cinnamon bark oil (7000 mg/kg) and in lavender oil and peppermint oil (20 mg/kg). Coumarin is also found in fruits (bilberry; 0.0005 mg/kg), green tea (1.2 – 1.7 mg/kg) and other foods, such as chicory . Many coumarin derivatives are also present in plants. The maximum limits for coumarin in food and non-alcoholic beverages are laid down in EC/1334/2008:

Compound food in which the presence of the substance is restricted Maximum level	**mg/kg**
Traditional and/or seasonal bakery ware containing a reference to cinnamon in the labelling	50
Breakfast cereals including muesli	20
Fine bakery ware, with the exception of traditional and/or seasonal bakery ware containing a reference to cinnamon in the labelling	15
Desserts	5

The theoretical maximum daily intake (TAMDI) of coumarin was calculated to be about 4.1 mg/day for a 60 kg person or 0.07 mg/kg/day.

Figure 1: Structure of coumarin

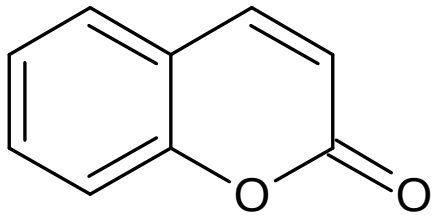
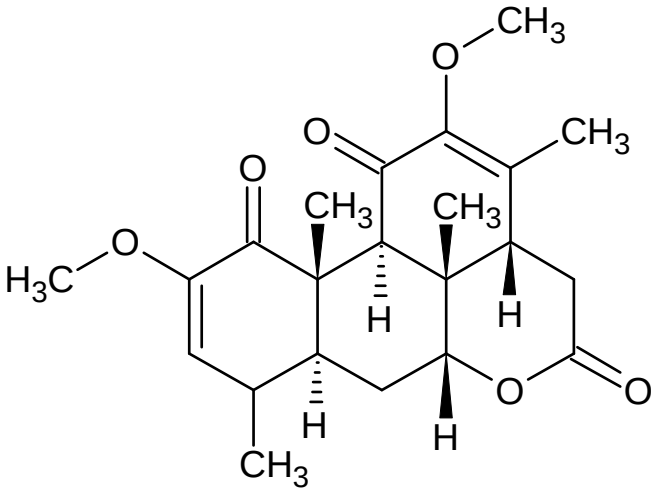


Figure 2: Structure of Quassine



3. SCOPE

This method describes the determination of coumarin and quassine involving a rapid clean-up with subsequent separation and detection using HPLC in raw and processed food and beverage products with an LOQ of 0.5 mg/kg or better.

4. PRINCIPLE

Coumarin and quassine are extracted from foods and beverages using water or a mixture of methanol and water. Prior to filtering, the extract may be cleaned up by means of dispersive solid phase extraction (SPE) if required, where the solvent is directly added to the extract and finally analysed by reverse-phase HPLC with photodiode-array (PDA) detection.

5. SAMPLING

Samples are homogenised and sub-sample taken and stored at an appropriate temperature.

6. EXTRACTION APPARTUS

6.1 Glassware and Disposable Plastic Ware

General laboratory glassware to be used except where stipulated. Unless otherwise stated volumetric glassware should be of grade 'A' quality. Laboratory equipment may be generic, e.g., balances and homogenization equipment.

