Determination of Natural Levels of Coumarin in Different Types of Tobacco Using a Mass Fragmentographic Method†

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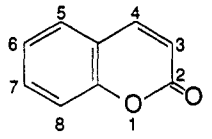
An accurate analytical method for qualitative and quantitative determination of endogenous coumarin in tobacco has been developed. Coumarin is extracted from the tobacco, and the extract obtained is purified by using high-pressure liquid chromatography or thin-layer chromatography. The quantification is performed by gas chromatography–mass spectrometry using a selected ion monitoring technique and ^{13}C-labeled coumarin as internal standard. The detection limit of the method is 0.050 μg of coumarin/g of tobacco. Coumarin was estimated in a number of different tobacco types. The concentrations were generally below 1 μg/g of tobacco. In fire-cured tobacco, however, the concentration varies between 1.1 and 4.7 μg/g. The higher concentrations in this tobacco may be explained by the presence of coumarin in the hickory smoke, which is condensed onto the leaf during the fire-curing process.

INTRODUCTION

Coumarin (1,2-benzopyrone) is a crystalline compound having a sweet and herbaceous odor. It is used as a flavoring and fragrance material in food, tobacco, cosmetics, and toiletries (Arctander, 1969).



Coumarin

Coumarin is widely distributed in the plant kingdom. It is noteworthy that as early as 1937 Späth published the occurrence of coumarin in 66 plants belonging to 24 families. A principal source is the tonka bean. Coumarin is also found in several species of clover (Haskins and Gosz, 1957, 1961), chicory plant (Sannai et al., 1982), tolu balsam, Peru balsam, lavender, cassia, and *Carphephorus odoratissimus* (Späth, 1937; Spector, 1956; Karlsson et al., 1972).

Coumarin occurs as a natural component in a few berries like raspberries, bilberries, cloudberries, and cherries (Maarse et al., 1989; Späth, 1937). It has also been isolated from other food products such as peppermint oil (Takahashi et al., 1980), cinnamon oil (Senanayake et al., 1978), and pouchong and longjing tea (Yamanishi et al., 1980; Kawakami and Yamanishi, 1983). The coumarin detected in bread is likely to originate from clover seeds occurring with the wheat used (Buttery et al., 1978).

Coumarin has also been identified as a natural constituent in burley (Fujimori et al., 1976), Turkish, and Virginia tobacco and in tobacco blends (Hoffmann and Woziwodzki, 1968).

The acute as well as subacute and/or chronic toxicity of coumarin has received considerable attention. Oral administration of coumarin to mice, rats, and guinea pigs has been reported to give LD_{50} values of 196, 290–680, and 202 mg of coumarin/kg of body weight, respectively (Kitagawa and Iwaki, 1963; Hazleton et al., 1956; Jenner et al., 1964).

Single doses of coumarin (ip, rats) have been reported to produce a depletion of hepatic glutathione, to increase liver weight, and to lead to centrilobular necrosis. The hepatotoxicity has been ascribed to 3,4-epoxycoumarin, which is a metabolic intermediate in rat, other metabolites being 3-hydroxycoumarin and 2-hydroxyphenylacetic acid (Lake et al., 1989).

By contrast, coumarin is metabolized to 7-hydroxycoumarin in man and baboon (Lake et al., 1989). The difference in the metabolism of coumarin is reflected in the toxicity. Thus, long-term administration of coumarin to rats has shown histological evidence of hepatotoxicity, but no such effects have been observed in baboon (Hazleton et al., 1956; Bär and Griepentrog, 1967; Hagan et al., 1967; Evans et al., 1979). Moreover, the murine marrow GM stem cell activity is suppressed at a 10 times lower concentration of coumarin than is the activity of corresponding human cells (Gallicchio et al., 1989).

Coumarin has previously been quantified in tobacco by the use of gas chromatography (GC) after purification of the samples by thin-layer chromatography (TLC) (Nesemann and Seehofer, 1970), while high-pressure liquid chromatography (HPLC) has been used to determine the content of coumarin and its metabolites in biological samples (Walters et al., 1980).

