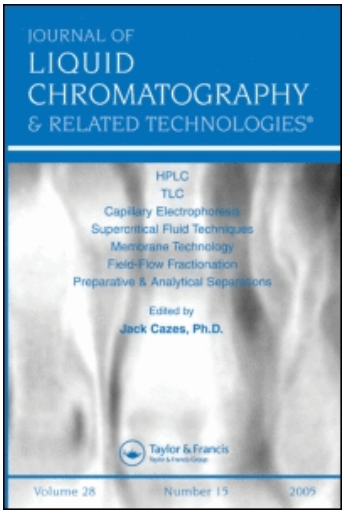

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DETERMINATION OF COUMARIN IN VANILLA FLAVORINGS BY QUANTITATIVE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

High performance silica gel TLC was used to qualitatively and quantitatively determine coumarin in real and artificial vanilla flavorings. Coumarin was detected as a fluorescent or colored zone and quantified by scanning with a densitometer. Recoveries and reproducibility values are reported for fortified samples. The method was applied to a qualitative survey of domestic vanilla samples and quantification of coumarin in an adulterated foreign sample. The result of this determination was verified by standard addition analysis. The method was also applied to the determination of coumarin in a spiked wine sample.

INTRODUCTION

Coumarin (2,3-dihydrobenzofuran) is a compound with a taste and aroma similar to vanillin that was formerly used as a flavoring agent in various foods. Since 1954, the use of coumarin as a food additive has been banned in the United States because it was found to cause liver damage in laboratory animals (1). However, in many

other countries coumarin is still added to commercial vanilla extracts, and some of these coumarin-contaminated products enter the United States.

The AOAC official analytical method for determination of coumarin in vanilla extract involves separation on a silica gel column and UV photometry (2). Qualitative thin layer chromatography (TLC) has been used to separate, detect, and identify coumarin in vanilla flavorings (1,3-5) and in vanilla-flavored food extracts (6). Densitometry was used to quantify coumarin in durgs and tonka bean tinctures or alcoholates by measurement of UV absorption of TLC zones (7).

This paper reports a convenient, accurate, and precise quantitative TLC method for analyzing coumarin-containing vanilla flavorings. Samples are directly applied to a high performance (HP) preadsorbent silica gel layer, and coumarin is separated, detected as a fluorescent or colored zone, and scanned with a densitometer. The advantages of quantitative analysis on preadsorbent thin layers were described in an earlier paper (8).

EXPERIMENTAL

Coumarin standard was purchased from Aldrich. A 1.00 mg/ml stock solution was prepared in methanol, and TLC standard solutions at concentrations of 0.500, 1.00, 10.0, and 100 ng/µl by appropriate dilution of the stock solution with methanol. Fortification of vanilla extracts was carried out using one of these standards or an additional 100 mg/ml spiking solution.

Analyses were performed on 10 x 10 cm or 10 x 20 cm Whatman LHPKD channeled, preadsorbent silica gel plates, which were predeveloped with methylene chloride-methanol (1:1) and allowed to air dry before spotting. Initial zones of samples and standards (1-10 µl) were applied to the preadsorbent area of the lanes using a 10 µl Drummond microdispenser. Three or four standards in the range of 100-800 ng for fluorescence scanning and 1-8 µg for absorbance scanning were spotted on each plate to prepare a calibration curve, along with duplicate sample aliquots having a concentration bracketed by the standards.

Plates were developed in a presaturated, paper-lined glass TLC tank with toluene-methanol (97:3) for a distance of 6-7 cm beyond the preadsorbent-silica gel junction. After air-drying, coumarin zones were detected by spraying with a 5-10% aqueous solution of sodium or potassium hydroxide and viewing fluorescence under long-wave (360 nm) UV light. Overspraying with diazotized sulfanilic acid reagent produced orange zones. The reagent was prepared by mixing 25 ml of 5% $NaNO_2$ with 100 ml of an aqueous solution containing 0.9 g of sulfanilic acid plus 9 ml of concentrated HCl, both solutions at $0^{\circ}C$.

Fluorescent zones were measured immediately using a Shimadzu CS-930 scanner with a 350 nm excitation wavelength from the deuterium source and emission filter setting 2. The absorbance of colored zones was measured with a Kontes Model 800 fiber optics scanner and HP model 3992a integrator/recorder using the 8 mm light beam and white phosphor (440 nm peak wavelength). Peak areas printed out by the densitometer systems were used for calculation

of the linear regression equation of the standard curve and for interpolation of sample concentrations from the calibration curve for each analysis.