6.1.1 Centrifuge tubes, glass or inert plastic, *ca.* 50 mL with cap
6.1.2 Glass vials, 40 mL with screw-cap

6.1.3 Glass vials, 12 mL with screw-cap
6.1.4 Glass vials, 4 mL with screw-cap
6.1.5 HPLC autosampler vials, 2mL with crimp cap

6.2 Equipment

6.2.1 Balance, 4-place (0.0001g) analytical
6.2.2 Balance, 2-place (0.01g) top-pan
6.2.3 Food mixer/blender, domestic or Waring type
6.2.4 Homogeniser, Ultra-Turrax with 5-8mm probe or equivalent
6.2.5 Sample shaker; AQS manufacturing Ltd., Cat. No. R100B, or equivalent
6.2.6 Ultrasonic bath
6.2.7 HPLC system capable of providing isocratic binary mobile phase and UV detection, preferably with photodiode array capability
6.2.8 HPLC column, Zorbax C_8 5 µm 250 x 4.6 mm, or equivalent
6.2.9 HPLC guard column, C_8 suitable for use with analytical column
6.2.10 Positive displacement pipettes e.g. Gilson Microman M25, M50, M250 and M1000 or equivalent
6.2.11 Solid phase extraction cartridges, C_{18} Varian Bond-Elut 500 mg/6mL or equivalent
6.2.12 SPE vacuum station (optional)

7. REAGENTS

7.1 Chemicals

7.1.1 Acetic acid, HPLC grade
7.1.2 Acetonitrile, HPLC grade
7.1.3 Methanol, HPLC grade
7.1.4 Water, HPLC grade
7.1.5 Coumarin
7.1.6 Quassine
7.1.7 Methyl umbelliferone

7.2 Solutions

7.2.1 Extraction solution, 90% methanol.
Add 450 mL methanol to 50 mL water and mix thoroughly.
7.2.2 Standards preparation solution, 80% methanol.
Add 400 mL methanol to 100 mL water and mix thoroughly.
7.2.3 SPE wash solution, 20% methanol.
Add 125 mL methanol to 375 mL water and mix thoroughly.
7.2.4 SPE elution solution, 60% methanol.
Add 300 mL methanol to 200 mL water and mix thoroughly.
7.2.5 HPLC mobile phase B: 0.5% Acetic acid in water.
From a 2.5 L HPLC grade water Winchester, remove 12.5 ml. Pipette 12.5 ml acetic acid into the Winchester bottle and mix thoroughly.

8. STANDARDS

8.1 Stock Standards

8.1.1 Coumarin ca. 1000 µg/mL.
Prepare a stock standard of coumarin at a concentration of *ca.* 1000 µg/mL by weighing out *ca.* 50 mg coumarin on a 4-place analytical balance and dissolve in 50 mL of 80% methanol in a volumetric flask. Calculate the actual concentration.

8.1.2 Quassine ca. 1000 µg/mL.
Prepare a stock standard of quassine at a concentration of *ca.* 1000 µg/mL by dilution of the Trifolio standard (ca 200 mg) in 80% methanol in a 100 mL volumetric flask. Calculate the actual concentration using the stated batch purity e.g. batch 5544 13.34% quassine and 41.2% neoquassine equivalent to 54.5% total purity, therefore multiply by a factor of 0.545 to obtain true concentration.

8.1.3 Coumarin and quassine mixed standard ca. 100 µg/mL.
Pipette 1 ml of coumarin 1000 µg/mL (8.1.1) and 1 ml of quassine ca. 1000 µg/mL (8.1.2) into a 10 ml volumetric flask. Make up to the mark with 80% methanol.

8.1.4 Methyl umbelliferone ca. 500 µg/mL.
Prepare a stock standard of methyl umbelliferone (MU) at a concentration of *ca.* 500 µg/mL by weighing out *ca.* 50 mg MU on a 4-place analytical balance and dissolve in 100 mL of 80% methanol in a volumetric flask. Calculate the actual concentration.

8.1.5 Methyl umbelliferone ca. 100 µg/mL.
Pipette 2 ml of methyl umbelliferone 500 µg/mL (8.1.4) into a 10 ml volumetric flask. Make up to the mark with 80% methanol.