The aim of the present study has been to develop an accurate method for the determination of coumarin and to establish the endogenous concentrations of coumarin in different tobacco types. The need for such a method has been prompted by ongoing discussions in some countries seeking to set a maximum admissible concentration of coumarin in tobacco products without considering that coumarin is endogenous to tobacco.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals used were of analytical grade if not otherwise stated. Thin-layer chromatography plates (Kieselgel 60) were purchased from Merck, Darmstadt, Germany. Sodium (2-^{13}C)acetate and (2,2′-^{13}C)acetic anhydride were from Cambridge Isotope Laboratories, Cambridge, MA. Unlabeled coumarin was purchased from Aldrich, Gillingham, Great Britain.

Stock solutions of unlabeled and ^{13}C-labeled coumarin were prepared by dissolving the appropriate amount of the compounds in acetone.

Synthesis of (3-^{13}C)Coumarin. Labeled coumarin was prepared using a modification of the Perkin reaction. A mixture of sodium (2-^{13}C)acetate (295 mg), 2-hydroxybenzaldehyde (500

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† This work was supported by grants from The House of Borkum Riff AB, Stockholm, Sweden.

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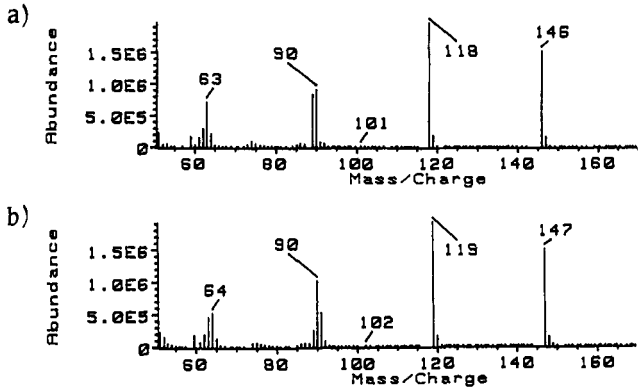

Figure 1. Mass spectra (EI) of (a) commercially available coumarin and (b) synthetic ^{13}C-labeled coumarin.

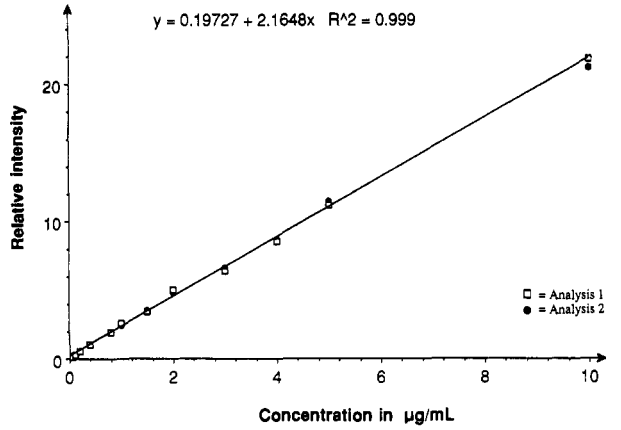

Figure 2. Standard curve obtained by analysis of various amounts (0.050–10 µg/mL) of unlabeled coumarin and a constant quantity (0.4 µg/mL) of ^{13}C-labeled coumarin.

µL), and (2,2′-^{13}C)acetic anhydride was heated at 180 °C under reflux for 15 h. The reaction mixture was cooled, diluted with dichloromethane, and washed with water, 1 M sodium hydroxide, 1 M hydrochloric acid, and water. Drying and evaporation gave a crude product, which was flash chromatographed on a silica gel column using dichloromethane as the eluent. After evaporation of the solvent and recrystallization from a mixture of diethyl ether–hexane, 220 mg of ^{13}C-labeled coumarin was obtained (yield 61%). Isotopic composition: (3-^{13}C)coumarin, 98.7%; unlabeled coumarin, 1.3%. The identity and purity were confirmed by the use of GC, nuclear magnetic resonance (NMR) and mass spectrometry (MS).

Tobacco Samples. Cured tobaccos from different countries were investigated.

Flue-Cured Tobacco. One type from Malawi, two types from the United States and Zimbabwe, and seven types from Brazil were used.

Air-Cured Tobacco. One type from Italy, one type from the United States, one type from Zimbabwe, and five types from Poland were studied.

Sun-Cured Tobacco. One type from Greece and one from Turkey were investigated.

Fire-Cured Tobacco. Two types from Poland and four types from the United States were used.

Sample Preparation. To 5 g of fine-cut tobacco (moisture content 12–15%) were added 20 µg of ^{13}C-labeled coumarin and 50 mL of hexane–chloroform (75:25). The sample was stirred for about 1 h and then left to equilibrate for another hour.

