Rapid and Simple Method for Estimation of Sugar Esters

Y. Lin and G. J. Wagner*

Plant Physiology/Biochemistry/Molecular Biology Program, Department of Agronomy, University of Kentucky, Lexington, Kentucky 40546-0091

A procedure is described for a simple and highly sensitive quantitative assay of various sucrose and glucose ester mixtures extracted from plant tissue surfaces and of certain synthetic sugar esters of commercial importance. The method involves staining sugar esters with Rhodamine B followed by spectrophotometric detection of the "complexed" stain. The only instrumentation required consists of a colorimeter, spectrophotometer, and microplate reader or, alternatively (for higher sensitivity), a spectrofluorometer. The minimum detectable level at A_{550nm} is 10 μg, while that measuring fluorescence is 0.1 μg. The stain does not appear to differentiate among sugar ester types.

Keywords: *Sugar esters; Rhodamine B; histochemistry; trichome exudate*

INTRODUCTION

Sucrose and glucose ester mixtures are principal components of trichome exudates produced by certain *Nicotiana*, *Lycopersicon*, *Solanum*, and *Datura* species. Sucrose esters (SE) of *Nicotiana* are shown to be synthesized exclusively in trichome glands (Kandra and Wagner, 1988) from sucrose and activated acids derived from branched-chain amino acid metabolism (Wagner, 1991). Numerous studies indicate that these compounds are involved in plant–pest interactions (Cutler et al., 1992; Goffreda et al., 1990; Severson et al., 1991). In addition, tobacco sucrose esters have important organoleptic properties (Severson et al., 1985).

In *Nicotiana*, SE mixtures are well characterized and are grouped into over eight types of acetylated or nonacetylated 2,3,4-tri-*O*-acyl-α-D-glucopyranoside β-D-fructofuranosides, where acyl groups are C_3–C_8 straight or branched aliphatic acids (Cutler et al., 1992). While all known *Nicotiana* SE contain three acyl groups on the glucose moiety, the nature of groups occurring at specific positions (2, 3, or 4) can vary, as can the nature of mixtures of acyl groups found in a SE type. Also, the number and position of acetyl groups occurring in SE containing them may vary. Thus, the number of distinct SE structures produced by members of the genus *Nicotiana* alone is tremendous. The biological significance of this species-specific diversity is not understood. Synthetic SE [i.e., sucrose diacetate hexaisobutyrate (SAIB), sucrose octaacetate, etc.] have commercial applications in emulsifiers, adhesives, detergents, denaturants, etc. Their usefulness principally lies in their amphipathic nature and viscous properties.

Quantitation of sugar ester mixtures is generally made using gas chromatography of their trimethylsilyl ethers or by HPLC. An advantage of the gas chromatography approach is that it can resolve SE types to some extent, as well as separate SE mixtures from leaf surface terpenes and hydrocarbons in a single analysis (Severson et al., 1985). Direct chemical ionization mass spectrometry has been reported to be a quantitative method for analysis of underivatized SE (Einolf and Chan, 1984). HPLC provides separation of terpenes and SE without the requirement for derivatization and—unlike gas chromatography—allows complete recovery. However, various SE types in mixtures are not well separated by this method using existing systems (Guo et al., 1994). All of the above methods require expensive instrumentation having limited portability. TLC methods can separate or partially separate terpenes and SE but are not quantitative.

Histochemical methods for detecting organic constituents in plant cells generally suffer from the fact that strong acids, organic solvents, heat, or clearing with chloral hydrate are often required to bring about staining, increasing the potential for artifact. A water-soluble stain that would selectively or semiselectively and rapidly complex a compound of interest at room temperature would be ideal as a histochemical stain for use with living tissue. Rhodamine B is extensively used as a TLC-spray-detection reagent for lipids, generally as a solution in 95% EtOH. However, its relatively high solubility in H_2O along with its high extinction and stability in solution makes it useful for staining lipophilic and amphipathic molecules with high sensitivity in the absence of tissue-damaging conditions and reagents. We have shown in qualitative experiments that this dye can be used to localize SE on the plant leaf surface, apparently without altering the natural disposition of exudate chemicals. We also find that Rhodamine B can be used to detect SE accumulated on insects which had walked on a stained leaf surface (Lin and Wagner, 1994). Thus, the stain can be used to monitor aspects of insect behavior and insect/surface–chemical interactions.