8.2 Standard dilutions for HPLC

Prepare the following dilutions for HPLC calibration using 80% methanol:

Standard	Volume of coumarin 1000 µg/mL (µL) (8.1.1)	Volume of quassine 1000 µg/mL (µL) (8.1.2)	Volume of MU 100 µg/mL (µL) (8.1.5)	Dilution volume (mL)	Coumarin and quassine (µg/mL)	MU (µg/mL)
1	0	0	250	5	0	5
2	50 *a*	*a*	250	5	1	5
3	100 *a*	*a*	250	5	2	5
4	50	50	250	5	10	5
5	100	100	250	5	20	5
6	250	250	250	5	50	5
7	350	350	250	5	70	5
8	500	500	250	5	100	5

[a = Solution of 100 µg/mL coumarin and quassine mixed standard (8.1.3)]

9. PROCEDURE

9.1 Sample preparation

9.1.1 Spice powders, tea leaves and liquid samples are usually adequately homogenous and do not normally require further treatment other than thorough mixing. Samples can be stored at room temperature.

9.1.2 Dry and semi-dry goods such as breakfast cereals, biscuits, cakes and boiled/jelly sweets should be homogenised using a domestic food processor or Waring-type blender. Some boiled or jelly sweets may need to be dissolved in warm water before sampling. Where this is the case, samples should be quantitatively mixed at a ratio of one part sample to 3 parts water. Gentle heating of the water up to 60 ^{o}C can be used to assist dissolution. Weights and volumes should recorded in the analysts laboratory notebook. Samples can be stored at room temperature.

9.1.3 Non-dry goods such as dairy products should be homogenised using a domestic food processor / blender or Ultra-Turrax homogeniser. Samples can be stored chilled or frozen depending on when extraction is to take place.

9.2 Extraction

The following procedures are intended for samples with a coumarin and/or quassine content of 1mg/kg (or mg/L) and above. For samples with a suspected analyte(s) content below ca. 1 mg/kg (or mg/L) SPE may be used to concentrate the extract (see section 9.3).

9.2.1 *Foods (other than as stated in 9.2.4 and 9.2.6)*

Weigh out 2-5 g ± 0.05 g of sample depending upon expected coumarin content, using a 3 decimal place balance, into a 50 mL centrifuge tube. Typical weights are 5 g for biscuits and cakes and 2 g for samples containing high levels of cinnamon. Using a pipette, add 250 µL of 500 µg/mL methyl umbelliferone internal standard (8.1.4) to the sample.

9.2.2 Using a measuring cylinder add 20 mL of 90% methanol extraction solution (7.2.1) to the vial. Blend the mixture using the Turrax dispersion probe for 30 seconds and place on a shaker for 30 minutes. Centrifuge for 5 minutes at ca. 3000 rpm. Carefully transfer the supernatant to a 25 mL volumetric flask.

9.2.3 Add 5 mL of fresh 90% methanol extraction solution (7.2.1) to the sample vial, shake vigorously by hand for ca. 30 seconds and centrifuge for 5 minutes at ca. 3000 rpm. Carefully transfer the supernatant to the 25 mL volumetric flask and dilute to volume with 90% methanol extraction solution (7.2.1).

Note: *For samples containing significant amounts of fat, this may be removed by transferring the sample extract to a 40 mL screw cap vial and freezing at ca. –18℃ for approximately 30 mins. Centrifuge for 5 mi nutes at ca. 3000 rpm and take an aliquot of the supernatant to 9.2.8.*

9.2.4 *Spices, concentrates and flavourings*

Weigh out 0.1-1 g ± 0.01 g of sample depending upon expected coumarin content, using a 3 decimal place balance, into a 50 mL centrifuge tube. Typical weight is 0.1 g for cinnamon powder. Using a pipette, add 250 µL of 500 µg/mL methyl umbelliferone internal standard (8.1.4) to the sample. Continue with steps 9.2.2 and 9.2.3.

9.2.5 *Beverages*

Using a pipette, add 250 μL of 500 μg/mL methyl umbelliferone internal standard (8.1.4) to 5 mL of the sample contained in a 25 mL volumetric flask and dilute to volume with 90% methanol extraction solution (7.2.1).

