but rather large in sample 2. The reason for this is not yet apparent and these differences will be studied more thoroughly.

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Quantitating the Recovery of Thiamine (Vitamin B_1) from Decalso in the Thiochrome Method for the Determination of Thiamine

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In the determination of thiamine (vitamin B_1) by the thiochrome method, purification of sample extracts on the cation exchanger, Decalso, is usually necessary. This purification step, as usually carried out, can be a major source of error because recovery of thiamine from Decalso is low (about 90%) and varies ±8.5% from the mean recovery value. A study of the variables involved showed that eluent temperature and volume are important factors affecting thiamine recovery. Determination of the quantitative relationship between these factors and thiamine recovery led to a minor modification of the purification step that gave nearly quantitative (97.5% average) recovery of thiamine from Decalso and thus substantially enhances the precision and accuracy of the thiochrome method for the determination of thiamine.

In a study at the Western Regional Research Laboratory, on the effect of air pollutants on nutrients in food, we have determined thiamine in hundreds of samples, principally vegetables, by the thiochrome method and, in the process, have critically reexamined some aspects of this widely used procedure.

Purification of the sample extract, with an artificial silicate cation exchanger or its commercially available equivalent, Decalso, is generally recommended in the thiochrome method (Freed, 1966; Strohecker and Henning, 1966). Because omission of this step left fluorescent impurities in our sample extracts, we also had to routinely include the Decalso purification step in our determination of thiamine in fruits and vegetables.

If sample extracts are treated with Decalso, the thiamine standards also should be treated (Freed, 1966; Strohecker and Henning, 1966). However, during 5 months in which we used recommended procedures, our recoveries of thiamine were low and inconsistent (Table I). Mean recovery (89.4%, Table I) was substantially less than the 92–96% recovery deemed satisfactory (Freed, 1966) and the wide range (16.7% of mean recovery value) and large standard deviation indicated that the Decalso purification step was a major source of error.

Previous investigators too have found that recovery of thiamine from Decalso may be incomplete and irregular. Jowett (1940) reported only 68% recovery over a wide range of concentrations, Harris and Wang (1941) recommended omission of the absorption step because it caused variable loss of thiamine, and Bechtel and Hollenbeck (1958) reported recoveries ranging from 84 to 100%.

Low recovery that is consistent may be acceptable in a quantitative method but with inconsistent recovery, precision and accuracy suffer in proportion to the magnitude of the inconsistency. We, therefore, investigated the Decalso purification step to determine if the variables could be manipulated to improve recovery of thiamine. The variables tested were thiamine/Decalso ratio, regeneration of Decalso, and temperature and volume of eluent.

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MATERIALS AND METHODS

Reagents. Decalso (Thiochrome Decalso, Permutit T, Fisher Scientific Co. No. T-97) was used as received except in one test with regenerated Decalso (Table II). Decalso was regenerated (or reactivated), after one cycle of thiamine adsorption and elution with acid-KCl, as described by Freed (1966). Water and isobutyl alcohol were redistilled in glass. A stock thiamine solution (100 μg of thiamine hydrochloride/ml of 0.01 *N* HCl) prepared from a dry reference standard was appropriately diluted for either direct fluorometric determination or for application to Decalso columns. For direct fluorometric determinations the stock was diluted with acid-KCl reagent. For application to Decalso columns, the stock was diluted so that the resulting solution contained the equivalent of 5.0 ml of 2.5 *M* sodium acetate per 100 ml and was about 0.075 *N* in HCl.

Thiamine in pinto beans was determined by extracting the ground beans with hot 0.1 *N* HCl, treatment with a dual enzyme mixture consisting of takadiastase and papain in sodium acetate buffer, filtration, Decalso treatment, etc. as described by Freed (1966).

All other reagents were prepared as described by Freed (1966).

Apparatus. The borosilicate glass columns consisted of an 8 mm o.d. × 14.5 cm tube having a capillary tube (3.5 cm long × 6 mm o.d. × 0.5 mm i.d.) sealed to one end and a reservoir (35 mm o.d. × 11.7 cm long) sealed to the other (top) end. Maizel-Gerson reaction flasks used were Wilkins-Anderson (1972) Catalog No. 15845-00. Fluores-

Table I. Recovery of Thiamine Analytical Standards from Decalso over a 5-Month Period[a]

Recovery, %	
94.2	96.1
84.0	92.6
96.1	81.0
88.0	86.6
84.3	91.2
Mean:	89.4
Range:	15.1
Std dev:	5.39

[a] Standard (25 ml) was applied to columns containing 3.0 g of Decalso followed by hot washes and hot elution with acid-KCl to a volume of 25 ml.

cence was measured on a Turner Model 110 fluorometer, operated in the least sensitive setting, and zeroed with the opaque (dummy) cuvette provided with the instrument.