Here we describe a rapid, simple, and inexpensive method for quantitative estimation of SE after formation of SE–Rhodamine B complexes.

EXPERIMENTAL PROCEDURES

Plant Materials and Assay Procedures. *Nicotiana tabacum* cv. T.I. 1068 plants were grown in pots in the greenhouse. Pieces of leaf lamina were cut from fully expanded leaves and dipped (20 s) in a vial containing 10 mL of 100% acetonitrile to dissolve trichome exudate. For most species tested 2–10 cm^2 of laminar tissue was sufficient for assay. After removal, the area of leaf pieces was determined.

Acetonitrile washes were evaporated to dryness *in vacuo*, and then 5 mL of 0.5% (w/v) Rhodamine B in H_2O (large excess) was added and the sample held for 10 min. The dye solution was decanted, and the sample was rinsed five times (~5 s each, without agitation) with 10-mL aliquots of H_2O to remove unbound stain. The SE–Rhodamine B complex remains as a red-blue film/syrup. To dissolve the complex, 2.0 mL of 50% acetonitrile in H_2O was added. The absorption (spectrophotometer) or fluorescence (fluorometer) of the solu-

0021-8561/94/1442-1709$04.50/0

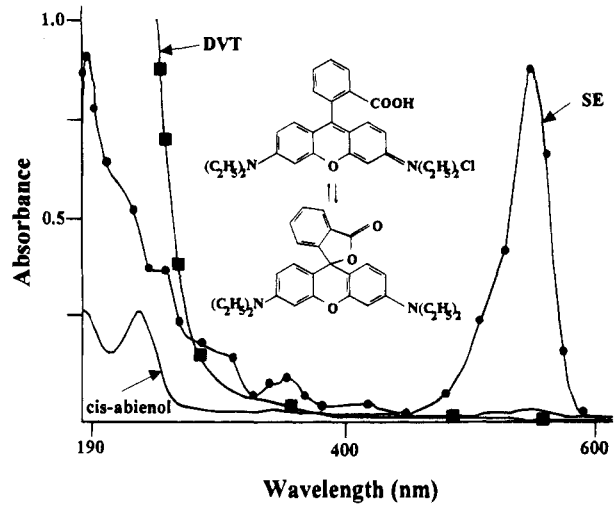

Figure 1. Absorption spectrum of SE mixture–Rhodamine B complex. SE were obtained from *N. tabacum* cv. T.I. 1068. Spectra represent dye retained by exudate and lack of retention by equivalent amounts of duvatriene diols (DVT) and *cis*-abienol, the other major components of T.I. 1068 trichome exudate.

tion was monitored at 550 nm or with excitation 550 nm/emission 582 nm, respectively. All manipulations and solutions were at room temperature (~23 °C). To stain SE on tissue surfaces, tissue pieces were submerged in 0.2% Rhodamine B in H_2O for 60 min and then washed by submersion in 4 separate volumes of H_2O (5 s in each) to remove unbound stain. Rhodamine B was purchased from Sigma Chemical Co., St. Louis, MO.

To establish the relationship between absorption at 550 nm and micrograms of SE, a sample was prepared from T.I. 1068 by washing mature leaves with acetonitrile. The extract was filtered through Whatman No. 1 paper and concentrated *in vacuo*. Total SE were then purified by HPLC, collected, and evaporated to dryness. A weighed portion was used to prepare a dilution series containing 5–200 μg of SE. Aliquots of known concentration were placed as drops on microscope slides, and solvent was allowed to evaporate. Slides were stained and washed, and SE–Rhodamine B complex was recovered with 50% acetonitrile in H_2O and assayed as described above.