9.2.7 *Spiked samples*
Spiked samples are prepared at the weighing out stage prior to solvent extraction. Add 50 μL of the 100 μg/mL mixed coumarin and quassine standard (8.1.3) per gram of sample, to a blank sample using a positive displacement pipette. At least one blank and spike of the appropriate sample must be extracted for each 10 unknown samples.

9.2.8 *All sample extracts*
Filter 1-2 mL of the extract through a PVDF syringe filter (0.2 μm) in to a 2mL crimp-top autosampler vial and analyse by HPLC (9.4).

9.3 SPE concentration / clean up (OPTIONAL)

If the sample is suspected of containing low levels of coumarin or quassine (i.e. < 1 mg/kg or mg/L), SPE concentration can be used (9.3.1). When co-extracted materials interfere significantly with coumarin or quassine determination by HPLC, SPE may be used to clean up the extract (9.3.7) or to concentrate the analytes (9.3.8).

9.3.1 Beverages
For aqueous samples such as beverages, add 50 μL of the 500 mg/L methyl umbelliferone standard (8.1.4) to a 25 mL volumetric flask and dilute to the mark with the sample.

9.3.2 Prepare an SPE cartridge by passing through 5 mL methanol followed by 5 mL water – do not allow the cartridge to dry out. Should the column run dry, repeat the preparation stages.

9.3.3 Load the sample from 9.3.1 onto the cartridge at a flow rate of ca 1-2 drops per second.

9.3.4 Wash the cartridge with 5mL water followed by 5mL 20% MeOH and allow the eluant to run to waste.

9.3.5 Elute the analytes with 5mL 60% methanol and collect in a 12mL vial. Allow the cartridge to run dry.

9.3.6 Filter 1-2 mL of the eluant through a PVDF syringe filter (0.2 μm) in to a 2mL crimp-top autosampler vial and analyse by HPLC (9.4).

9.3.7 Sample methanolic extracts
For SPE cleanup, dilute 5mL of the methanolic sample extract (9.2.1-9.2.7) with 20 mL of water, mix thoroughly and carry out steps 9.3.3-9.3.6.

9.3.8 For samples with suspected analyte levels below 1 mg/kg (or mg/L), dilute a greater aliquot volume with a 4-fold volume of water, mix thoroughly and carry out steps 9.3.3-9.3.6.

Note: *An adjustment to the calculation of results must be made for the increase in the mass of the internal standard when using this procedure (see10.1).*

9.4 HPLC analysis

The following HPLC parameters have been found to be suitable:

Injection volume: 20µL
Mobile phase composition: Initial 25:75 (A:B) 1 mL/min
Column: Zorbax C_8 5 µm C_{18} 250 x 4.6 mm
Gradient:

Time	A Acetonitrile (%)	B Water 0.5% acetic acid (%)
0	25	75
9	25	75
20	80	20
20.2	25	75
28	25	75

PDA conditions: Monitoring wavelength: 280 x 4 nm (coumarin/MU)
Monitoring wavelength 256 x 4 nm (quassine/MU)
Reference wavelength: 600 x 4 nm
Spectrum range: 190-400 nm

Peak retention times: (see Figure 3) Methyl umbelliferone *ca.* 10.7 mins
Coumarin *ca.* 12.5 mins
Quassine ca. 13.4 and 14.1 mins (2 peaks).

Note: *It may be necessary to remove co-extracted interferences from the column if an unstable baseline, high background and or ghost peaks are observed. This may be achieved e.g. by increasing the mobile phase acetonitrile content to 95% at the end of the run and holding for 10 minutes*

10. DATA PROCESSING

10.1 Calculation of coumarin and quassine levels in samples

Identify the coumarin, quassine and MU internal standard peaks on the basis of their retention time in single standards and measure the respective peak areas: MU internal standard ~12.3 mins, coumarin ~14.9 mins and quassine ~16.3/16.8 mins. Divide the coumarin and total quassine peak areas by the internal standard peak area to obtain the peak area ratios. From these calculate coumarin and quassine concentrations of the extract from the regression equation of the appropriate calibration graph.

