Application Note 1128

Determination of Coumarins in Cosmetics

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Key Words
Personal Care Product Analysis, Cosmetic Quality, Cosmetic Safety, HPLC, Acclaim 120 C18 Column

Goal
To develop an efficient HPLC method for the determination of coumarins in cosmetics due to the risk of toxicity to the liver and kidneys. The coumarins to be determined are coumarin, acenocoumarol, 7-methoxycoumarin, 6-methylcoumarin, dicoumarol, 7-ethoxy-4-methylcoumarin, and pyranocoumarin.

Introduction
Coumarins are fragrant compounds. They belong to the benzopyrone chemical class and have been widely used as aroma enhancers in cosmetics, foods, and drinks. Toxicity studies reveal that coumarins are moderately toxic to the human liver and kidneys and may cause liver cancer in rats and lung tumors in mice.[1-3] Therefore, the use of coumarins has been regulated, despite the fact that they are usually found naturally in many edible plants such as strawberries, black currants, apricots, and cherries.[4] Coumarin was banned as a food additive in the United States in 1954, largely because of the hepatotoxicity results in rodents.[4]

Table 1 shows the requirements of some coumarins for cosmetic products in the US, European Union (EU), and China. The US FDA asked manufacturers of suntan and sunscreen products to discontinue the use of 6-methylcoumarin;[5] while in China, the permitted content of 6-methylcoumarin in cosmetic products is up to 30 mg/kg.[6] In the EU, seven coumarins (dicoumarol, 7-ethoxy-4-methylcoumarin, acenocoumarol, 7-methoxycoumarin, dihydrocoumarin, 7-methylcoumarin, and pyranocoumarin) are forbidden in cosmetic products. Six of those seven (all except pyranocoumarin) are forbidden in China.[6,7] Therefore, effective methods for the determination of coumarins in cosmetics are necessary. HPLC has been extensively applied to the determination of coumarins in cosmetics.[8,9] Figure 1 shows the structures of coumarins that will be determined in this work.

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Table 1. Permitted and forbidden coumarin compounds in cosmetics in the EU, US, and China.

Coumarins	USA[5]	China[6]	EU[7]
Dicoumarol	No restriction	Forbidden	Forbidden
7-Ethoxy-4-methylcoumarin			
Acenocoumarol			
7-Methoxycoumarin			
Dihydrocoumarin*			
7-Methylcoumarin*			
Pyranocoumarin		No restriction	No restriction
Coumarin			
6-Methylcoumarin	The FDA has encouraged manufacturers not add to cosmetics	< 30 mg/kg	

*These compounds were not available when this study was conducted.

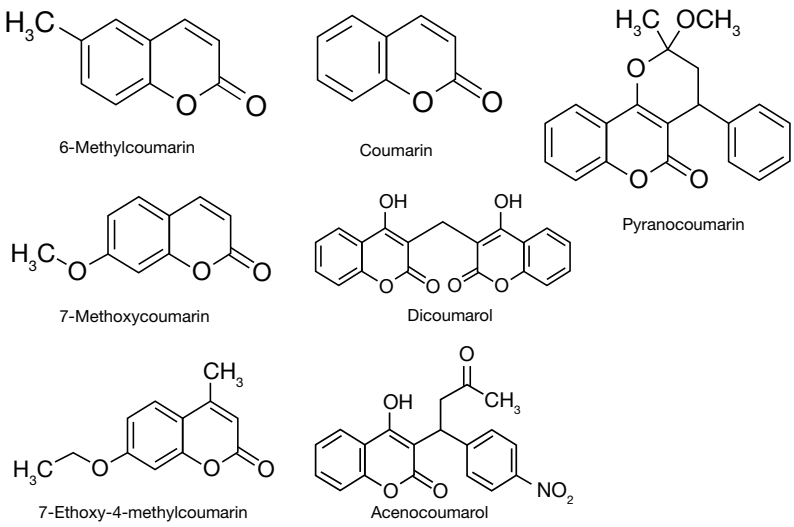

Figure 1. Structures of coumarin compounds.