Pure and imitation vanilla flavorings were purchased from a local grocery store. Samples were fortified with 0.10 to 20% coumarin by adding a calculated volume of one of the coumarin standards or spiking solutions to a weighed sample of flavoring. The solution was then diluted to an appropriate volume so that a 1-10 µl aliquot of sample would be bracketed by standards. For example, to prepare a 10% solution, 1 ml of spiking solution (containing 0.10 g of coumarin) was mixed with 0.90 grams of vanilla. When this mixture was diluted with methanol in a 100 ml volumetric flask, a 5 µl aliquot had a theoretical value of 5 µg, which was within the range of standards used for scanning absorbances of the colored spots. Percent recovery was calculated by comparing the average coumarin weight obtained for duplicate samples to the theoretical value, and reproducibility was evaluated by calculating the percentage difference between the duplicate sample results.

RESULTS AND DISCUSSION

Coumarin had an R_F value of about 0.54 when developed with toluene-methylene chloride (97:3). The zones appeared as bright, fluorescent bands across the lanes when sprayed with the methanolic base and viewed under longwave UV light. The zones became orange on a white background when the layer was oversprayed with

diazotized sulfanilic acid. The ability to detect coumarin as either a fluorescent or colored zone allowed quantification to be carried out by scanning fluorescence or visible absorbance, and both approaches were studied.

The lower limit for reliable scanning of coumarin was about 5 ng for fluorescence and 500 ng for absorbance. As little as 100 ppm of coumarin in vanilla could be easily analyzed by fluorescence and 0.1% by absorbance. Both of these values are below the level expected to be present in actual adulterated flavoring products.

Both fluorescent and colored zones changed intensity with time, and plates must, therefore, be scanned immediately after spraying the detection reagents. Calibration curves typically had linearity (R) values of 0.990 for 5 to 400 ng of coumarin standards for fluorescence and 1-8 μg for absorbance. To correct for small variations in slope and intercept values for different plates, three to five standards were usually chromatographed together with duplicate sample aliquots on each plate. For some analyses, samples were bracketed by only two standards, or were compared to a single standard of very similar coumarin content known to be within the linearity range of the method.

Four different vanilla flavorings were purchased and found by TLC analysis to be coumarin free: a major brand pure vanilla extract (35% alcohol); two major brand imitation vanilla flavors, one with 20% vanillin and one with less than 20% vanillin; and a generic brand artifically flavored baking vanilla containing unspecified amounts of vanillin and ethyl vanillin. To test the

fluorescence method, each of these samples was spiked with 2% and 20% (w/w) of coumarin. Recoveries for the four 2% samples averaged 96.6% with a range of 91.0-105%, and for the four 20% samples averaged 101% with a range of 94.4 to 107%. The percentage difference between duplicate sample aliquots was usually 3% or less. Figure 1 shows scans of coumarin zones from the 20% spike of one of the imitation vanillas. Only coumarin zones were visible on sample lanes when layers were viewed under UV light. However, scanning produced several other peaks, none of which interfered with the coumarin.

The absorbance method based on scanning colored zones was evaluated by spiking the vanilla samples with 10, 5, 3.3, 1, 0.5, 0.33, and 0.1 percent of coumarin. Recoveries within ±15% of theoretical were obtained at all concentrations, and the great majority were within ±10%. The average recovery of these analyses was 99.8%. Differences between duplicate samples were typically within 15%. The accuracy and precision of the absorbance method were poorer than the fluorescence method, probably because of the difficulty in applying two spray reagents as consistently as one. Figure 2 shows scans from the analysis of a 0.5% spike of the generic vanilla. Zones in addition to coumarin were visible on sample chromatograms only for the 0.1% concentration. However, as seen in Figure 2, an extra peak sometimes appeared when samples were scanned, but it did not interfere with quantification of coumarin.

