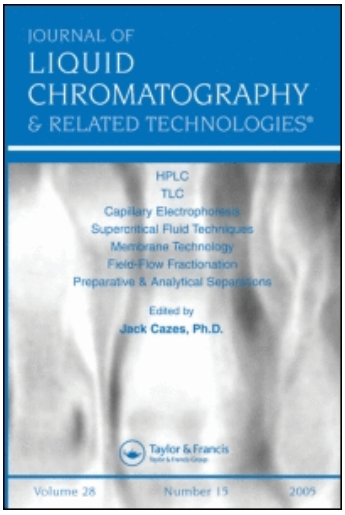


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# Determination of Natural and Synthetic Colors in Alcoholic And Non Alcoholic Beverages by Quantitative HPTLC

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# DETERMINATION OF NATURAL AND SYNTHETIC COLORS IN ALCOHOLIC AND NON-ALCOHOLIC BEVERAGES BY QUANTITATIVE HPTLC

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## ABSTRACT

Methods based on high performance thin layer chromatography with scanning densitometry and solid phase extraction were developed for quantification of lycopene and permitted synthetic food dyes in alcoholic and nonalcoholic beverages. Results of analyses of real samples are reported, and the methods were validated by determining the precision of replicate analyses and accuracy (recovery) by analyzing spiked samples.

## INTRODUCTION

Thin layer chromatography (TLC) has been used to separate and detect natural colorants added to food products to fraudulently intensify colors, such as lycopene, bixin, canthaxanthin, and beta-apo-8'-carotenal added to products derived from red pepper. The pigments were quantified by scraping the zone

off the layer and elution from the silica gel with petroleum ether followed by spectrophotometry or by gradient elution high performance liquid chromatography (HPLC) after saponification.[1] TLC combined with solid phase extraction (SPE) has been used for the identification of synthetic color additives (permitted FD&C food dyes) in nonalcoholic[2,3] and alcoholic beverages,[4] but quantification, which is usually performed by column liquid chromatography (HPLC)[5,6] was not carried out.

The purpose of the present study was to develop quantitative high performance TLC (HPTLC) methods for natural and synthetic food colors, to demonstrate the application of the methods to actual samples, and to evaluate their sensitivity, selectivity, accuracy, and precision. A direct-spotting method is described for the determination of lycopene, the natural red pigment in tomatoes, in tomato juice and paste, which would be applicable for quantitative analysis of other sample types[1] to which this pigment had been added.



Methods are also described for the quantification of synthetic food colors in nonalcoholic beverages such as fruit drinks and soda and alcoholic wine coolers by SPE combined with HPTLC. SPE is required for these samples in order to concentrate the colors and remove interferences that cause analyte zones in sample chromatograms that are streaked or do not line up with standards.

## EXPERIMENTAL

### Standards and Standard Solutions

Lycopene (92.5% purity) and carotene standards were obtained from Sigma Chemical Co. (St. Louis, MO). TLC standard solutions (92.5 ng/μL were prepared in methylene chloride. Standards of the synthetic certified food colors listed in Table 1 were obtained from Warner Jenkinson Co. (St. Louis, MO, USA) with certificates of analyzed purity.

TLC standard solutions were prepared at concentrations of 180 ng/μL in ethanol-water (1:1) for $C_{18}$ solid phase extraction (SPE) of nonalcoholic beverages and for amino-column SPE of alcoholic beverages, and in ethanol-0.2 M sulfuric acid (1:1) for amino-column SPE of nonalcoholic beverages. The Blue No. 1 solutions were diluted 1:10 (18.0 μg/μL) to prepare the TLC standard.