Experimental

Equipment and Software

- Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation LC (RSLC) system, including:
  - LPG-3400RS Quaternary Pump (P/N 5040.0036)
  - SRD-3400 Integrated Solvent and Degasser Rack (P/N 5035.9245)
  - WPS-3000TRS Well Plate Sampler, Thermostatted (P/N 5840.0020), with 25 µL sample loop (P/N 6820.2415) and a 25 µL syringe (P/N 6822.0001)
  - TCC-3000RS Thermostatted Column Compartment (P/N 5730.0000)
  - DAD-3000RS Diode Array Detector (P/N 5082.0010), with 2.5 µL flow cell (P/N 6082.0300)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2
- Fisher Scientific™ CPXH Series Digital Ultrasonic Cleaners (P/N 15-337-410)
- Thermo Scientific™ Sorvall™ ST16 Centrifuge (P/N 75004240)

Consumables

- Thermo Scientific™ Target2™ Polypropylene Syringe Filters (0.45 µm, 30 mm, P/N F2502-9)
- Thermo Scientific™ Nunc™ Disposable Plastic Centrifuge Tubes, (10-mL, P/N 12-565-201)

Reagents and Standards

- Deionized (DI) water, 18.2 MΩ×cm resistivity (generated from the Thermo Scientific™ GenPure™ Pro UV-TOC, P/N 50131948)
- Acetonitrile (CH_3CN), HPLC Grade (Fisher Scientific™ P/N AC610010040)
- Acetic acid (CH_3COOH), 99.5% (Fisher Scientific P/N AC12404-0010)
- Sodium hydroxide (NaOH), ≥97.0 % (Fisher Scientific P/N S318-500)
- Methylene chloride (CH_2Cl_2), HPLC Grade (Fisher Scientific P/N D143SK-4)
- Dicoumarol, >98% (Fisher Scientific P/N 50-014-39502)
- Pyranocoumarin (Fisher Scientific P/N 50-767-92)
- Acenocoumarol, ≥98% (Sigma, SML0074, CAS 152-72-7)
- Coumarin (Fisher Scientific P/N AC11053-0050)
- 7-Methoxycoumarin (Fisher Scientific P/N AC20512-0500)
- 6-Methylcoumarin (Fisher Scientific P/N 50-730-881)
- 7-Ethoxy-4-methylcoumarin (Fisher Scientific P/N 50-908-723)

Preparation of Standard Solutions

Stock Standard

Dissolve 0.01 g of each standard in 10 mL of a mixture of acetonitrile and methylene chloride (95:5, v/v), respectively. The concentration of each stock standard is 1000 mg/L.

Mixed Stock Standard

Add 2.5 mL of each stock standard solution to a 25-mL volumetric flask and bring to the volume with acetonitrile. The concentration of each coumarin component in the mixed stock standard is 100 mg/L.

Mixed Standard Solutions for Calibration

For calibration, prepare nine mixed working standard solutions with different concentrations by diluting the proper amount of the mixed stock standard solutions with acetonitrile. The volumes of each solution needed to make the calibration standards are shown in Table 2.

Chromatographic Conditions	
Column:	Thermo Scientific™ Acclaim™ 120 C18 analytical column, 3 µm, 3 × 150 mm (P/N 063691)
Mobile phase:	Acetonitrile / DI water
In gradient:	0–5 min, 56% acetonitrile; 5.5–7.5 min, 100% acetonitrile; 7.6–10 min, 56% acetonitrile
Injection volume:	1 µL
Flow rate:	0.425 mL/min
Temperature:	30 °C
Detection:	UV absorbance, 306 nm

Table 2. Preparation of mixed standards for calibration (each mixed standard contains 7 coumarins).

Stock Standards	Volume of Each Stock Standard (mL)	Volume of Acetonitrile (mL)	Final Volume (mL)	Final Concentration of Each Analyte (mg/L)
Mixed Stock Standard (100 mg/L for each analyte	5.00	5.00	10	50
	2.00	8.00		20
	1.00	9.00		10
	0.50	9.50		5.0
	0.20	9.80		2.0
	0.10	9.90		1.0
	0.05	9.95		0.5
	0.02	9.98		0.2
	0.01	9.99		0.1

Sample Preparation

Two cream cosmetic samples were provided by a customer from Jiangsu, China.