Purification by TLC. About 200 µL of the hexane–chloroform extract was applied on a silica gel thin-layer chromatography plate. The TLC plate was developed in pentane–ethyl acetate (70:30 v/v), and the coumarin-containing part of the silica gel was recognized by the use of UV light. The coumarin-containing spot was scraped into a 10-mL centrifuge tube. About 2 mL of acetone was added, and the sample was extracted for 5 min in an ultrasonic bath and thereafter centrifuged for 5 min

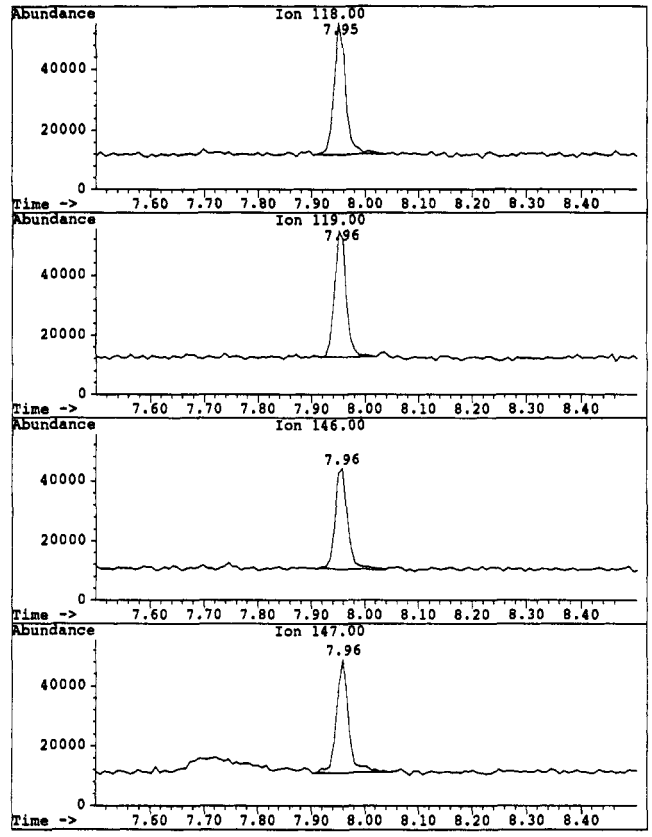

Figure 3. Mass fragmentogram of a standard consisting of 0.4 µg/mL of unlabeled and ^{13}C-labeled coumarin.

at 5000*g*. The extract was transferred to another tube and evaporated to about 10 µL. About 2 µL of this solution was analyzed by the use of GC–MS.

Purification by HPLC. An alternative sample purification was performed by using a Varian 5000 HPLC equipped with a 50-µL loop (Rheodyne 7125) and an UV detector (Varian 2550); 50 µL of the extract was injected into the HPLC and gradient-eluted (T_0 = 6%, T_{18} = 40%, T_{24} = 6% chloroform in hexane) with a flow rate of 1.2 mL/min. The samples were chromatographed on a column system consisting of a precolumn (Varian NH2-10, 50 × 4.6 mm i.d.) and an analytical column (Supelco LC NH2-2, 220 × 4.6 mm i.d., particle size 5 µm). The wavelength used for detection was 273 nm. The coumarin-containing fraction was collected in a 5-mL conical vial, and the volume was reduced to about 10 µL under a stream of nitrogen. About 2 µL of the sample was analyzed by GC–MS.

Gas Chromatography–Mass Spectrometry Determination of Coumarin. Qualitative and quantitative analyses of coumarin in the different tobacco types were performed using a gas chromatography (HP 5890), connected to a mass-selective detector (HP 5970), which was controlled by a microprocessor work-station (HP 59970c). The gas chromatograph was equipped with a Supelco (SP 2330) capillary column, 30 m × 0.32 mm i.d., 0.2-µm film thickness. Helium was used as a carrier gas with a linear flow of about 30 cm/s. The samples were injected in the splitless mode, the split valve being closed for 1 min after injection. The injector temperature was 220 °C; the GC oven was maintained at 100 °C for 1 min. The temperature of the oven was programmed at 18 °C/min to 200 °C and kept at this temperature for 10 min. The mass spectrometer was operated in the full scan mode (mass range 40–200 for qualitative analysis of coumarin) or in the selected ion monitoring (SIM) mode for quantitative analysis. In the latter case, the mass spectrometer was focused at m/z 146, 118, and 147, 119, the dominating fragments ($M^{\cdot+}$ and $M - CO^{\cdot+}$) for coumarin and its ^{13}C-labeled analogue, respectively. After correction for the contribution from naturally occurring ^{13}C in the peaks at m/z 147 and 119, quantification was based on peak areas relative to those of the internal standard. To correct for a possible drift in the ratio of the recorded fragments, two of the samples from the calibration curve were reanalyzed on each analytical occasion.