### Thin Layer Chromatography

TLC was carried out on 10 x 20 cm Whatman (Clifton, NJ) LHPKDF laned, high performance preadsorbent silica gel plates prewashed by development with methylene chloride-methanol (1:1). Standards and samples were applied to the preadsorbent using a 10 μL Drummond (Broomall, PA) digital microdispenser, and plates were developed in a paper-lined, vapor saturated Camag (Wilmington, NC) HPTLC twin-trough chamber. After drying with warm air from a hair drier or in an oven for ca. 5 min., the areas of the colored zones were scanned with a Shimadzu (Columbia, MD) CS-930 densitometer in the single beam, reflectance mode at the wavelength of maximum absorption for each compound, as determined from the in situ spectra recorded between 370 and 700 nm with the spectral mode of the densitometer. A calibration curve was constructed by linear regression of standard area and weight data, and the weights of analyte in the spotted sample aliquots were interpolated from the curve. Accuracy was determined by adding a known amount of dye to a blank sample not containing that dye or spiking a preanalyzed sample with an additional amount of dye naturally contained. Recovery from these spiked samples was calculated by comparing the experimental concentrations of the dye with the theoretical concentration based on the level of fortification.



### Analysis of Samples

#### Determination of lycopene in tomato juice and paste

Tomato juice was spotted directly with no sample preparation and tomato paste (6.32 g) was dissolved in 30.0 mL of deionized water prior to spotting. The lycopene TLC standard was spotted in 4.00, 6.00, and 8.00 μL aliquots (370, 555, and 770 ng, respectively) to prepare the calibration curve, along with duplicate 5.00 μL aliquots of the tomato juice and paste samples. Development of the plate with petroleum ether-methylene chloride (85:15) for a distance of 7 cm beyond the preadsorbent-silica gel interface required ca. 20 min., and the zones were scanned at 385 nm. The concentration of lycopene in tomato juice (mg/mL) was calculated by dividing the weight interpolated from the calibration curve by the volume of sample spotted.

For tomato paste (mg/g), the interpolated weight was divided by the sample volume spotted and multiplied by the volume of water in which the paste was dissolved; this weight was divided by the weight of paste used.

**Determination of Synthetic Food Colors in Non-Alcoholic Beverages by $C_{18}$ SPE**

The applicability of the quantitative TLC method was demonstrated by determining Red No. 40 and Blue No. 1 food colors in fruit drinks, and the method was vaildated by analyzing a colorless citrus soda spiked with Red No. 40.

Samples were analyzed by use of a J. T. Baker (Phillipsburg, NJ) glass manifold (no. 7018-00) operated with sufficient vacuum (ca. 15-20 mm of mercury) to produce a flow rate of ca. 3 mL/min. Bakerbond 3 mL light-loaded $C_{18}$ columns (no. 7189-03) were prewashed with 3 mL of isopropanol and 12 mL of deionized water. Columns were inserted into the manifold through stopcock-adaptors, which were adjusted to prevent the column from becoming dry during or after the conditioning stages. Following addition of 5.00 mL of sample with a pipet, the column was washed with 5 mL of 5% aqueous acetic acid to elute polar impurities, dried by drawing vacuum for 10 minutes, and removed from the manifold. Dyes were eluted into a 5 mL graduated vial with a tapered bottom by forcing an appropriate volume of the eluent through the column with gentle pressure from a rubber bulb. The eluent was adjusted to a definite volume prior to spotting for TLC analysis by dilution to a known volume with isopropanol or concentration by evaporation with nitrogen gas flow.



For the determination of Red No. 40 in a fruit punch, the dye was eluted from the SPE column with 2-2.5 mL of isopropanol-water (17:83), and the eluate volume was adjusted to 2.00 mL to prepare the TLC sample. Standard aliquots of 1.00, 2.00, 4.00, and 6.00 μL (18.0-108 ng) and duplicate 2.00 μL aliquots of sample were applied to a silica gel layer, which was developed with butanol-acetic acid-water (4:1:5). Zones were scanned at 500 nm, and the concentration of dye (mg/mL) was calculated by dividing the interpolated weight by the volume of TLC sample spotted, multiplying by the total volume of sample, and dividing again by the volume of beverage placed on the column.