Add 0.5 g of a cream sample and 7.5 mL of a mixture of 0.1 mol/L NaOH (dissolve 0.4 g of NaOH in 100 mL of DI water) and acetonitrile (1: 9, *v/v*) to a 10-mL centrifuge tube. Extract in an ultrasonic bath for 30 min, cool to room temperature, add 0.08 mL of 1 M acetic acid (dilute 4 mL of acetic acid to 100 mL with DI water), and centrifuge the extract for 20 min at 8000 rpm. Remove the supernatant, add 7.5 mL of the mixture of 0.1 mol/L NaOH and acetonitrile (1: 9, *v/v*) to the residue, and extract and acidify a second time in the same manner. Combine the two supernatants (total volume <20 mL) in a 25-mL volumetric flask, and bring to volume with the mixture of 0.1 mol/L NaOH and acetonitrile (1: 9, *v/v*). Filter the sample solution through a 0.45 µm syringe filter prior to injection.

Add 0.5 g of a cream sample, 0.5 mL of the mixed calibration standard with concentration of 50 mg/L for each analyte, and 7.5 mL of the mixture of 0.1 mol/L NaOH and acetonitrile (1: 9, *v/v*) to a 10-mL centrifuge tube. Sample preparation is completed using the procedure above. The spiked concentration of each analyte in the cream sample will be 1 mg/L.

Results and Discussion

Chromatography

The seven analytes are all ideal candidates for reversed-phase chromatography with UV detection. Figure 2 shows chromatograms using Acclaim Phenyl-1 and 120 C18 columns under their individually optimized chromatographic conditions. 7-Methoxycoumarin (peak 3), 6-methylcoumarin (peak 4), and dicoumarol (peak 5) were eluted in a different order on the two columns, and separations of all analytes were completed within 10 min using either column. The Acclaim 120 C18 column was chosen because it provides baseline separation for all analytes with peak resolutions ≥2.5.

Calibration linearity for UV detection of coumarins was investigated by making three consecutive 1 µL injections of a standard prepared at nine different concentrations (i.e., 27 total injections). Each analyte exhibited a linear relationship in the concentration range of 0.2–50 mg/L when plotting concentration (c) versus peak area (A). The calibration data are listed in Table 4. Those calibrations were used to quantify the coumarins in the cosmetic samples. Five replicate injections of a mixed calibration standard with a concentration of 0.2 mg/L for each analyte were used for estimating the method detection limit (MDL) using a signal-to-noise ratio of 3. The measured MDLs are also listed in Table 4.

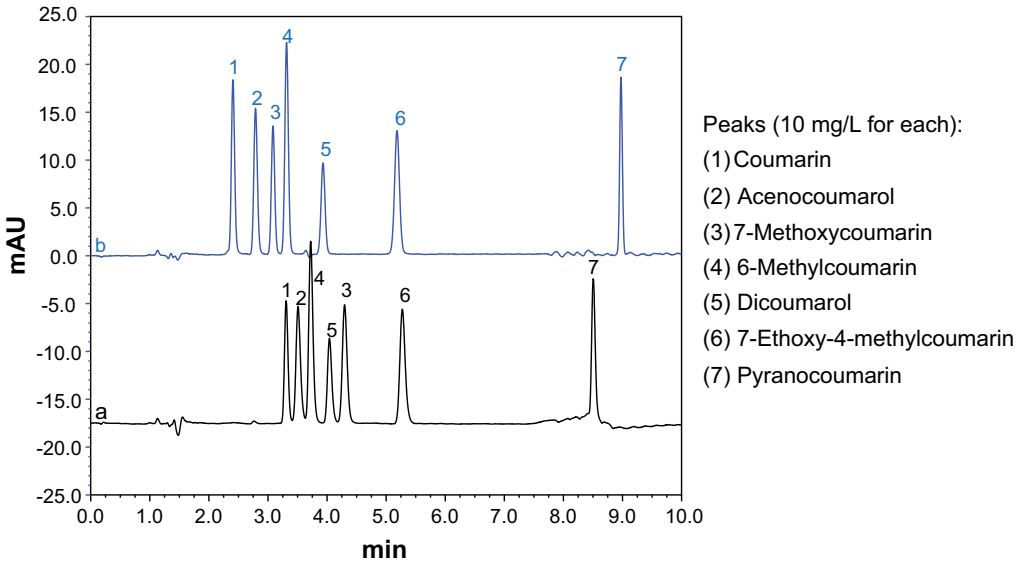

Figure 2. Chromatograms of coumarins on (a) Acclaim Phenyl-1 (3 µm, 3 ×150 mm) and (b) Acclaim 120 C18 (3 µm, 3 ×150 mm) columns.