Preparations of Columns. A 3-g (±0.02 g) portion of dry Decalso per column was weighed into a 30-ml beaker. Each column was plugged with a bit of glass wool just above the inlet to the capillary. Water sufficient to fill the column up to about one-half of reservoir capacity was added and any air bubbles in the water were eliminated. From this point, a flow of water through the column was maintained until the sample was added. The 3-g portion of Decalso in the beaker was covered with water, stirred to release air, then transferred, with water rinses, to the column where the Decalso was allowed to settle and pack solely by gravity and the flow of water through the column. A head of water was maintained above the Decalso up to the time the thiamine or sample extract was added.

Addition of Samples to Prepared Decalso Columns. Most of the water above the Decalso was aspirated out and 25.0 ml of the thiamine-containing sample was pipetted into the reservoir just as the remaining water disappeared into the Decalso. By gravity only, the sample was then allowed to run into the column followed by three 10-ml hot (90–95°) water washes.

Elution of Thiamine from Decalso. Thiamine was eluted from Decalso with acid-KCl reagent applied to the column in approximately 10-ml portions. For "cold" elutions, acid-KCl reagent was at room temperature. For "hot" elutions, the reagent was heated to 90–95° on a hot plate, but, to reduce concentration, boiling was avoided. The eluate was collected in either a 25-ml or a 50-ml volumetric flask and elution was carried out to the almost full volume. Eluates were diluted to the mark with acid-KCl; hot eluates were first permitted to cool to room temperature.

Formation and Measurement of Thiochrome. Thiochrome was formed and extracted into isobutyl alcohol essentially as described by Freed (1966). A blank was run, as nearly simultaneously as possible, with each sample. Duplicate 5.0-ml portions of the thiamine solution were placed into each of two Maizel-Gerson reaction flasks. The alkali blank was first formed in one flask by adding to it 3.0 ml of 15% sodium hydroxide with a Labindustries repipet, and swirling for about 2 sec. Then, 15.0 ml of isobutyl alcohol was immediately added, with a Labindustries repipet; the tube was shaken vigorously by hand for about 2 sec and set aside. The second flask was similarly treated except 3.0 ml of alkaline potassium ferricyanide was added instead of 15% sodium hydroxide. The two flasks were then shaken for 90 sec on a mechanical shaker and centrifuged briefly to speed phase separation, and the water layers removed by aspiration. Anhydrous sodium sulfate, used to dry the remaining isobutyl alcohol layer, was added with a small measuring cup (about 2-g capacity), the flasks were shaken for 30 sec and centrifuged briefly, and the isobutyl alcohol decanted into matched cuvettes for fluorescence determination. Net fluorescence of the sample equals its fluorescence minus blank fluorescence.

Table II. Effect of Thiamine/Decalso Ratio on the Recovery of Thiamine Analytical Standard from Decalso Columns[a]

μg of thiamine/g of Decalso	Thiamine recovery from: Decalso used as received, %	Thiamine recovery from: Decalso, used once, regenerated,[b] %
0.208	87.7	87.7
0.417	94.4	96.1
1.67	87.2	90.8
2.50	87.8	90.4
3.33	92.6	94.4
6.67	91.4	89.3
Mean[c]	90.18	91.45
Range	7.2	8.4
Std dev	3.03	3.18

[a] Standard (25 ml) applied to columns containing 3.0 g of Decalso followed by hot washes and hot acid-KCl elution to a volume of 25 ml. [b] Regeneration is also referred to as "reactivation" or "purification." [c] Comparison of means by t test: t(calcd) = 1.46; $t_{0.05}$(10 df) = 2.23.

Table III. Effect of Eluent Temperature on the Recovery of Thiamine Analytical Standard from Decalso[a]

Aliquot no.	Eluted from Decalso: Hot, net fluor.	Eluted from Decalso: Cold, net fluor.	Eluted from Decalso: Cold/hot, %	Recovy of thiamine std from Decalso[b]: Hot, %	Recovy of thiamine std from Decalso[b]: Cold, %
1	67.1	61.0	90.9	88.1	80.1
2	66.9	59.4	88.8	87.8	78.0
3	65.1	56.3	86.5	85.4	73.9
4	70.3	58.5	83.2	92.3	76.8
Mean	67.4	58.8	87.4	88.4	77.2

[a] Thiamine adsorbed onto the Decalso column from 25-ml aliquots and then eluted to a volume of 25 ml. [b] Based on a net fluorescence of 76.2 which the thiamine standard (0.075 μg/ml) showed before adsorption on Decalso.