The determination of Blue No. 1 in a raspberry drink was carried out as above except that the dye was eluted from the column with 2.5-3 mL of isopropanol-water (23:77), the eluate was adjusted to 3.00 mL, the 1.00-6.00 μL standard aliquots contained 180 ng-1.08 ug of dye, duplicate 4.00 μL aliquots of sample solution were spotted (delete if same as above), and zones were scanned at 630 nm. For the determination of Red No. 40 and Blue No. 1 in a grape drink, both dyes were eluted from the SPE column with 2.5-3 mL of isopropanol-water (2:8) and the eluate adjusted to 3.00 mL to prepare the TLC standard. After TLC, as described above for the samples containing the

individual dyes, the separated dye zones in the sample chromatograms were scanned at their respective wavelengths of maximum absorption and quantified against calibration curves prepared from standards of each dye chromatographed on the same plate.

To determine the accuracy of the TLC method, a 0.0500 mg/mL spiked sample was prepared by diluting 50.0 μL of a 10.0 μg/μL standard solution of Red No. 40 to 10.0 mL with decarbonated, colorless citrus soda, and a TLC standard was prepared containing 200 ng/μL of the dye in ethanol-water (1:1). Five μL of the spiked sample was analyzed by eluting the SPE column with 5 mL of isopropanol-water (17:83), adjusting the eluate volume to 5.00 mL, and chromatographing duplicate 6.00 μL aliquots of sample together with 1.00, 1.50, and 2.00 μL aliquots of the TLC standard. The theoretical weight in the sample aliquots was 300 ng for 100% recovery, which would match the 1.50 μL standard.



**Determination of Synthetic Food Colors in Non-Alcoholic Beverages by Amino Column SPE**

The applicability of the method was demonstrated by determining Yellow No. 5 and Blue No. 1 food colors in a lemon-lime drink, and the method was vaildated by analyzing a colorless citrus soda spiked with Blue No. 1.

SPE and TLC were performed in general as described in the last section with the following changes. SPE was performed on Fisher (Pittsburgh, PA) PrepSep $NH_2$ columns (no. P456), which were prewashed with 5 mL of ethanol-water (1:1) and 10 mL of deionized water. Following addition of 10.0 mL of sample with a pipet, the column was washed with 10 mL of ethanol and 40 mL of water to elute interferences, and the column was not dried by drawing vacuum. The colors were eluted with 3.5-4.0 mL ethanol-0.20 M sulfuric acid (1:1) into a 10 mL vial, and the eluate was blown down just to dryness under a nitrogen stream and reconstituted in 1.00 mL of ethanol-0.2 M sulfuric acid (1:1). The mobile phase was butanol-ethanol-water-ammonia (50:25:25:10), and 1.00, 2.00, 4.00, and 8.00 μL of the TLC standards (18.0-108 ng for Blue No. 1 and 0.180-108 μg for Yellow No. 5) were spotted with duplicate 7.00 μL sample aliquots. Blue and yellow dye zones were scanned at 630 and 420 nm, respectively.

To determine the accuracy of the TLC/amino SPE method, a 0.0500 mg/mL spiked sample of Blue No. 1 in decarbonated colorless citrus soda and corresponding TLC standard were prepared as described above for the Red No.

40 spike. Recovery analyses were performed as for described for the unknown lemon-lime drink except that 5.00 mL of sample was applied to the column; the dye was eluted with 5.0 mL of ethanol-0.2 M sulfuric acid (1:1) and the eluate volume was adjusted to exactly 5.00 mL; and 2,00, 4.00, and 6.00 μL (40.0-120 ng) and duplicate 2.00 μL of sample were applied for TLC.

### Determination of Synthetic Food Colors in Alcoholic Beverages by Amino Column SPE

Red No. 40 and Yellow No. 5 were determined in strawberry- and lemon-flavored wine coolers, respectively, using the SPE technique described above for nonalcoholic beverages except that 30.0 mL of sample was applied to the amino column, the column was washed with 10 mL of water instead of 40 mL to elute interferences, the colors were eluted with 4.0 mL of ethanol-0.5 M sulfuric acid (1:1), and the evaporated eluate was reconstituted in 1.00 mL of ethanol-water (1:1). TLC was carried out using 0.50-8.00 μL of standards and duplicate 9.00 μL (yellow) and 2.00 μL (red) aliquots of samples. Mobile phases were butanol-ethanol-water ammonia (50:25:25:10) (yellow) and butanol-acetic acid-water (4:1:5) (red), and zones were scanned at 420 and 500 nm, respectively. Accuracy was determined by analyzing a wine cooler containing Yellow No. 5 and then spiking the sample with a sufficient volume of a 1.83 mg/mL stock solution to double the dye concentration. The analysis was repeated and duplicate 4.5 μL aliquots of the spiked sample were spotted with duplicate 9.0 μL aliquots of the original sample on the same plate. The average scan areas of the two samples would be equal for 100% recovery.