Method Reproducibility, Linearity, and Detection Limit

Short-term method reproducibility was estimated by making eight consecutive injections of the mixed calibration standard with concentration of 10 mg/L for each analyte (Figure 3). As shown in Table 3, method reproducibilities (RSDs) for retention time were all <0.1%, and those for peak area were all <2%, demonstrating good short-term precision for this method.

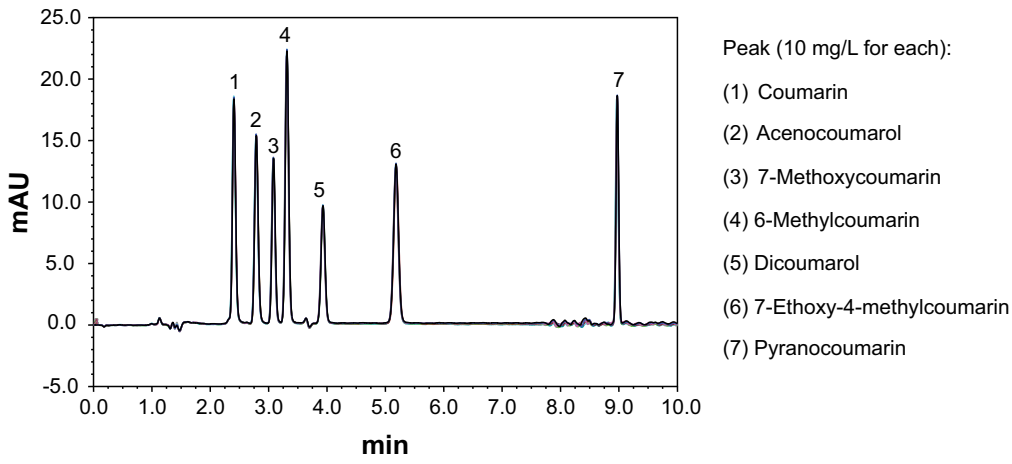

Figure 3. Overlays of chromatograms of eight consecutive injections of a mixed coumarins standard.

Table 3. Method reproducibility data.

Coumarins	Retention Time RSD	Peak Area RSD
Coumarin	0.09	1.13
Acenocoumarol	0.03	1.97
7-Methoxycoumarin	0.09	1.30
6-Methylcoumarin	0.08	1.02
Dicoumarol	0.08	1.16
7-Ethoxy-4-methylcoumarin	0.06	1.19
Pyranocoumarin	0.07	1.28

Table 4. Calibration data and MDLs.

Analyte	Regression Equation	r^2	Range (mg/L)	MDL (µg/L)
Coumarin	A = 0.0854c - 0.5105	0.9995	0.2–50	37
Acenocoumarol	A = 0.0860c + 0.0416	0.9998		40
7-Methoxycoumarin	A = 0.0959c - 0.1348	0.9998		32
6-Methylcoumarin	A = 0.0985c - 0.0461	0.9999		37
Dicoumarol	A = 0.0804c + 0.0437	0.9998		45
7-Ethoxy-4-methylcoumarin	A = 0.0728c - 0.0173	0.9997		36
Pyranocoumarin	A = 0.0858c + 0.0703	0.9999		41

Sample Analysis

Using acetonitrile as the extractant in an ultrasonic bath efficiently extracts the coumarins and removes oil components from a cream sample.[10,11] The cream sample was analyzed after such an extraction using the HPLC method described here, and the analysis results are summarized in Table 5. Figure 4 shows chromatograms of the two cream samples, and Figure 5 shows chromatograms of sample number 1 and the same sample spiked with standards. No coumarins were detected in either cream sample. To judge method accuracy, recoveries from cream sample number 1 spiked with a mixed standard were investigated. The recoveries ranged from 80% to 94% for the seven coumarins (Table 5), demonstrating that this method is suitable for the determination of coumarins in cosmetics.

