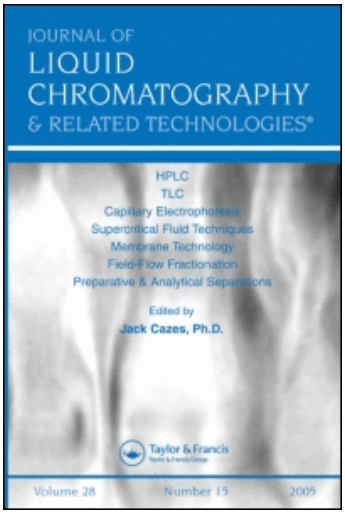

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Determination of Coumarin in Food Using Ultra-Performance Liquid Chromatography–Electrospray-Tandem Mass Spectrometry

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Abstract: A method based on the use of ultra performance liquid chromatography (UPLC) tandem mass spectrometry interfaced with electrospray (UPLC/MS/MS) was devised for the determination of coumarin residues in food samples. Sample treatment includes accelerated solvent extraction (ASE) using dichloromethane followed by a clean up on a gel permeation chromatograph. UPLC was performed on an Acquity UPLC BEH C_{18} (100 mm × 2.1 mm), the mobile phase was water–acetonitrile (50/50 v/v) (each component containing 0.1% formic acid) at a flow rate of 0.3 mL min^{-1}. For unequivocal identification of the substance, two ions were detected and chosen for multiple reactions monitoring (MRM). Validation was carried out on a spiked sample of vanilla milk rice and liquorice sweets. The described method meets all the criteria of Decision 2002/657/EC and is easy to use in routine analyses.

Keywords: Accelerated solvent extraction (ASE), Coumarin, Tandem mass spectrometry interfaced with electrospray (UPLC/MS/MS), Ultra performance liquid chromatography (UPLC)

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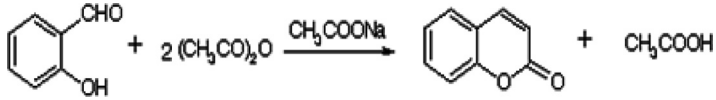
INTRODUCTION

Coumarin is a naturally occurring benzopyrone. It occurs in various plants including tonka bean and sweet clover and in several natural flavouring source materials. Coumarin itself was originally used as a flavouring substance until the direct use of coumarin in food was prohibited in the USA in 1954 following reports of hepatotoxic effects in rats and dogs.[1] It is still used in fragrances and tobacco. More recently, it has been used in the medical treatment of high protein lymphoedema and chronic infections such as brucellosis and tuberculosis.

Coumarin has also been investigated in the treatment of renal cell carcinoma, malignant melanoma, and prostate cancer.[1] The biosynthesis of coumarin in plants is via hydroxylation, glycolysis, and cyclisation of cinnamic acid. Coumarin can be prepared in a laboratory in a Perkin reaction between salicylaldehyde and acetic anhydride.[8]



The Pechmann condensation provides another synthesis of coumarin and its derivatives.[8]

The Council Directive 88/388/EC set maximum limits for certain substances obtained from flavourings and other food ingredients with flavouring properties present in foodstuffs as consumed.[2]

Synthetic coumarin is used in cosmetics.[2] The smell if that of fresh hay. Coumarin is also used for medicinal purposes to treat oedemas.[2] Isolated coumarin may not be added to foods. If it is contained in parts of plants added to flavour foods (as is the case with cinnamon), the amount of coumarin is limited to 2 milligrams per kilogram food in accordance with the Flavourings Ordinance.[3] Several analytical approaches have been used to determine coumarin in different matrixes: gas chromatography with electron capture detection (GC-ECD),[4] gas chromatography with mass spectrometry (GC-MS),[4] gas chromatography,[16] high performance liquid chromatography (HPLC),[5,13] HPLC UV,[6] HPLC FLD,[7] HPLC/MS/MS,[10,12,14] thin layer chromatography,[15] capillary zone electrophoresis,[19] polarographic determination.[11]

The aim of this work was to develop a method applicable to different matrices like milk rice, sweets, roll, cereal breakfast, etc. Tandem mass spectrometry (MS/MS) has proven to be a useful and a time saving analytical tool, with many applications for direct detection

of target molecules in food samples. When coupled with chromatographic techniques, it combines the separation capabilities of chromatography and the power of MS/MS as an identification and confirmation method. The validation has been realised on milk rice, biscuits, and liquorice sweets.

