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QUANTITATIVE ANALYSIS OF SUGARS BY DENSITOMETRIC INSPECTION OF THIN-LAYER CHROMATOGRAMS: ANALYSIS OF METHOD

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SUMMARY

This paper describes a method for quantifying thin-layer chromatograms, presents data supporting the importance of running separate standards for each sample replicate, discusses possible variation due to spotting errors, and supplies statistical data for comparisons to other studies.

INTRODUCTION

The basic methods used to analyze thin-layer chromatograms quantitatively can be divided into four groups: (1) elution before measurement[1-5]; (2) *in situ* densitometric comparisons[1,6-9]; (3) visual evaluation[1,10]; and (4) measurement of spot area[1,11,12].

The immediate problem is to determine which of these basic methods will yield the required degree of precision. Precision can be estimated by the variance of repeated samples. Also, precision may be expressed in terms of the ratio of the standard deviation to the mean as the coefficient of variation. Unfortunately, many workers[2,9-11] have used the statistics "percent of error" or "percent accurate", which are probably a measure of bias and not of precision. Others[6,12] have made no statistical attempt to test the precision of their method. If all workers had expressed precision in terms of the coefficient of variation, more meaningful comparisons could be made. It is important also to record the number of replications because as replications increase to a certain level, coefficient of variation may become less.

In this report we include a detailed description of *in situ* densitometric quantitation of free sugars on TLC sheets. Also, we present data supporting the importance of cochromatography of standards for each constituent, discuss variation due to spotting

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errors, and supply the basic statistical data so that this method may be compared to others.

EXPERIMENTAL

Materials and methods

Samples of one-year-old needles were collected randomly from the entire crowns of eight 12–16-year-old western white pine trees (*Pinus monticola* Dougl.). Immediately after collection, the needles were submerged in liquid nitrogen and placed in dry ice for transporting to laboratory freezers. A motorized mortar and pestle was used to pulverize the samples which were submerged in liquid nitrogen; then the samples were dried by reduced pressure for 48 h.

Extraction and clearing. All samples were extracted by using chloroform in Soxhlet extractors for 5 h to remove waxes, fats and pigments. Free sugars were extracted using 80% methanol in Soxhlet extractors for 16 h. 80% methanol was added to the extract to equal 100-ml volume. A 25-ml aliquot of each sample was treated with 4 ml of a saturated solution of neutral lead acetate to precipitate substances that interfere with chromatography. 7 ml of saturated sodium phosphate were added to precipitate the excess lead. All precipitated materials were removed by centrifugation. Desalting of the samples was accomplished with ion-exchange columns (Amberlite IR-120 and IRA-400*). After desalting, all samples were concentrated by reduced pressure to 10-ml volume and stored at —10°. At this point the samples were ready for chromatography.

Chromatography. Eastman 20 × 20 cm chromatogram sheets (6060 Silica Gel with fluorescent indicator) were cut into 5 × 20 cm strips and dipped in a 0.1 *M* solution of monobasic potassium phosphate[2,13–15]. The wet chromatograms were dried at 85° for 90 min and then stored over calcium chloride at room temperature. Each chromatographic strip received a standard and sample spot. MARTIN AND WELCH[15] found streaking was a problem when spot concentrations were above 4 μg per sugar**. 2 μg of each sugar standard was therefore considered satisfactory for this experiment. To control spot size[16,17], it was necessary to spot 0.1 μl at a time with a precision microsyringe (Kensington Scientific Co.). Forced air from a hair dryer, without heat, was used to dry between 0.1-μl aliquots.

After spotting, the chromatograms were developed in either solvent system A (ethyl acetate–pyridine–water, 8:2:1) or solvent system B (isoamyl alcohol–pyridine–water, 4:4:1)[15].

Solvent system A was used to separate fructose, glucose, and sucrose (two multiple developments)[15] and solvent system B, raffinose and stachyose (two multiple developments). After development, the sugars were localized by dipping the chromatograms in a solution containing 2 g diphenylamine, 2 ml aniline, 20 ml of 85% phosphoric acid in 200 ml acetone. The chromatograms were then air dried for 15

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** Spot concentrations of 1–3 μg of each sugar resulted in a linear relationship between sugar quantity and integral number.

min, excess phosphoric acid wiped from the chromatograms' backs and then placed in a 100° oven for 5 min.

Densitometer. A Joyce Loebel MK.11 densitometer equipped with a 300–400 nm filter and a 1 × 9 mm slit was adjusted for transmission scanning of the strips. A drive gear ratio of 1:3 was used. This particular densitometer has an automated integrating system which yields an integral number that was used in comparisons of standards to samples.