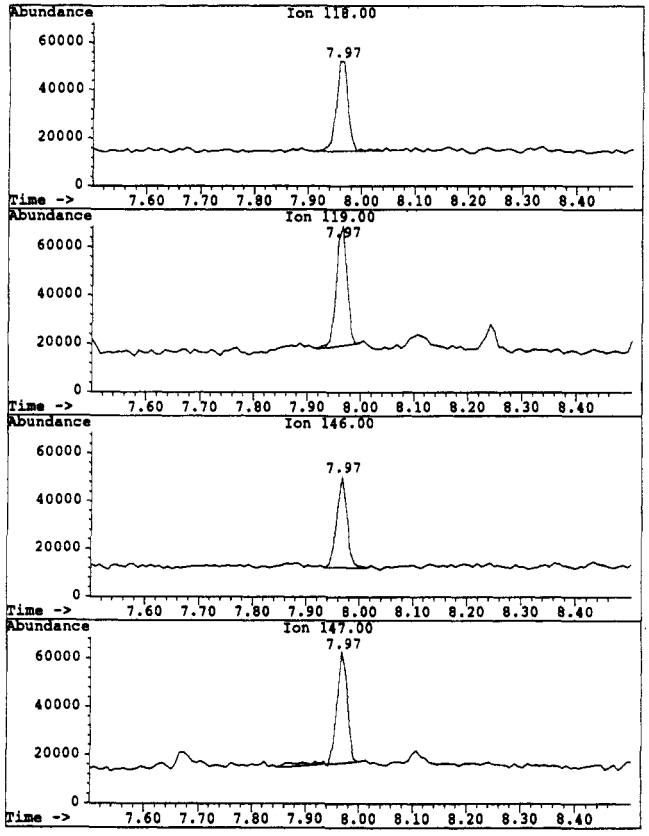

Figure 4. Mass fragmentogram of naturally occurring coumarin in a sample of American fire-cured tobacco using ^{13}C-labeled coumarin (0.4 μg/mL) as internal standard.

Table I. Comparison of Coumarin Concentrations in the Same Tobacco Sample (Spiked with Unlabeled Coumarin) Using Two Different Purification Techniques: TLC and HPLC

purification by TLC concn, μg/g	purification by HPLC concn, μg/g
7.5	7.4
7.3	7.4
7.4	7.4
7.4	7.4
7.4	7.2
$X = 7.40 \pm 0.10$	$X = 7.36 \pm 0.09$
$n = 5$	$n = 5$

Calibration. A standard curve for coumarin was prepared by adding to 50 mL of solvent (hexane–chloroform 75:25) various amounts of unlabeled coumarin (0.050–10 μg/mL) to a constant amount (0.4 μg/mL) of ^{13}C-labeled coumarin. The mixtures were worked up according to the TLC procedure described above before the analysis.

RESULTS AND DISCUSSION

The methods previously used for identification and quantification of coumarin lack in sensitivity and selectivity, which results in insufficient precision of the analytical results. The GC–MS–SIM method (Gilbert, 1987) presented here is accurate and sensitive enough for determination of natural concentrations of coumarin in tobacco. The method compensates for errors caused by the gas chromatograph (injection, column absorption), mass spectrometer (instability of the instrument), and incomplete recovery of the extraction procedure. The treatment of the tobacco samples is, however, critical; our experiments show that as much as 70% of the coumarin present may be lost by sublimation on inappropriate drying and grinding of tobacco samples. Therefore, the tobacco samples should not be dried or ground prior to the workup procedure. The analytical data thus obtained are then corrected for the moisture content of the tobacco and are expressed on a dry weight basis below.