## RESULTS AND DISCUSSION

### Determination of Lycopene

Lycopene standard formed a red-orange flat, oval-shaped zone with an $R_f$ value of 0.32 when developed with petroleum ether-methylene chloride (85:15) on preadsorbent HPTLC silica gel plates. Development with petroleum ether-acetonitrile-methanol (2:2:4) on a Whatman LKC-18F reversed phase bonded silica gel layer produced a compact lycopene standard zone with an $R_f$ value of 0.24, but directly-spotted samples did not chromatograph as well on the $C_{18}$ plate as on silica gel, which was used in all analyses. No sample preparation was required for analysis of tomato juice or dissolved tomato paste because directly spotted samples produced zones that matched standards in migration distance and shape. The in situ visible spectrum of a standard zone exhibited a

major absorption peak at 385 nm and a smaller peak at 440 nm. This spectrum differed markedly from the spectrum recorded in petroleum ether, which had three roughly equal peaks at 446, 475, and 505 nm.[7] Carotene, which is also present in tomatoes, was separated from lycopene as a yellow v-shaped zone with an $R_f$ value of 0.57. The red zones from the samples and the lycopene standard had identical $R_f$ values and in situ spectra, which confirmed the identity of lycopene in the sample.

For quantification, samples and standards were scanned at 385 nm immediately after TLC because zones faded with increased time of standing due to photochemical air oxidation, resulting in decreased scan areas. The calibration curve prepared from the area of the three standard aliquots had a linear regression correlation coefficient (r value) of 0.99. A sample of tomato juice was analyzed four times and the average concentration of lycopene found was 0.123 mg/mL with a coefficient of variation (CV) of 3.7%, while dissolved tomato paste assayed at 0.483 mg/g with a CV of 0.72%. As another indication of the excellent precision of the quantitative TLC method, the differences between the scan areas of the duplicate 5.00 μL sample aliquots were 1.2% and 1.8%, respectively.



Although the applicability of the new direct TLC method was demonstrated for the analysis of two tomato products, it is equally appropriate for the quantification of lycopene in red pepper products to which it has been added as a coloring agent. The same extraction and solvent partition cleanup methods already described for indirect quantification by spectrometry after scraping and elution of TLC zones and proven to provide quantitative recoveries[1] would be used prior to the much simpler described densitometric TLC method. The method can also be combined with the alumina column chromatographic isolation of lycopene in tomato paste described in university laboratory manuals[7] to form the basis of an excellent organic/analytical student experiment.

**Determination of Synthetic Food Colors**

The $R_f$ values of 11 synthetic colors encountered in beverages are shown in Table 1 for the two mobile phases used in this study. Red No. 40, Blue No. 1, and Yellow No. 5, which are among the most widely used colors, were chosen to test the quantitative methods for nonalcoholic beverages. With both mobile phases, the dyes formed compact, flat, v-shaped zones that produced narrow, symmetrical scan peaks. Calibration curves typically had r values of 0.96-0.99.

**Table 1**

**$R_f$ Values of Synthetic Colors**

| Synthethic Color | System 1* | System 2** |
|---|---|---|
| Blue No. 1 | 0.25 | 0.63 |
| Green No. 3 | 0.25 | 0.62 |
| Yellow No. 5 | 0.06 | 0.60 |
| Yellow No. 6 | 0.29 | 0.66 |
| Amaranth | 0.06 | 0.62 |
| Red No. 3 | 0.94 | 0.64 |
| Red No. 4 | 0.35 | 0.86 |
| Red No. 40 | 0.32 | 0.64 |

* System 1: butanol-acetic acid water (4 : 1 : 5).
** System 2: butanol-ethanol-water-ammonia (50 : 25 : 25 : 10).
Blue No. 2 fades quickly during TLC development.



