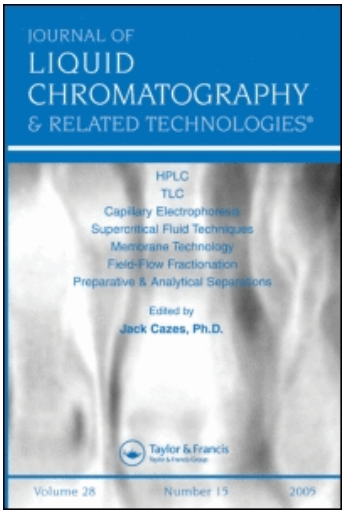


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# Quantitative TLC Determination of Stevioside and Rebaudioside A in Beverages

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# QUANTITATIVE TLC DETERMINATION OF STEVIOSIDE AND REBAUDIOSIDE A IN BEVERAGES

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## ABSTRACT

A method for the determination of stevioside or rebaudioside A in beverages by TLC is described. The natural sweeteners are determined directly at concentrations of 100-200 ppm; below 100 ppm, the sample is purified by a reversed phase silica gel 60 silanized column chromatographic procedure. Quantification by scanning densitometry is carried out after TLC on C-18 preadsorbent layers and detection with anisaldehyde-sulfuric acid reagent. Recoveries from various beverages spiked at 10-200 ppm ranged from 81.8 to 111.0% (stevioside) 87.5 to 111.6% (rebaudioside A). TLC on HP silica gel allows separation of stevioside and rebaudioside A from each other, qualitative identification of the sweeteners, and rapid screening of beverages for their presence.

## INTRODUCTION

Stevioside and rebaudioside A are natural sweeteners that have been commercially used in a variety of beverages and foods in Japan, including pickles, soft drinks, ices, and

candy (1). The compounds have been detected in such products in the range of 3-440 ug/g (2).

The only specific method reported for determination of these compounds in beverages and foods involves extraction with water from the sample, purification of the extract on a reversed phase silica gel 60 silanized column, qualitative detection by silica gel TLC using anisaldehyde-sulfuric acid reagent for visualization, and quantification by HPLC on an amino-bonded column using a mobile phase of acetonitrile-water (200:45) containing tetrabutylammonium phosphate and UV detection at 210 nm (1). The HPLC method was verified at stevioside and rebaudioside A concentrations of 10 and 100 ppm.



Since we had used anisaldehyde-sulfuric acid detection reagent earlier as the basis of a quantitative TLC method for determination of pulegone in foliage, stem, roots, and flowers of Hedeoma pulegioides (3), it was decided to apply this reagent to the TLC quantification of stevioside and rebaudioside A in beverages. Only two previous papers reporting the TLC/densitometry of steviosides were found in the literature (4, 5), and neither of these involved the analysis of beverages or foods. No earlier papers on the densitometry of rebaudioside A were found.

The quantitative TLC method was tested using tea and soft drinks fortified with 10-200 ppm of stevioside and rebaudioside A. Determination at 100-200 ppm was carried out directly without extraction or cleanup, while extraction and cleanup by column chromatography preceded TLC at 10-50 ppm.

## EXPERIMENTAL

### Standards

Standards of stevioside and rebaudioside A were obtained from Wako Pure Chemical Industries, Ltd. (Richmond, VA USA). Stock standard solutions containing 1.00 mg/ml and 10.0 mg/ml of each sweetener in 80% aqueous ethanol were diluted appropriately to prepare TLC standards and spiked beverages.

### TLC analysis

Normal phase TLC was carried out on 20 x 10 cm Whatman (Clifton, NJ USA) LHPKDF preadsorbent high performance silica gel plates containing 19 lanes; reversed phase TLC was performed on 20 x 20 cm Whatman LKC18DF preadsorbent, laned C-18 bonded silica gel layers.

Sample and standard zones were applied using a 25 ul Drummond (Bromall, PA USA) digital microdispenser, and plates were developed in a solvent-equilibrated, paper-lined glass chamber with chloroform-methanol-glacial acetic acid (90:60:12) (development distance 8 cm) on silica gel and with methanol-water (7:3) (15 cm) on C-18 bonded silica gel. The mobile phase was removed by drying at room temperature in a fume hood after development.

Analyte zones were detected by spraying the layer with a fresh solution prepared by mixing, in order, 90 ml of ethanol, 5 ml of conc. sulfuric acid, 1 ml of glacial acetic acid, and 5 ml of p-anisaldehyde and heating the plate immediately for 10-15 minutes in an oven set at 110-115°C. The reagent was uniformly sprayed twice, with brief air

drying between applications. If spots are not seen after heating, the warm plate should be sprayed again and returned to the oven.

The sample and standard zones were scanned at 648 nm using a Shimadzu CS-930 densitometer in the single beam, reflectance mode. The densitometer was zeroed at the same Y-coordinate position just above the zone to be scanned in each lane.

Sample preparation and spotting protocol

Spiked samples were prepared at nominal concentrations of 200, 150, and 100 ppm by combining 0.20, 0.15, and 0.10 ml of the stevioside or rebaudioside A 1.0 mg/ml standard with 0.80, 0.85, and 0.90 ml of beverage, respectively. The volumes were measured with a 1000 ul Drummond digital dispenser. Standards were prepared at the same concentration by substituting 80% ethanol for the beverages. Analyses were performed without any preparation by direct spotting of duplicate 3.0, 4.0, and 6.0 ul aliquots of each sample and the same volume of the respective standard on adjacent lanes. Using this procedure, the theoretical weight spotted for each sample was 600 ng. Percent recovery was calculated as (average sample scan area/standard area)x100.