To assay using microplates, extracts (250 μL) were placed into wells of standard polystyrene microplates (96 wells, Fisher Scientific Co.) and solvent was evaporated under the heat of an incandescent lamp (~15 min). Alternatively, solvent may be evaporated overnight without heat. Staining and washing was as described above (scaled down), and color was read in with a microplate reader at A_{543nm}. Alternatively, leaf disks (1-cm diameter) were placed into wells of a 24-well poly(vinyl chloride) plate, and exudate was extracted with 2 mL of acetonitrile for 15 s. Leaf pieces were removed, and solvent was evaporated and stained as above. This method (all steps made directly in the microplates) may be applicable for assay of large numbers of samples collected in the field (for example, in a plant breeding study). Although polystyrene and poly(vinyl chloride) are said to be unstable to acetonitrile, we observed minimal deterioration of plastics under the conditions described here. Deterioration increases as the volume of acetonitrile used is increased.

HPLC analysis of free unstained SE mixtures employed a C_{18} column in reversed-phase mode and elution as described earlier (Guo et al., 1994). Briefly, SE in 100% CN were fractionated using an Econsphere C_{18}, 5-μm column (250 × 4.6 mm, Alltech Associates Inc., Deerfield, IL). Elution at 1.2 mL/min was with 70% CN in H_2O, and detection was at A_{214nm}.

RESULTS AND DISCUSSION

Figure 1 shows the absorption spectrum for a SE–Rhodamine B complex (80 μg of SE/mL) of T.I. 1068 SE. A single absorption peak at 549.6 nm with a shoulder

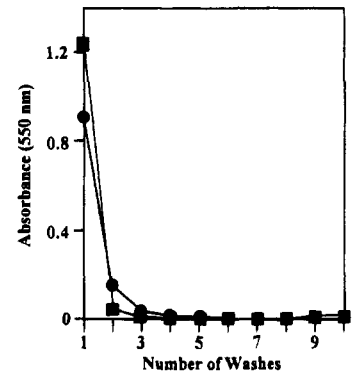

Figure 2. Effectiveness of sequential washing to remove unbound Rhodamine B from SE. Washes 1–8 were as described under Experimental Procedures (5 s each), while washes 9 and 10 were for 5 min each. Squares represent HPLC purified SE (100 μg) and circles total exudate (T.I. 1068), 100 μg of each. Data represent Rhodamine B occurring in wash solutions.

at 513 nm was observed. The absorption spectrum of Rhodamine B alone in 50% acetonitrile was virtually identical to that of SE–Rhodamine B in the region of ~A_{550nm} (not shown). Absorption at ~A_{550nm} is attributed to the anhydride form of Rhodamine B (Figure 1 insert). Similar quantities of T.I. 1068 exudate duvatriene diols (DVT) and the labdane *cis*-abienol (the other two major trichome exudate components of T.I. 1068) had no absorption at 550 nm after staining (Figure 1). Similar results were obtained for the tobacco exudate labdanes sclareol, manool, and 2-hydroxymanool (not shown). Virtually no A_{550nm} was obtained with leaves prewashed with 100% acetonitrile to remove exudate. The color of complexed SE–Rhodamine B in solution was stable at room temperature for at least 4 days when stored in a closed vial. There was no significant difference in absorption when staining was for 5, 10, or 20 min (0.5% dye). Between 0.02 and 0.5% dye concentration, the intensity of staining 200 μg of T.I. 1068 SE increased in proportion to dye concentration but was not increased thereafter (data not shown). An uncomplexed dye washout curve is shown in Figure 2.

Analysis of exudates from various tobaccos (about 100 μg/mL) showed that the absorption maxima for SE–Rhodamine B complexes were similar and like that of Rhodamine B alone (Table 1). SE mixtures of the tobaccos analyzed are mixtures of compounds that vary in their acyl acid composition (Severson et al., 1985); therefore, extinction coefficients could not be determined. Petunia SE–Rhodamine complexes also had an absorption maximum similar to that of tobaccos tested. Unlike tobacco, petunia SE contain substantial straight-chain C_6–C_8 acyl groups. The synthetic SE sucrose laurate also had a similar absorption maximum. Thus, the nature of acyl groups does not appear to influence greatly the qualitative aspects of SE–Rhodamine B complexes. It was not possible to apply the assay described to measure sucrose octaacetate because this compound is too water-soluble and was dissolved during washing (with H_2O or EtOH) to remove excess dye. With the synthetic SE sucrose diacetate hexaisobutyrate, stain did not penetrate beyond the surface layer. A similar result was obtained using myristic acid, a major epidermal wax acid of tobacco epidermis. Glucose esters from tobacco had an absorption spectrum like that of SE. Rhodamine 6G (also used to stain lipids after TLC) also bound to SE and could be used in the assay. SE–