EXPERIMENTAL

Chemicals and Material

The certified standard of coumarin for study was obtained from Sigma. Individual standard solutions were prepared by placing approximately 10 mg of standard in a glass tube and adding an approximate amount of solvent to reach a concentration of 100 mg L^{-1}. These solutions were stored in the dark at 4°C.

Grade solvents acetonitrile, methanol, and dichloromethane and also formic acid were obtained from Merck (Germany). Acetone, p.a. was obtained from Mikrochem, Slovakia, acetone was rectified. Water was freshly prepared from a Milli-Q with a specific resistance 18 MΩ and a total carbon value <5 ppb. Hydromatrix, which was used for ASE was obtained from Varian, (USA). Food samples were obtained from the trade network.

Apparatus

The following devices were used for extraction and clean up: a balance, a mechanical shaker, Dionex ASE® 200 with 22 mL stainless extraction cells (Dionex, USA), gel permeation stainless steel column, 500 × 8 mm, BIO BEADS BIO BEADS S X, rotary evaporator (Buchi, Switzerland). The UPLC equipment consisted of a Waters Acquity UPLC system (Waters USA). For mass spectrometry, a Micromass Quattro Premier XE tandem mass spectrometer (Micromass UK, Altrincham UK) was used.

Glassware and other recipients were chosen as was suitable for each step of the procedure.

Sample Preparation

Samples were dried and ground before filling the extraction cells. Samples, which contained water (greater than 10%) were mixed in equal proportion with sodium sulphate (anhydrite) or Hydromatrix® (Varian, USA).

Table 1. Extraction conditions of coumarin by using ASE® 200

System pressure	10.34 MPa
Oven temperature	100°C
Oven heat-up time	5 min
Static time	5 min.
Static cycles	2
Solvent	Dichloromethane/acetone (2:1), (v/v)
Flush volume	60% of extraction cell volume
Nitrogen purge	1.034 MPa for 60 s

A sample of 2 g was mixed with 5 g sodium sulphate (anhydrite) or 2 g Hydromatrix® in mortar and filled to 22 mL extraction cell. Dead volume was filled by sea sand. Residues of coumarin were extracted in ASE® 200 according to conditions described in Table 1.

Extract was collected to a 40 mL extraction vial and after that evaporated to dryness by the rotary evaporator, temperature 40°C.

The volume was adjusted to 2 mL with dichloromethane. Therefore, 0.5-mL of extract was injected on BIO BEADS S X-3 column in the following conditions: Column: steel column 8×500 mm; Packing: Bio Beads S X-3; Eluent: dichloromethane; Flow rate: 0.6 mL/min; Pressure: 0.6 MPa (max.).

Sixteen 36 mL of dichloromethane fraction was collected and evaporated by the rotary evaporator (40°C). The residues were dissolved in 10 mL of acetonitrile: water (50:50) both containing 0.1% formic acid.

Chromatographic Conditions

UPLC separations were carried out on a ACQUITY UPLC™ system using a reversed phase column Acquity UPLC BEH C_{18} (100 mm × 2.1 mm) with 1.7 μm spherical porous particles. The isocratic elution was performed using 50% of water and 50% of acetonitrile, each containing 0.1% formic acid. The flow rate was 0.3 mL min^{-1}. Column temperature was 30°C. Injected volume was 10 μL. Time of analyses, which involved separation and reconditioning of the column was 3 min.