RESULTS AND DISCUSSION

The quantity of Decalso used per column, the nature of the sample, and the volume of sample extract applied determine the load of adsorbables per gram of Decalso (LA/g of Decalso). The nature and quantity of adsorbables in extracts of many samples, such as fruits and vegetables, are seldom precisely known; therefore, in such samples the LA/g of Decalso is also imprecisely known. But, when an aliquot of standard thiamine solution is applied to a column containing a known weight of Decalso, the LA/g of Decalso can be calculated precisely. This ratio, as reported by Bechtel and Hollenbeck (1958), can be critical to the recovery of thiamine. Therefore, we carried out an experiment to determine if variations in this ratio, in a range we were likely to use, influenced thiamine recovery. In this experiment we used Decalso as received and after regeneration.

Considering first the effect of variations in the thiamine/Decalso ratio, we see (Table II) that a 32-fold difference in this ratio, in a range bracketing our practical

Table IV. Effect of Eluent Temperature on the Recovery of Thiamine Adsorbed on Decalso from Pinto Bean Extracts[a]

Sample no.	Eluted from Decalso: Hot, net fluor.	Cold, net fluor.	Cold/hot, %
Low thiamine levels			
1	35.8	32.1	89.7
2	35.7	31.4	88.0
3	30.8	26.9	87.3
4	33.1	30.3	91.5
5	31.4	27.5	87.6
6	32.7	30.7	93.9
		Av	89.7
High thiamine levels			
1-A	80.9	72.2	89.2
2-A	77.2	70.3	91.1
3-A	77.7	67.5	86.9
4-A	80.9	66.9	82.7
5-A	75.2	68.2	90.7
6-A	80.2	72.5	90.4
		Av	88.5

[a] Per column extract (25 ml) and 25 ml of eluent.

analytical concentrations, did not affect thiamine recovery. Therefore, variation of this ratio does not explain the low recovery of thiamine which is evident in Table II as it was in Table I.

Considering next the comparison between Decalso as received and regenerated Decalso, we see (Table II) that the mean recoveries of 90.18 and 91.45% do not differ significantly (see *t*-test calculation at bottom of Table II). Therefore, regenerated Decalso offered no advantage over Decalso as received.

Temperature of the acid-KCl solution used to elute thiamine from Decalso is mentioned in most publications on the thiochrome method, but hot elution is suggested only to increase rate of eluent flow. In the literature we found no reference to the effect of eluent temperature on recovery of thiamine from Decalso, so we investigated this.

With hot eluent, mean recovery was 88.4% (Table III) which again is comparable to our previous recoveries, under similar conditions, of 90.2 and 91.4% (Table II) and 89.4% (Table I). But, with cold (room temperature) eluent mean recovery was only 77.2% (Table III) so, on the average, cold elution recovered only about 87% as much thiamine as hot. Results were similar when hot and cold eluents were used to elute thiamine adsorbed on Decalso from pinto bean extracts containing two levels of thiamine (Table IV). Hence eluent temperature definitely affects recovery of thiamine from Decalso with hot eluent recovering about 11% more thiamine than cold (Table III). Decalso columns are seldom thermostated and since the columns usually flow at different rates some will run hotter than others. It is now apparent that such temperature variations can be one cause of variation in the recovery of thiamine from Decalso.

However, our recoveries with hot eluent were still only about 87-90% (Tables I-III). Hence it was obvious there was another important factor affecting thiamine recovery. Incomplete adsorption of thiamine onto Decalso, pH of sample added to Decalso, decomposition of thiamine during the process, or incomplete elution of thiamine from Decalso appear to be the only remaining possibilities. Of these four factors, incomplete elution of thiamine from the resin seemed the most likely possibility and was therefore investigated.

Table V. Effect of Eluent Volume on Recovery of Thiamine Analytical Standard from Decalso[a]

Determ. no.	Net fluorescence: Std before application to Decalso (I)	Decalso treated: 50-ml eluent (II)	25-ml eluent (III)	Recovery from Decalso[b]: 50-ml eluent, %	25-ml eluent, %
1	78.9	77.0	71.5	99.9	92.7
2	77.8	75.2	68.6	97.5	89.0
3	75.5	76.2	74.1	98.8	96.1
4	76.0	72.2	68.5	93.6	88.8
5	74.5	74.6	67.7	96.8	87.8
6	77.8	74.8	67.8	97.5	87.9
7	76.1	75.8	70.0	98.3	90.8
8	79.3	73.8	68.3	95.7	88.6
9	77.8	76.8	68.0	99.6	88.2
Mean	77.078	75.156	69.389	97.5	90.0
Range	4.8	4.8	6.4		
Std dev	1.62	1.53	2.15		

Means compared	*t* Test: *t* (calcd)	$t_{0.05}$ (16 df)	$t_{0.01}$ (16 df)
I vs. II	2.59	2.120	2.92
II vs. III	6.56	2.120	2.92
I vs. III	8.56	2.120	2.92

[a] Twenty-five milliliters applied to Decalso followed by hot washes and hot elution. [b] Percent recovery = 100 × net fluorescence of eluate/mean net fluorescence of standard (77.1) before application to Decalso.