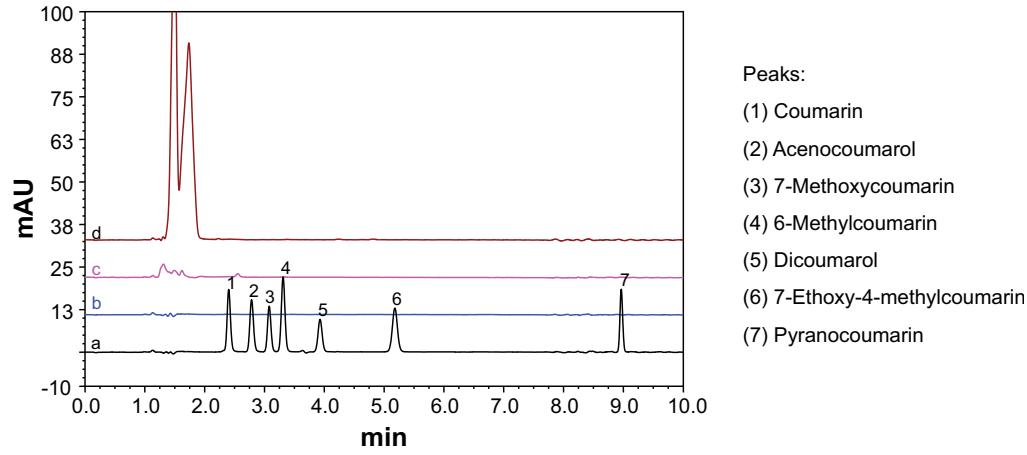

Figure 4. Chromatograms of (a) a mixed coumarins standards with concentration of 10 mg/L for each, (b) blank, (c) cream sample 1, (d) cream sample 2.

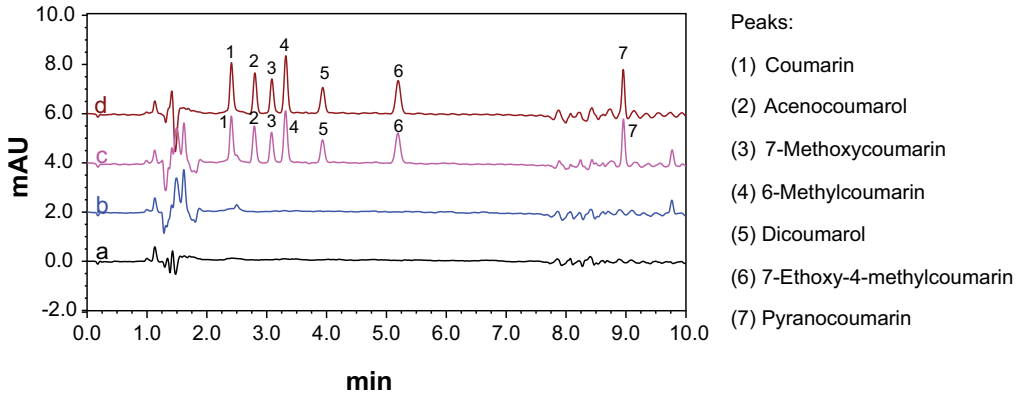

Figure 5. Chromatograms of (a) blank, (b) cream sample 1, (c) the same sample spiked with coumarins standards with concentration of 1.0 mg/L for each, (d) a mixed coumarins standards with concentration of 1.0 mg/L for each.

Table 5. Detected amounts of coumarins in cosmetics.

Analyte	Sample 1				Sample 2
	Detected mg/g	Added mg/L	Found mg/L	Recovery (%)	Detected mg/g
Coumarin	ND*	1.0	0.89	89	ND
Acenocoumarol		1.0	0.89	89	
7-Methoxycoumarin		1.0	0.94	94	
6-Methylcoumarin		1.0	0.85	85	
Dicoumarol		1.0	0.90	90	
7-Ethoxy-4-methylcoumarin		1.0	0.92	92	
Pyranocoumarin		1.0	0.80	80	

* “ND” represents “Not detected”

Conclusion

This work describes an efficient HPLC method with UV detection for a simultaneous determination of seven coumarins in cosmetics with the advantages of good method reproducibility and a wide linearity range.

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