Table 2. MRM Methods parameters

	MRM transitions	Dwell time (s)	Cone voltage (V)	Collision energy (eV)
Coumarin (quantification)	146,8 → 90,8	0,1	35	24
Coumarin (confirmation)	146,8 → 102,7	0,1	35	17

MS–MS Parameters

A Quattro Premier XE tandem quadrupole instrument was used in UPLC-MS/MS analysis. The instrument was operated in positive ESI mode. ESI parameters, as well as selection and tuning of MS/MS transitions and analyte dependent parameters (collision energy and cone voltage are given in Table 2), were performed by direct infusion of coumarin standard solution (concentration 1 mg L^{-1}) into the mobile phase flow. Nitrogen was used as a desolvation gas at a flow rate of 600 L h^{-1} and also as a cone gas at a flow rate of 60 L h^{-1}. MS/MS parameters: capillary voltage 2.8, extractor voltage 4 V, source temperature 120°C, desolvation temperature 400°C, collision gas pressure 0.5 Pa.

RESULTS AND DISCUSSION

The aim of this work was to develop a method applicable to different matrices like milk rice, sweets, roll, cereal breakfast, etc.

The first step was to develop a procedure for extraction of coumarin. Published methods were not suitable for the type of matrices which were studied. Investigated samples contained enough amounts of fat.

The best results were achieved using the combination of accelerated solvent extraction (ASE) using dichloromethane followed by gel

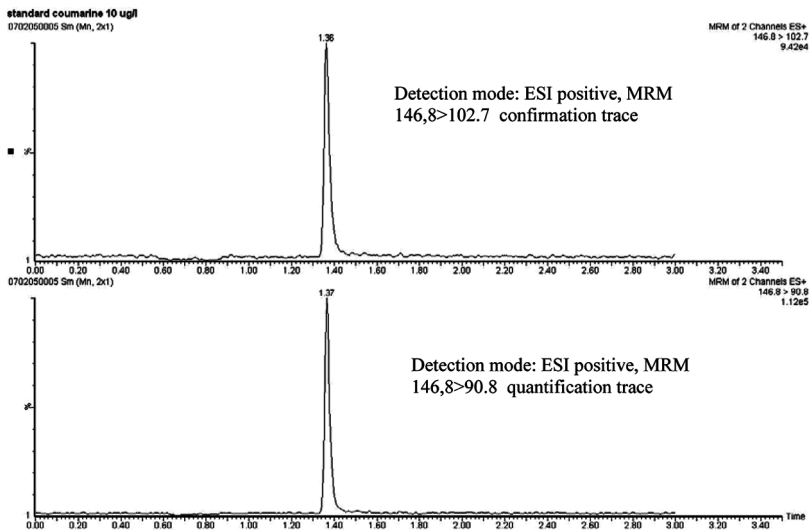


Figure 1. Chromatogram of standard coumarin solution (concentration 10 mg/l), on Acquity UPLC BEH C_{18} column using isocratic UPLC/MS/MS method, flow-rate 0.3 mL/min, positive ESI mode, MRM.

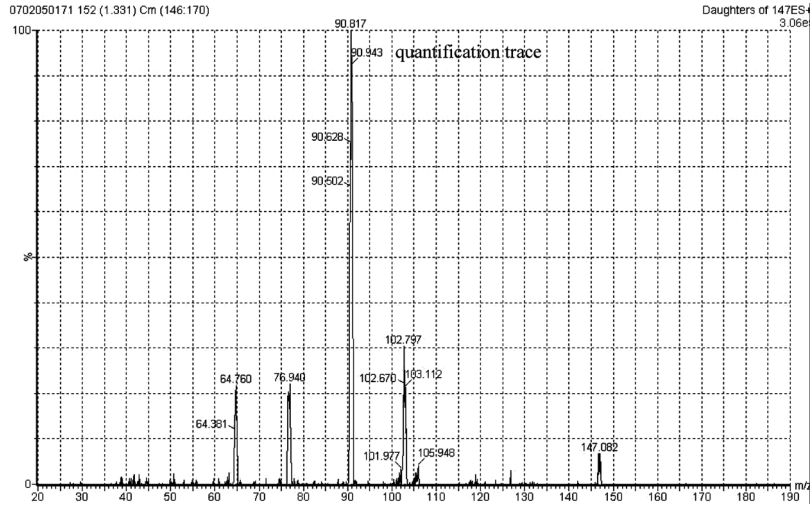


Figure 2. Daughter scan of standard coumarin solution (concentration of coumarin 1 mg/L), on Acquity UPLC BEH C_{18} column using isocratic UPLC/MS/MS method, flow rate 0.3 mL/min, positive ESI mode.