Table II. Concentrations of Coumarin in Various Tobacco Types[a]

tobacco type	no. of replicates	concn, μg/g
American flue-cured		
type 1	2	0.3, 0.3
type 2	2	0.3, 0.2
Brazilian flue-cured		
type 1	2	0.8, 1.0
type 2	2	0.2, 0.2
type 3	2	0.3, 0.3
type 4	2	0.2, 0.1
type 5	2	0.3, 0.4
type 6	2	0..3, 0.2
type 7	2	nd, nd
Malawi flue-cured		
type 1	2	1.3, 1.2
Zimbabwe flue-cured		
type 1	2	0.3, 0.3
type 2	2	0.2, 0.2
Italian air-cured		
type 1	2	0.2, 0.3
Polish air-cured		
type 1	2	nd, nd
type 2	2	0.5, 0.3
type 3	2	nd, nd
type 4	2	1.4, 1.2
type 5	2	0.6, 0.6
American air-cured		
type 1	2	0.3, 0.2
Zimbabwe air-cured		
type 1	2	0.2, 0.2
Greek sun-cured		
type 1	2	nd, nd
Turkish sun-cured		
type 1	2	0.3, 0.2
Polish fire-cured		
type 1	2	1.7, 1.5
type 2	4	2.6, 1.7, 1.2, 1.1
American fire-cured		
type 1	4	4.3, 3.7, 2.8, 3.3
type 2	2	4.4, 3.5
type 3	4	4.7, 3.7, 4.0, 2.8
type 4	2	3.4, 3.7

[a] The results are based on replicate analysis and are expressed on dry weight basis. [b] nd, not detected.

Mass spectra of unlabeled and ^{13}C-labeled coumarin are shown in Figure 1. Coumarin exhibits an abundant molecular ion at m/z 146. As discussed by Porter (1985), loss of carbon monoxide from the molecular ion is an important fragmentation process resulting in the base peak at m/z 118. Elimination of a second molecule of carbon monoxide followed by loss of a hydrogen radical gives the peak at m/z 89. In agreement with this, the corresponding peaks are shifted to m/z 147, 119, and 90 in the mass spectrum of (3-^{13}C)coumarin.

The standard curve shows a good linearity up to a concentration of 10 μg/mL in the extract (Figure 2). The deviation observed above this concentration is probably caused by saturation and instrumental effects. Therefore, if high concentrations are to be measured (10 μg/mL in the extract; 100 μg/g in tobacco), a smaller tobacco sample should be used. The detection limit of the method is 0.050 μg of coumarin/g of tobacco.

Figure 3 displays the mass fragmentogram of a standard consisting of 0.4 μg/mL of unlabeled and ^{13}C-labeled coumarin. A mass fragmentogram of naturally occurring coumarin in American fire-cured tobacco together with internal standard (0.4 μg/mL) is shown in Figure 4. It can be seen that the retention times of the standard (Figure

3) and the endogenous coumarin in the tobacco sample (Figure 4) are in complete agreement. Moreover, the area ratios of the m/z 118/146 and 119/147 peaks are the same in the standard and in the tobacco sample, thus excluding the existence of possible interferences in the samples.

Coumarin values (in a tobacco sample spiked with coumarin) obtained by using TLC and HPLC as purification steps and subsequent mass fragmentography are shown in Table I. It can be seen that the two methods give virtually equivalent results.

Table II presents coumarin concentrations in different tobacco types including types used in the production of pipe tobacco. The levels of coumarin vary between less than 0.050 (nd) and 4.7 μg/g. The highest concentrations are found in fire-cured tobacco, a result that is most likely explained by contribution from coumarin present in the hickory smoke used in the fire-curing process. There are, however, great variations in concentrations between the replicates of some fire-cured tobaccos. The scattered values may be due to an uneven condensation of the hickory-containing smoke onto the tobacco leaf. Therefore, to establish the coumarin concentrations in such tobacco samples, several replicates must be analyzed.

ACKNOWLEDGMENT

We are grateful to Mr. Tore Pettersson for the synthesis of ^{13}C-labeled coumarin and valuable discussions and to Dr. Arne Björnberg for his interest in this work.

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Received for review February 11, 1992. Accepted May 18, 1992.

Registry No. Coumarin, 91-64-5; (3-^{13}C)coumarin, 142237-29-4; sodium (2-^{13}C)acetate, 13291-89-9; 2-hydroxybenzaldehyde, 90-02-8; (2,2′-^{13}C)acetic anhydride, 17830-01-2.