Procedure

A sample was spotted adjacent to standards on each of sixteen chromatograms (5 × 20 cm); a set of eight chromatograms was developed in solvent system A and the remaining in solvent system B. A given set of eight chromatograms was developed within the same chromatography tank, thus keeping tank effects and development time constant.

RESULTS AND CONCLUSIONS

Fig. 1A illustrates the TLC separation of fructose, glucose, and sucrose, using solvent system A. Separation of raffinose and stachyose, using solvent system B, is depicted in Fig. 1B. The accompanying traceouts with integral numbers for the respective chromatograms are included in these figures.

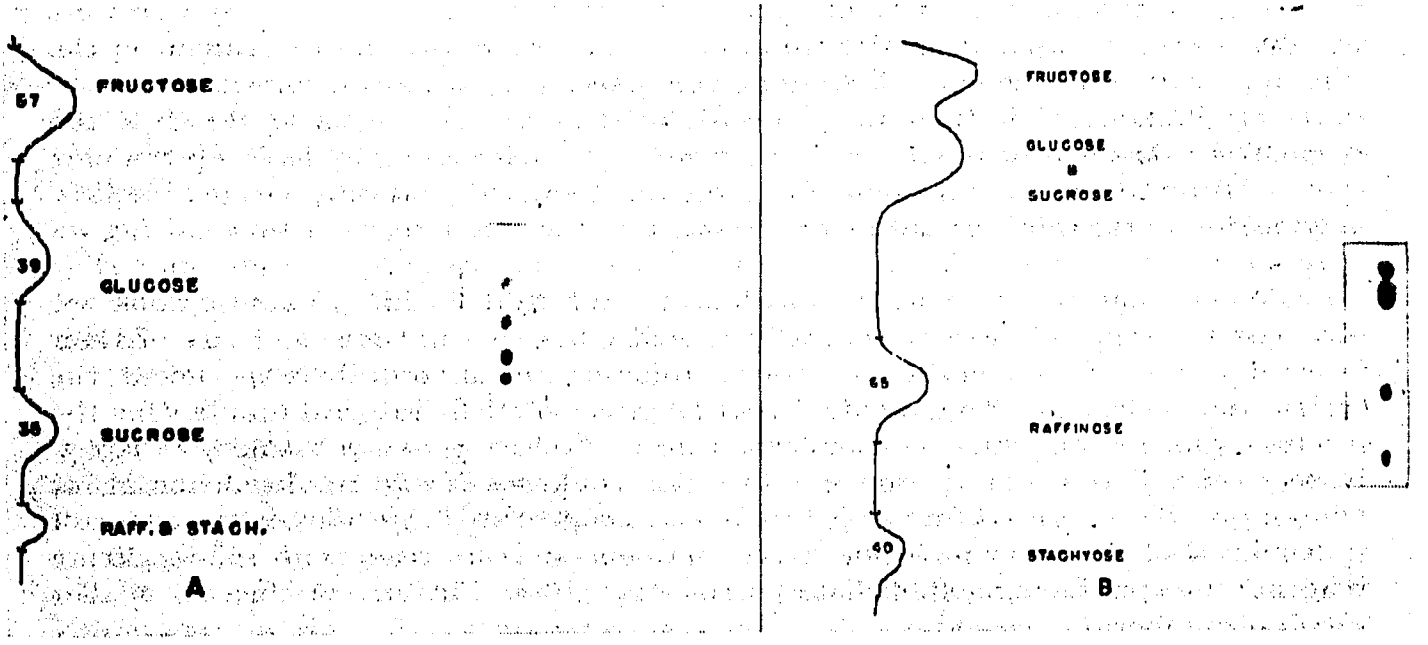

Fig. 1. (A) TLC separation of fructose, glucose, and sucrose with two developments in system A (ethyl acetate–pyridine–water, 8:2:1); (B) TLC separation of raffinose and stachyose with two developments in system B (isoamyl alcohol–pyridine–water, 4:4:1).

The means ($\bar{X}$) and coefficients of variation (CV) for concentrations of each sugar in the eight needle samples are given in Table I. All values were based on eight chromatographic replications. The coefficients of variation ranged from 4.5 to 11.0% for fructose; 5.5 to 14.5% for glucose; 7.2 to 15.1% for sucrose; 7.9 to 12.7% for

TABLE I

MEANS ($\bar{X}$)[a] AND COEFFICIENTS OF VARIATION (CV) OF SUGAR CONCENTRATION BASED ON EIGHT CHROMATOGRAPHIC REPLICATIONS OF EACH NEEDLE SAMPLE