permeation chromatography. Extraction is applicable for a wide range of matrices. Fats and oils easily soluble in organic solvents are simply transferred into dichloromethane prior to the GPC cleaning step. This procedure is very advantageous to samples with a high content of fat. Procedure is time consuming, but ASE is an automated process with a

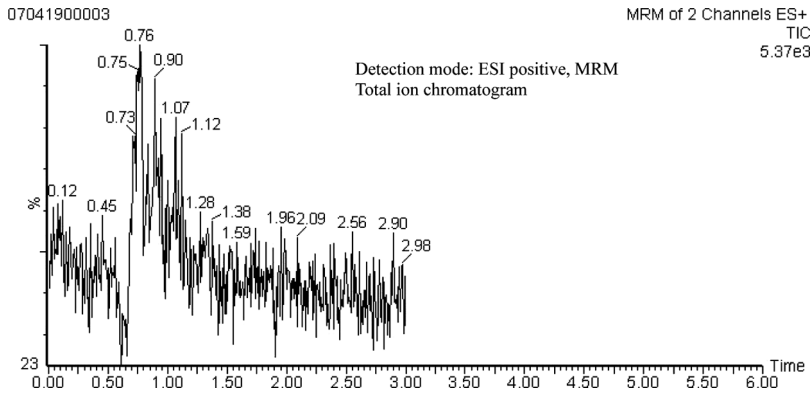


Figure 3. Chromatogram of milk rice extract without presence of coumarin, on Acquity UPLC BEH C_{18} column using isocratic UPLC/MS/MS method, flow rate 0.3 mL/min, positive ESI mode, MRM.

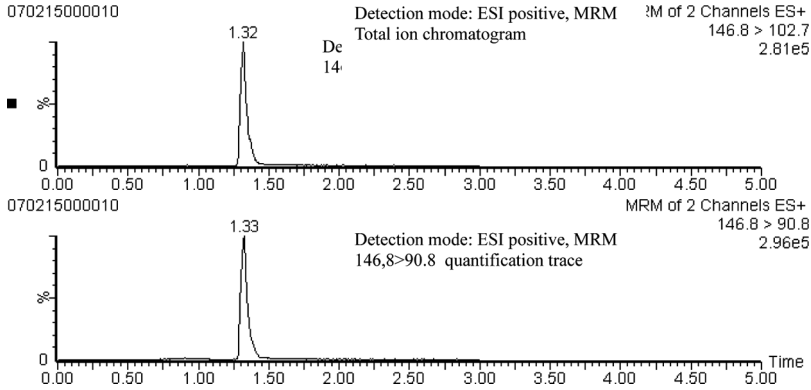


Figure 4. Chromatogram of Haribo sweets extract spiked with coumarin on level 2 mg/kg, on Acquity UPLC BEH C_{18} column using isocratic UPLC/MS/MS method, flow rate 0.3 mL/min, positive ESI mode, MRM.

limitation of 25 samples within one run. GPC is also an automated process with a limitation on 25 samples within one run.

Optimal fractions from GPC, which contained coumarin, were selected using a coumarin standard with a concentration of 10 mg/kg. This standard was injected into the GPC and each 2 mL fraction was retained, evaporated to dryness under stream of nitrogen, and measured. Fractions of 17–18 mL, 19–20 mL, and 21–22 mL contained coumarin.