Spiked samples at levels of 10, 30, and 60 ppm were prepared by adding respectively 0.10, 0.30, or 0.60 ml of the 10 mg/ml stevioside or rebaudioside A standard to 100 ml of beverage. Analyses of these samples were performed by adapting the silanized (C-2 bonded) silica gel 60 (EM Science, cat. # 7719, Cherry Hill, NJ) column cleanup

procedure described earlier (1). Columns (2.5 g, 22 mm id) were prewashed with methanol and water. Sample sizes applied were 4.0 g for the 60 ppm spike, 8.0 g for 30 ppm, and 24 g for 10 ppm, each dissolved in 30 ml of deionized water. Impurities were eluted in turn with pH 7 phosphate/borate buffer, water, and 20% aqueous methanol. The analytes were eluted with 30 ml of 80% methanol, the solution was evaporated just to dryness, and the residue was reconstituted with 2.0 ml of 80% ethanol. Duplicate 5 ul aliquots of each test solution (representing 600 ng of analyte for 100% recovery in all cases) were spotted adjacent to a standard sample containing 600 ng for determination of recoveries.



## RESULTS AND DISCUSSION

Stevioside and rebaudioside A were detected as blue zones on a pink background on both C-18 and silica gel layers. The background and zone colors were stable over a period of at least several hours. On C-18 developed with the methanol-water mobile phase, both compounds formed flat, elliptical-shaped zones with $R_f$ values of 0.34 and limits of visual detection and scanning of 50 ng and 100 ng, respectively. No solvent system could be found that provided separation of the two analytes on the C-18 layer.

On silica gel developed with chloroform-methanol-acetic acid, stevioside and rebaudioside A were resolved with $R_f$ values of 0.34 and 0.25, respectively, but the zones were diffuse and not completely resolved from interfering

TABLE 1

AVERAGE PERCENTAGE RECOVERIES FROM FORTIFIED SAMPLES

| Sample | | S | R |
|---|---|---|---|
| 200 ppm | 1 | 97.3 | 88.3 |
| | 2 | 106.8 | |
| | 2 | 102.3 | |
| | 3 | 89.6 | 111.6 |
| | 3 | | 96.7 |
| | 3 | | 89.8 |
| | 4 | 106.8 | |
| | 5 | | 89.4 |
| 150 ppm | 1 | 87.7 | 104.8 |
| | 2 | 111.0 | 110.9 |
| | 3 | 93.3 | 101.8 |
| | 4 | 88.4 | |
| | 6 | 89.8 | 101.8 |
| 100 ppm | 1 | 100.0 | 95.5 |
| | 1 | 105.7 | |
| | 2 | 87.5 | 100.2 |
| 60 ppm | 1 | 85.2 | 87.5 |
| | 2 | 81.8 | 106.3 |
| | 5 | 99.5 | 94.4 |
| 30 ppm | 1 | 86.2 | 99.0 |
| | 2 | 110.5 | |
| | 5 | | 91.5 |
| 10 ppm | 1 | 93.3 | 92.1 |
| | 2 | 88.1 | |
| | 5 | 93.4 | 100.4 |
| | 5 | 93.2 | |

S=stevioside, R=rebaudioside A; Samples: 1=cola, 2=brand 1 of citrus soda, 3=commercial diet iced tea, 4=brand 2 of citrus soda, 5=brewed tea, 6=diet cola; multiple entries indicate that different samples of the same beverage were analyzed.

compounds in chromatograms of directly spotted samples. Even after column chromatography, analyte zones were too diffuse to allow reliable measurement by scanning, although few additional zones appeared on the chromatograms. On C-18, the additional detected zones from directly spotted samples formed a heavy streak from $R_f$ 0.75 to the solvent front, and

they were well resolved from the analytes and did not interfere with scanning.

The wavelength used for densitometric quantification (648 nm) was the absorption maximum noted on the in situ spectra recorded for 200 ng zones of stevioside and rebaudioside A. Calibration curves generated from standards of both compounds were linear (correlation coefficient 0.99) from 100-1600 ng. The amounts of samples and standards applied during analyses were, therefore, within the linear range, allowing a single standard to be used to quantify samples.



Table 1 shows average recoveries obtained for samples of various beverages spiked with 100-200 ppm (direct application method) and 10-60 ppm (column cleanup method). Values ranged from 88.4 to 111.0% for the direct application method and 81.8 to 110.5% for the lower concentrations requiring column concentration and cleanup. The effectiveness of the column procedure when combined with C-18 TLC was indicated by the recoveries obtained as well as the absence of zones interfering with scanning of the analyte in any of the chromatograms at the three concentration levels. All beverage samples were not spiked with both sweeteners, but rather a reasonable variety of samples was tested in order illustrate the level of accuracy it is possible to achieve by use of the TLC method. As a measure of precision, there was typically a 5-7% difference between the scan areas of duplicate samples.

In summary, the C-18 TLC method allows quantification of stevioside or rebaudioside A in a variety of common

beverages. Although reported recoveries were more consistent for the earlier HPLC procedure (1), the accuracy and precision of the TLC method are reasonable for samples containing ppm levels of the sweeteners. Beverages can be directly spotted if the concentration is 100 ppm or higher, but cleanup and concentration by C-2 column chromatography is required at lower concentrations. The ability to spot multiple samples on a single plate provides high sample throughput. The silica gel TLC method allows rapid screening of samples and qualitative identification of the sweeteners, but quantification must be carried out by the C-18 TLC or previously-reported (1) HPLC method.



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