Table 1. Absorbance Maxima of Sugar Ester–Rhodamine B Complexes[a]

sample	peak absorbance (nm)	sample	peak absorbance (nm)
sucrose esters from *N. tabacum*		sucrose esters from *N. glutinosa*	
cv. Kentucky black	549.4	cv. 24B	549.8
cv. Kentucky 14	549.4	cv. 24A	550.0
cv. Red Russia	549.4	sucrose esters from petunia,	549.6
cv. T.I. 1068	549.6	cv. Falcon Red and White	
cv. KDH 960	549.6	sucrose monolaurate	550.2
cv. Samsun	549.6	myristic acid	550.0
cv. NC 2326	549.6	glucose ester[b]	549.0
cv. NFT	549.8		
cv. TI. 1223	549.8		

[a] Absorbance peaks of Rhodamine B in H_2O and in 50% acetonitrile are 553.6 and 549.2 nm, respectively. [b] Glucose esters purified from *N. tomentosiformis* were a gift from R. Severson.

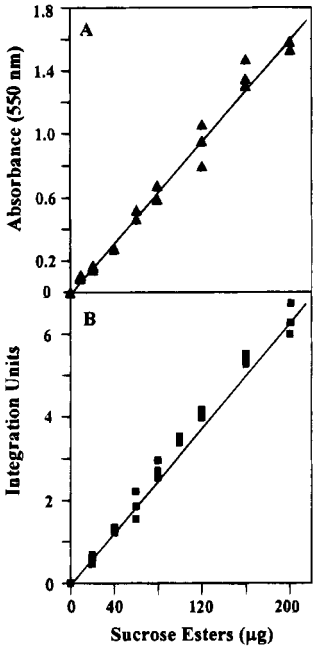

Figure 3. Comparison of the precision of Rhodamine B and HPLC methods for quantitation of SE from *N. tabacum* cv. T.I. 1068: (A) determined using the Rhodamine B method, detection as A_{550nm}; (B) determined using HPLC, detection as A_{214nm}.

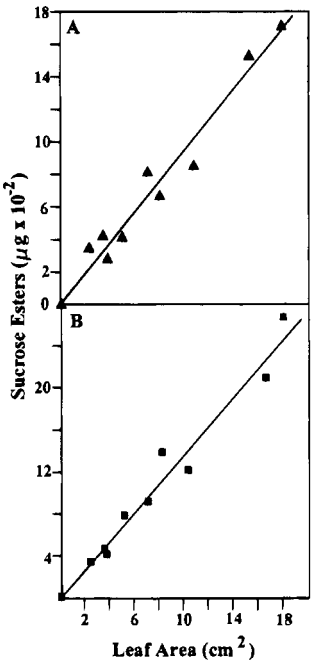

Figure 4. Sucrose ester mixture quantity vs leaf area, *N. tabacum* cv. T.I. 1068: (A) Rhodamine B method (A_{550nm}); (B) HPLC method (A_{214nm}).

Rhodamine 6G had an absorption maximum at 527.4 nm.

The relationship of absorption at 550 nm and sucrose ester content (T.I. 1068) was reproducible (correlation coefficient, 0.98) and linear between 0 and 200 μg/mL (Figure 3A). Using T.I. 1068 exudate, the mean of 12 separate estimations was 14.6 ± 1.6 μg/mL, a total procedural variability at a single concentration of about 11%. The standard curve for SE of T.I. 1068 monitored at A_{214nm} using HPLC is shown in Figure 3B. With this method, a correlation coefficient of 0.99 was observed.