Recoveries were performed for each step separately and also for all processes using a standard of coumarin. Recoveries for ASE ranged from 80% to 92%, recoveries for GPC ranged from 83% to 95%. Recoveries for all processes ranged from 80% to 90%. Some examples are given in Tables 3 and 4. Because there is no CRM available, the recovery was determined by experiments using a fortified blank matrix using % Recovery = 100 × measured content/fortification level.[9]

The second step of work was to develop a quick and sensitive UPLC/MS/MS procedure. UPLC conditions were simple. The isocratic elution was sufficient. Retention time of coumarin using these conditions

Table 3. Recovery and RSDs for milk rice

Level (mg/kg)	Recovery (%)	RSD (%)
1	97,0	3,13
2	100,0	3,92
3	95,3	7,25

Table 4. Recovery and RSDs for biscuits

Level (mg/kg)	Recovery (%)	RSD (%)
1	102,0	8,86
2	96,0	6,15
3	89,6	8,68

was 1.3 min. The instrument was operated in ESI positive mode. ESI parameters, as well as selection and tuning of MS/MS transitions and analyte dependent parameters (collision energy and cone voltage), were performed by direct infusion of coumarin standard solution into the mobile phase flow. The positive ion daughter scan spectrum of coumarin is shown in Figure 2. Mass spectrometry was optimised to obtain an MRM transition for the precursor ion, Figure 1.

The third step was validation of the method. Validation was done according to Commission Decision 2002/657/EC,[9] implementing Council Directive 96/23/EC concerning the performance of analytical methods, and the interpretation of results on different type of matrices, e.g., milk rice, biscuits.

Twenty representative blank samples were analysed to check for interfering peaks in the region where the target analyte was expected to elute. No interference was observed. The chromatogram of blank milk rice extract is shown in Figure 3, the chromatogram of spiked extract is presented in Figure 4.

The method was found useful over a wide concentration range of coumarin. Limit of detection (LOD) and limit of quantification (LOQ) were calculated from the calibration curve, uncertainty, repeatability, linearity, were calculated using software Effivalidation 3.0, which is laboratory software for validation of analytical methods according ISO 17025 for accredited laboratories and GMP quality control regulations.[20] The validation parameters of metho are given in Table 5. The applicability of this method was tested by analysing

Table 5. Validation parameters of method

Parameters	
Limit of detection	20 μg/kg
Limit of quantification	50 μg/kg
Scale	50–5000 μg/kg
Retention time	1,3 min
Analysis time	3 min
Correlation coeficient	0,9948

Table 6. Results of controls from Slovak market

Category	Number of samples	Average level (mg/kg)	Max. level (mg/kg)	Numbers of sample over maximum permitted concentration
pastry	13	6,6	18,5	10
biscuits	16	2,8	11,4	3
tea with cinnamon	6	4,7	11,5	—[a]
cinnamon sugar	3	39,4	67,5	—[a]
cereal breakfest	23	2,2	9	3
musli	1	1,5	1,5	0
cinnamon	11	1180	2363	—[a]
christmas biscuits	2	6,9	6,9	2
chocolate glaze	1	1,9	1,9	0

Comments:
a-cinnamon, cinnamon sugar, tea with cinnamon were not evaluated.
Pastry–involved typical sponge pastry filled or sprinkled with cinnamon.
Biscuits-involved dry biscuits, tea biscuits and biscuits spilled with chocolate glaze.
Cereal breakfast-involved e.g. cini minis and others cereals.
Cinnamon-includes whole and ground cinnamon.
Christmas biscuits–typical biscuits with honey.

large numbers of cinnamon product samples obtained from the Slovak market (Table 6).

CONCLUSIONS

Using UPLC under isocratic conditions and ESI positive detection mode, a sensitive method for quantitative and qualitative analysis of coumarin residues in food was devised. The method is suitable for different matrices. The entire extraction process is fully automated and performed in minutes for fast and easy extraction with low solvent consumption. ASE was developed to meet the new requirements for reducing solvent usage in the preparation of solid food samples.

REFERENCES

1. EFSSA J. **2004**, *104*, 1–36.
2. Frequently Asked Questions about coumarin in cinnamon and other foods, Federal Institute for Risk Assessment 2006.
3. Council Directive 88/388/EEC (OJ L 184, 1988, p. 61).