Triplicate analysis of fruit punch containing Red No. 40 yielded an average concentration of 0.0620 mg/mL with a CV of 2.0%. Duplicate analysis of blue raspberry drink gave an average Blue No. 1 concentration of 0.00825 mg/mL and a 3.6% difference between trials. Duplicate analysis of grape drink found an average of 0.0923 mg/mL of Red No. 40 with a 1.5% difference between trials and 0.00120 mg/mL of Blue No. 1 with an 8.3% difference. Triplicate analysis of the spiked soda yielded an average concentration of 0.488 mg/mL with a CV of 2.6%. The average recovery was 97.5%. During these analyses, the percent difference in area between scans of duplicate sample aliquots ranged from 0.70-2.8%.

These accuracy and precision results show that the $C_{18}$ SPE/TLC method is truly quantitative when used for the analysis of nonalcoholic beverages. Recovery of Red 40 from the spiked sample was excellent, and visual observations during the analysis of Blue No. 1 indicated that no color was left on the columns. The method will most likely be applicable to beverages containing the other dyes in Table 1. Mobile phases for elution of the dyes from the SPE column are obtained from the identification scheme presented by Young.[3] For analysis of beverages containing a mixture of colors, the compounds must be separated by selective elution from the SPE column, or eluted together and separated on the TLC plate. As an example of separation on a column, 5.00 mL of a colorless soda spiked with Yellow No. 5 (0.0200 mg/mL) and Red No. 3 (0.0700 mg/mL) was analyzed, and the mixture was

completely separated by elution of the yellow dye with 3.0 mL of isopropanol-water (2.5:97.5) and the red dye with the same volume of isopropanol-water (1:1). If a dye mixture is not separated either on the column or the TLC plate, it is still be possible to analyze the compounds individually by scanning at specific wavelengths where one absorbs and the other does not.

For analyses of nonalcoholic beverages using amino-column SPE, standards were prepared in the same ethanol-sulfuric acid solution as used to elute the column and the basic mobile phase (solvent 2, Table 1) so that the sample and standard zones lined up and sample zone streaking was minimized. The dyes are retained on the SPE column because of ionic interactions with the amino groups,[4] rather than hydrophobic interactions as in $C_{18}$ SPE, and the dyes are all eluted together so that selectivity must be achieved by TLC separation or scanning at specific wavelengths.



Duplicate analysis of the lemon-lime drink resulted in identical concentration values of 0.118 mg/mL for Yellow No. 5, and a mean of 0.000747 mg/mL with a difference of 1.2% for Blue No. 1. For these analyses, typical differences between the scan areas of the duplicate sample aliquots was 4.5%. Duplicate analysis of the spiked sample gave an average of 0.0485 mg/mL with a difference of 2.1%, which corresponded to a recovery of 97.0%.

For analysis of wine coolers using amino column SPE, preparation of standards and reconstitution of evaporated SPE column eluates in ethanol-water (1:1) resulted in the best line-up of sample and standard zones and sample least zone streaking. Triplicate analyses of a wine cooler containing Red No. 40 gave an average value of 0.00282 mg/mL with a CV of 8.1%. Analysis of a cooler containing Yellow No. 5 gave a similar dye content value, 0.00192 mg/mL. The spiking experiment on this sample yielded a percent difference of 2.8% between areas of the original and fortified samples, proving the accuracy of the analysis.

The purpose of these studies was to demonstrate the quantitative determination of natural and synthetic food colors using HPTLC and SPE. The optimal approach to analyzing unknown samples involves spotting a series of dye standards providing linear response along with a range of sample volumes, in order to find at least one sample whose scan area is bracketed by standards. Quantification can then be done more reliably by spotting duplicate aliquots of a single sample volume and a smaller range of standard volumes within the linear response region that more closely bracket the sample. Because of this method of in-system calibration, the quantitative results obtained will be at

least as accurate and precise as those achieved by spectrophotometry or HPLC, and the ability to spot multiple samples on the same plate and the small solvent volume used for plate development provide very favorable sample throughput and cost-effectiveness.

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