A qualitative screen of some 15 brands and types of domestic vanilla flavorings showed all to be negative for coumarin. One

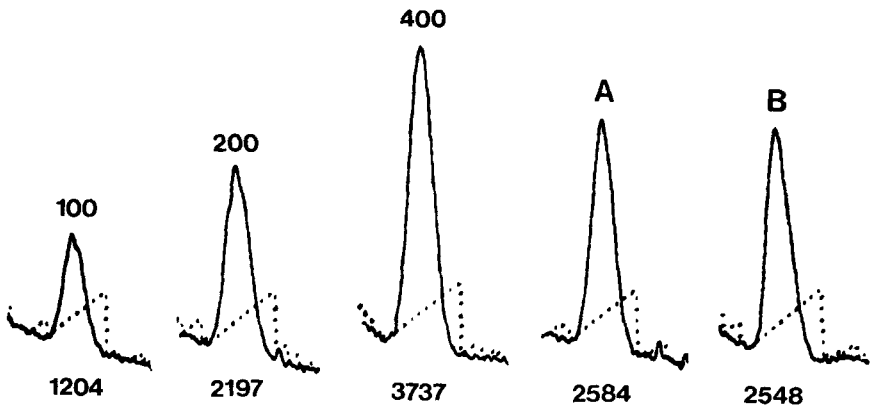

Figure 1. Shimadzu densitometer scans of the fluorescence of 100, 200, and 400 ng coumarin standards and duplicate 5 µl aliquots of a 20% spiked vanilla sample after spraying with alcoholic base. Integrator areas are shown below the peaks. The linearity (R) value of calibration curve was 0.99, and recoveries were 105% (A) and 103% (B) for the samples.

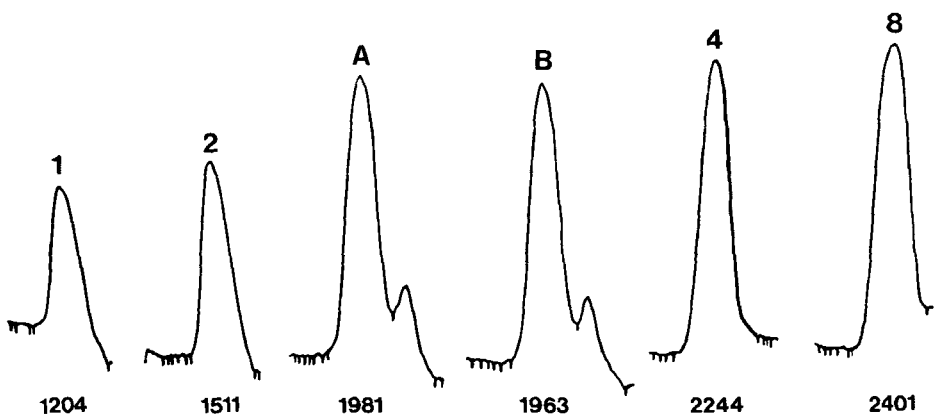

Figure 2. Kontes densitometer scans of the absorption of 1-8 µg coumarin standards and duplicate 5 µl aliquots of a 5% spiked vanilla sample after spraying with alcoholic base and diazotized sulfanilic acid. Integrator areas are shown below the peaks. The R value of the calibration curve was 0.99 and recoveries were 94.6% (A) and 92.3% (B) for the samples.

bottle of vanilla flavoring that was purchased in Mexico in 1985 tested positive and was quantified by the fluorescence TLC method: exactly 1 g of the flavoring was diluted to 20 ml with methanol in a volumetric flask. One ml of this solution was further diluted to 100 with methanol, and 3 µl was spotted along with 100–400 ng of coumarin standard. The coumarin zone in the sample was found to contain 330 ng, representing 22.0% based on the original 1 g sample. To further check the accuracy of the method, an amount of coumarin was added to the sample solution to increase the percentage by 50%, and 2 µl was spotted for TLC analysis. The 325 ng value obtained in this standard addition analysis represented 98.5% recovery of the added coumarin.

The TLC method described is a simple and convenient qualitative and quantitative screen for coumarin in vanilla flavorings. No sample preparation is necessary except dilution with methanol. Coumarin is also used as an additive in numerous perfumes and cosmetics at about the 400 ppm level (9). Attempts to apply the TLC method to these products were unsuccessful because the sample chromatograms had numerous zones, several of which interfered with scanning of the coumarin. An isolation step prior to spotting would be necessary for these samples.

Coumarin is an allowed constituent in May wine, up to a concentration of 5 ppm, if its presence is the natural result of the use of woodruff herbs, leaves, or essence for flavoring (10). The AOAC official method for coumarin in wines (11) involves chloroform extraction followed by GC with flame ionization detec-

tion. Application of the fluorescence-TLC method was tested using a May wine pre-analyzed to insure the absence of coumarin. A 100 ml sample was spiked with 0.500 mg of coumarin (5 ppm) and extracted with 20 ml of $CHCl_3$, and 20 µl of the extract was spotted for TLC. Recovery was 88.7% based on a 500 ng theoretical amount of coumarin applied to the plate.

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