Figure 4 compares SE mixture content of T.I. 1068 extract on a leaf area basis using the Rhodamine B assay (part A) versus the HPLC method of quantitation (part B). In these experiments, aliquots of the same tissue washes were used for the HPLC and Rhodamine B assays. As shown, the HPLC assay indicated about a 50% higher SE/cm^2 leaf area than did the Rhodamine B assay. This may, in part, be due to detection of other A_{214nm} absorbing substances in the region of SE eluting from HPLC. Alternatively, SE may be solubilized and lost during staining and washing steps required to remove noncomplexed stain. To determine the occurrence and/or extent of loss, samples of standard T.I. 1068 SE on glass slides were carried through the procedure, but Rhodamine B was omitted. SE remaining on slides and that recovered from all solutions used were quantitated by HPLC. Loss from slides was found to be ~10% and that recovered in stain-mimicing and -washing solutions was ~10%. Therefore, only a portion of the difference between Rhodamine B and HPLC methods of quantitation (Figure 4, part A vs part B) can be attributed to losses in the Rhodamine B procedure. We conclude that a portion of the A_{214nm} found in the region of SE elution from HPLC represents compounds other than SE and that the Rhodamine B method may be more representative of SE mixture quantity than the HPLC method used. For a given SE analysis task, the percent loss of SE in the Rhodamine B method may be established (as above) and factored into the quantitation. The relationship between the HPLC and Rhodamine B methods is compared in Figure 5. Regression analysis showed results obtained using the two methods were well correlated with regard to response versus concentration range.

In an attempt to clarify the observed differences between HPLC and Rhodamine B derived results, samples were partitioned with $CHCl_3$–H_2O (1:1) prior to analysis. This resulted in about 30% loss of amphipathic SE to the H_2O phase. Higher HPLC versus Rhodamine B derived values were again obtained. The

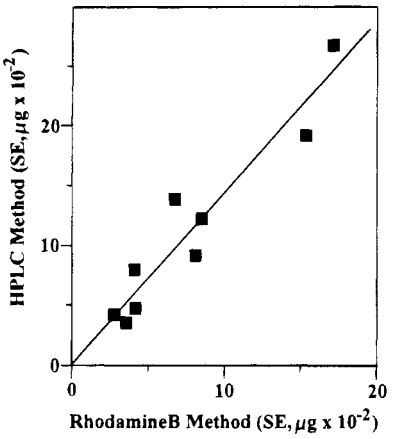

Figure 5. Comparison of the Rhodamine B and HPLC methods for quantitation of SE mixtures. For the Rhodamine B assay, designated amounts of T.I. 1068 SE were stained, dissolved in 50% (v/v) acetonitrile, and monitored using a 1.5-mL glass cuvette.

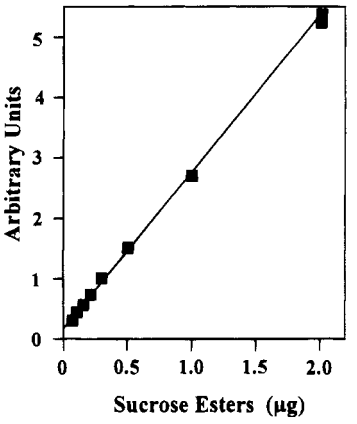

Figure 6. Detection of Rhodamine B–SE mixture by fluorescence, concentration vs relative fluorescence. SE in 50% acetonitrile were monitored using a 1.5-mL glass cuvette.

phenomenon was not unique to T.I. 1068 SE, as it was observed using extracts from five other tobacco types (data not shown).

Literature values for SE content of mature, field-grown T.I. 1068 leaf determined using GLC (trimethylsilyl derivatives) range from 19 to 35 $\mu g/cm^2$ leaf (Severson et al., 1985). This range compares better to the mean value of 95 $\mu g/cm^2$ obtained here using the Rhodamine B method than to the mean of 150 $\mu g/cm^2$ found with the HPLC method (the latter two from intermediate-age, greenhouse-grown plants).

As shown in Figure 6, detection sensitivity when measuring SE–Rhodamine B fluorescence is about 100 times greater than that using A_{550nm} (Figure 3A). Minimal detectable SE using fluorescence is about 0.1 μg (Figure 6), while that at A_{550nm} is about 10 μg (Figure 3A). The intensity of the fluorescence measurement is such that SE associated with a single aphid which had walked on a stained T.I. 1068 leaf surface for 15 min was easily quantitated (not shown). This result is not surprising given that the extremely high extinction of Rhodamine B makes this dye suitable as a deposition tracer in environmental studies (Sundaram, 1988).