4. Wisneski, H.H. Determination of coumarin in fragrance products by capillary gas chromatography with electron capture detection. J. AOAC Int. **2001**, *84* (3), 689–692.
5. Bogan, D.P.; O'Kennedy, R. Simultaneous determination of coumarin, 7-hydroxycoumarin and 7-hydroxycoumarin glucuronide in human serum and plasma by high-performance liquid chromatography. J. Chromatogr. B Biomed. Appl. **1996**, *686* (2), 267–273.
6. Chen, J.; Hu, G. The determination of coumarin in foods by high performance liquid chromatography (HPLC). Se Pu. **1999**, *17* (2), 203–205.
7. Hunter, K. Determination of coumarin anticoagulant rodenticide residues in animal tissue by high-performance liquid chromatography. I. Fluorescence detection using post-column techniques. J. Chromatogr. **1983**, *270*, 267–276.
8. Wikipedia.
9. Commission Decision 2002/657/EC.
10. Lowri, S.; de Jager, G.A.; Perfetti, G.W.; Diachenko, W. Determination of coumarin, vanillin, and ethyl vanillin in vanilla extract product: liquid chromatography mass spectrometry method development and validation studies, J. Chromatogr. A. **2007**, *1145*, 83–88.
11. Polarographic determination of coumarin and cinnamic acid in the preparation fibs. *Chemistry of Natural compounds*; 1992; Vol. 27.
12. Rychlik, M. Quantification of free coumarin and its liberation from glucosylated precursors by stable isotope dilution assays based on liquid chromatography-tandem mass spectrometric detection. J. Agric. Food Chem. **2008**, Epub ahead of print.
13. Avula, B.; Joshi, V.C.; Reddy, V.L.N.; Choi, Y.W.; Khan, I.A. Simultaneous determination of eight coumarins in Angelica gigas and in various other Angelica species by high performance liquid chromatography and comparative micro-morphology study of Angelica species. Planta. Med. **2007**, *73* (14), 1509–1516.
14. Ahn, M.J.; Lee, M.K.; Kim, Y.C.; Sung, S.H. The simultaneous determination of coumarins in Angelica gigas root by high performance liquid chromatography-diode array detector coupled with electrospray ionization/mass spectrometry. J. Pharm. Biomed. Anal. **2008**, *46* (2), 258–266.
15. Waksmunzka–Hajnos, M.; Petruczynik, A.; Hajnos, M.L.; Tuzimski, T.; Hawryl, A.; Bogucka–Kocka, A. Two-dimensional thin-layer chromatography of selected coumarins, J. Chromatogr. Sci. **2006**, *44* (8), 510–517.
16. Kim, M.R.; Abd El-Aty, A.M.; Kim, I.S.; Shim, J.H. Determination of volatile flavor components in danggui cultivars by solvent free injection and hydrodistillation followed by gas chromatographic-mass spectrometric analysis. J. Chromatogr. A. **2006**, *1116* (1–2), 259–264.
17. Liu, R.; Sun, Q.; Shi, Y.; Kong, L. Isolation and purification of coumarin compounds from the root of Peucedanum decursivum (Miq.) Maxim by high-speed counter-current chromatography. J. Chromatogr. A. **2005**, *1076* (1–2), 127–132.
18. Pashkova, A.; Chen, H.S.; Rejtar, T.; Zang, X.; Giese, R.; Andreev, V.; Moskovets, E.; Karger, B.L. Coumarin tags for analysis of peptides by MALDI-TOF MS and MS/MS. 2. Alexa Fluor 350 tag for increased peptide

and protein Identification by LC-MALDI-TOF/TOF MS. Anal. Chem. **2005**, *77* (7), 2085–2096.
19. Yue, M.E.; Jiang, T.F.; Liu, X.; Shi, Y.P. Separation and determination of coumarins from Cacalia tangutica by capillary zone electrophoresis, Biomed. Chromatogr. **2005**, *19* (3), 250–254.
20. Method Validation and Uncertainty software EffiValidation 3.0 is designed to support accredited ISO 17025 testing and calibration laboratories and quality control GMP laboratories. EffiChem, Lesni 593, CZ-679 71 Lysice, Czech Republic, EU.

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