Calculate the coumarin or quassine concentration in the sample from:

W_s = Sample weight or volume (g or mL)
V_{ext} = Methanolic extract volume (mL)
V_{aqt} = Methanolic extract aliquot volume taken for SPE (mL)
V_{spe} = SPE elution volume (mL)
C_{ext} = Concentration of coumarin/quassine in HPLC vial from calibration graph

***(i) Without SPE cleanup/concentration*:**

$$\text{Coumarin (mg/kg) (or quassine)} = \frac{C_{ext} \times V_{ext}}{W_s}$$

***(ii) With SPE concentration (beverages)*:**

$$\text{Coumarin (mg/kg) (or quassine)} = \frac{C_{ext} \times V_{spe}}{W_s}$$

(iii) With SPE cleanup/concentration (methanolic extracts):

$$\text{Coumarin (mg/kg) (or quassine)} = \frac{C_{ext} \times V_{ext} \times V_{spe}}{W_s \times V_{aqt}}$$

11. QUALITY ASSURANCE

11.1 Recovery of spiked samples within the batch should ideally be in the range of 70 – 110%.

11.2 Reagent blank samples should be prepared with each batch. Any analyte in the blank should be blank subtracted from the samples.

11.3 Peak spectrum matching should be applied with caution. Evidence of co-eluting impurities should be referred to line management.

Figure 3. Typical HPLC-PDA separation of methylumbelliferone, coumarin and quassine at 280nm (top), 256 nm (middle) and 320nm (bottom).

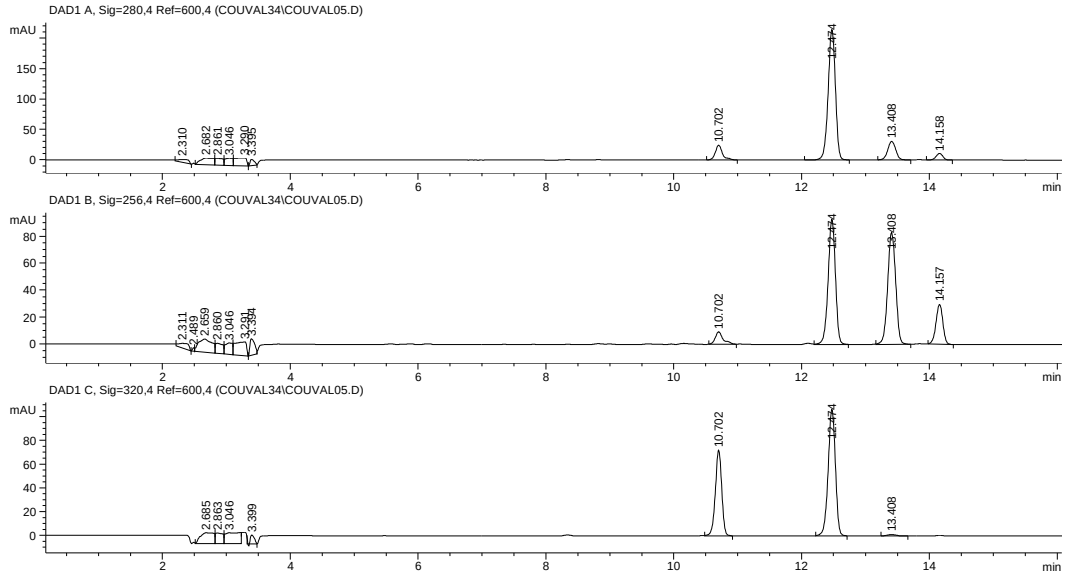


Figure 4. PDA spectra of methyl umbelliferone (left), coumarin (centre) and quassine (right).