In an effort to examine the nature of the SE–Rhodamine B complex, we determined the effect of pH on A_{550nm}. Samples of T.I. 1068 SE–Rhodamine B complex were suspended in 50% acetonitrile–H_2O titrated to pH 2.5–11.5 with HCl or NaOH. We observed the same pH-dependent difference in absorption maximum of complexed and free dye. At pH 2.5 and 11.5, absorption maxima were 556.8 and 550.8 nm, respectively. The fluorescence of Rhodamine dyes was somewhat enhanced at alkaline pH. Thus, association of dye with SE does not appear to alter spectroscopic properties of Rhodamine B. This result is consistent with the apparent lack of effect of varying SE type (acyl group composition) on SE–Rhodamine B absorption spectrum (Table 1). Further study is needed to establish the chemical and steric nature of the SE–Rhodamine B association.

In summary, we describe a simple, sensitive, rapid, stable, and inexpensive method for monitoring sugar esters, one that is easily adapted to large numbers of samples. Sensitivity is further increased when the fluorescence of sugar ester–Rhodamine B complexes is measured. The method represents a histochemical technique as well as an analytical one. The assay may be easily taken to the field and is amenable to microplate technology.

ACKNOWLEDGMENT

We thank Dr. L. Shain, Department of Plant Pathology, University of Kentucky, for assistance with microphotography and Dr. M. Jackson, USDA Crops Research Laboratory, Oxford, NC, for discussions concerning insect–exudate interactions. We also thank I. Deaton for typing the manuscript.

LITERATURE CITED

Cutler, H. G.; Severson, R. F.; Cole, P. D.; Sisson, V. A.; Jackson, M. D.; Stephenson, M. G. The biological activity of some mixed sucrose and glucose esters from exotic tobacco cultivars. *PGRSA Q.* **1992**, *20*, 18.

Einolf, W. N.; Chan, W. C. Estimation of sucrose esters in tobacco by direct chemical ionization mass spectrometry. *J. Agric. Food Chem.* **1984**, *32*, 785–789.

Goffreda, J. C.; Steffens, J. C.; Mutschler, M. A. Association of epicuticular sugars with aphid resistance in hybrids of wild tomato. *J. Am. Soc. Hortic. Sci.* **1990**, *115*, 161.

Guo, Z.; Severson, R. F.; Wagner, G. J. Biosynthesis of the diterpene *cis*-abienol in cell-free extracts of tobacco trichomes. *Arch. Biochem. Biophys.* **1994**, *308*, 103–108.

Kandra, L.; Wagner, G. J. Study of the site and mode of biosynthesis of tobacco trichome exudate components. *Arch. Biochem. Biophys.* **1988**, *265*, 425–432.

Lin, Y.; Wagner, G. J. Surface disposition and stability of pest-interactive, trichome exudated diterpenes and sucrose esters of tobacco. *J. Chem. Ecol.* **1994**, *308*, 103–108.

Severson, R. F.; Johnson, A. W.; Jackson, D. M. Cuticular constituents of tobacco: Factors affecting their production and their role in insect and disease resistance and smoke quality. *Recent Adv. Tob. Sci.* **1985**, *11*, 105–174.

Severson, R. F.; Jackson, D. M.; Johnson, A. W.; Sisson, V. A.; Stephenson, M. G. Ovipositional behavior of tobacco budworm and tobacco hornworm. In *Naturally Occurring Pest Bioregulators*; Hedin, P. A., Ed.; American Chemical Society: Washington, DC, 1991.

Sundaram, K. M. S. High performance liquid chromatographic determination of three tracer dyes used in forestry spray formulations. *J. Environ. Sci. Health (B)* **1988**, *23*, 53.

Wagner, G. J. Secreting glandular trichomes: More than just hairs. *Plant Physiol.* **1991**, *96*, 675.

Received for review January 19, 1994. Revised manuscript received April 28, 1994. Accepted May 25, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1994.