Sugar	*Sample*							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
Fructose								
$\bar{X}$	16,983	13,584	15,358	18,275	13,327	17,498	15,129	12,850
CV	7.8	10.3	7.6	11.0	6.2	4.9	5.4	4.5
Glucose								
$\bar{X}$	16,553	11,712	12,605	14,761	11,259	14,030	13,166	10,287
CV	7.6	14.5	10.6	10.5	9.1	7.5	10.5	5.5
Sucrose								
$\bar{X}$	5,996	8,907	10,605	9,809	10,153	7,605	7,141	14,731
CV	9.5	10.8	11.3	12.5	14.5	7.2	8.1	15.1
Raffinose								
$\bar{X}$	9,032	10,016	9,672	10,604	9,410	10,470	10,213	11,993
CV	11.1	12.0	12.7	11.7	7.9	8.0	8.8	8.3
Stachyose								
$\bar{X}$	2,096	2,772	2,962	3,282	3,058	2,405	3,134	3,039
CV	14.8	13.8	8.8	22.2	13.2	10.9	12.1	12.8

[a] μg of sugar/g dried needle tissue.

raffinose; and 8.8 to 22.2% for stachyose. It appears that quantitative analyses of those sugars developed in solvent system A (fructose, glucose, sucrose) were less variable as the distance from the origin increased*. Fructose, the lead sugar on the chromatogram, was less variable than its neighboring sugar glucose, which was closer to the origin. Glucose, in turn, was less variable than sucrose, the sugar closest to the origin. These observations could mean two things: (1) each sugar may have a particular spot diffusion that may be optimum for its measurement; or (2) the degree of variability may be due to the inherent nature of the sugar molecule reactivity with localizing reagents.

When quantity of sugar standards was a constant for all chromatograms, we observed a strong relationship between spot lengths, or diffusion, and size of their integral number. Based on the sixty-four chromatograms used in this experiment, the correlation coefficients for individual spot lengths and their integral number for the fructose, glucose, and sucrose standards were $\gamma = 0.879$, $\gamma = 0.708$, and $\gamma = 0.963$, respectively. This strong degree of association could be due to the localizing agent being more efficient on a diffused spot than on a compact spot; perhaps, in the compact spot not all of the sugar molecules have an opportunity to react with the localizing reagent, thus producing a spot that absorbs less light and in turn produces a smaller integral number.

It is evident in the above that differences between spot diffusions, resulting from chromatography of standards and samples in separate runs (developments), may introduce quantitation error. In order to minimize this error standards and samples were cochromatographed in sets of eight replications. Analysis of variance and test of significant difference[18] between means was undertaken on the integral numbers obtained from the eight standard sets of fructose (each set based on eight chromato-

* *i.e.*, as spot size increased.

TABLE II

ANALYSIS OF VARIANCE AND TEST OF SIGNIFICANT DIFFERENCES AMONG THE MEAN INTEGRAL NUMBERS OF THE FRUCTOSE STANDARD SETS

	Analysis of variance			
	Degrees freedom	*Mean squares*	*F*[a]	
Among sets of standards	7	1,725.06	47.33	
Within sets of standards	56	36.45	—	

	Test of significant differences							
	7	1	4	3	6	2	5	8
Spot length								
$\bar{X}$	38.5	37.1	45.5	45.5	45.6	48.4	50.5	61.1
CV	4.4	7.0	9.1	6.5	9.9	6.8	11.1	7.4
Integral number								
$\bar{X}$	67.3	68.4	78.6	83.1	84.1	89.3	90.0	113.9
CV	4.2	7.9	10.1	6.9	4.6	9.7	6.0	6.4
Significant difference[b]								

[a] $\alpha = 0.01$.

[b] Any two integral means not underscored by the same line are significantly different at the 99% level, Scheffe's test[18].

graphic replications). Fructose was considered typical for all sugars. The results of these tests plus the means and coefficients of variation for spot lengths are given in Table II. Some means were significantly different ($\alpha = 0.01$)[18]. For example, the mean integral number for standard set 7 was 67.3 compared to 113.9 for standard set 8. Therefore *gross error* would occur if the integral numbers of standard set 8 were used to calculate the concentration of 7's samples. This clearly points out the importance of running separate standards for each constituent within a sample set as a means of lowering error. We believe that the major cause of these significant differences between sets was due to the differences in spot diffusions caused by uncontrollable tank and development variations.

A possible source of variation within a chromatographic replication of eight could be due to spotting errors[16,17]. To demonstrate this, a single chromatogram within a sample set would have to contain all the lowest or highest values for each sugar measured. From the analyses of eight sample sets (each set equals eight chromatographic replications) the expected number of single chromatograms having either all the low or high values for each sugar measured would be, in both cases, eight. After examining the sixty-four chromatograms, on a per sample set basis, we found that four sample sets contained single chromatograms having all low values and one sample set contained a single chromatogram having all high values. Therefore, we do not agree with FAIRBAIRN AND RELPH[16] and SAMUELS[17] that spotting error need be a significant source of variation in quantifying thin-layer chromatograms.

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