Table VI. Comparative Time Required to Elute Thiamine from Decalso to Eluent Volumes of 25 and 50 ml[a]

	Time elapsed (min) between application of sample to Decalso and elution to a vol of: 25 ml	50 ml
	62	69
	61	83
	56	70
	52	67
	43	79
	49	54
	67	78
	54	71
Mean	55.5	71.4

[a] Hot elution.

Recommended eluent volumes range from 10 ml (Hennessy and Cerecedo, 1939), 15 ml (Bechtel and Hollenbeck, 1958), to as much as 50 ml (Strohecker and Henning, 1966), but 25 ml is most commonly recommended (Freed, 1966; Strohecker and Henning, 1966; AOAC, 1970). The obvious implication is that thiamine recovery will be either quantitative, or if not quantitative, suitably reproducible, in 10-25 ml of eluent. But, adsorption and elution of thiamine from Decalso are ion exchange pro-

cesses and eluent volume should be an important factor influencing its displacement.

Increasing eluent volume from 25 to 50 ml significantly increased thiamine recovery (mean recovery increased from 90 to 97.5%) and decreased the standard deviation from 2.15 to 1.53 (Table V). Hence, eluent volume is an important factor affecting both the quantity and reproducibility of thiamine recovery from Decalso.

Elution to 50 ml requires more reagent and time than for 25 ml. However, the acid-KCl eluent is not expensive so time is the more important consideration. It averaged 15.9 min longer (about 29% longer) per column to elute to 50 ml than to 25 ml (Table VI). However, the additional time is not cumulative when 6–12 columns are run at once.

Hence, for maximum precision and accuracy in the thiochrome method the important effects of eluent temperature and volume on thiamine recovery from Decalso must be recognized and controlled. Both factors could cause inaccuracy when sample extracts are purified on Decalso by hot elutions on unthermostated columns to 25-ml eluent volume. The nearly quantitative recovery of thiamine possible on unthermostated columns by hot elution to 50 ml suggests a standard curve may now be used in the thiochrome method instead of standards run for every set of determinations as is now generally recommended.

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Gel Filtration and Disc Gel Electrophoresis of Tomato Pectic Substances

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Pectic substances were extracted from tomato alcohol insoluble solids (AIS) according to their solubility in water, 0.2% ammonium oxalate, 0.05 *N* hydrochloric acid, and 0.05 *N* sodium hydroxide. Gel filtration with Sephadex G-200 indicated that all fractions were heterogeneous in molecular size, the majority having molecular weights of 2×10^5 or more. Small differences were noted in the gel filtration patterns of all the solubility fractions indicating differences in molecular weights of pectic substances from the Chico III and Homestead-24 cultivars. Disc gel electrophoresis also revealed a heterogeneous set of molecules with respect to molecular charge. At least three distinct bands or zones were easily distinguishable in each solubility fraction, and discernible differences in electrophoretic patterns were noted in pectic substances of the two cultivars.

Pectic substances of fruits and vegetables have been associated with the texture and firmness of fresh and processed products (Kertesz, 1951) as well as with the viscosity of juices (Kertesz and Loconti, 1944) and purees (McColloch and Kertesz, 1949). The softening of certain fruits upon ripening has been attributed to a decrease in the molecular size of the pectic substances (McCready and McComb, 1954). Both quality and quantity of pectic substances influence the viscosity of tomato pastes (McColloch et al., 1950) and the consistency of pureed tomato products (Luh et al., 1954).

The characterization of tomato pectic substances has dealt mainly with the composition of those found primarily in the water soluble fraction. Methods for extracting pectic substances have not been standardized, but a common procedure involves the sequential extraction of tissue with hot water, dilute mineral acid, and dilute alkali or ammonium oxalate (Kertesz, 1951; King and Bayley, 1963; Owens et al., 1952). Molecular weights of pectic substances have been shown to exhibit a high degree of variability and are dependent on both source and isolation technique (Worth, 1967). Furthermore, the shape of tomato has been implicated in the quantity of pectic substances (McColloch et al., 1950) and could possibly affect the molecular distribution of pectic substances. Therefore, it was the purpose of this study to determine and compare the molecular species of pectic substances occurring in the alcohol-insoluble solids following hot water, dilute mineral acid, dilute ammonium oxalate, and dilute sodium hydroxide extraction of two tomato cultivars, Chico III (pear-shaped) and Homestead-24 (round-type) tomatoes.

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MATERIALS AND METHODS

Preparation of Tomato Sample. Chico III and Homestead-24 tomato cultivars were planted in the spring of 1970 and 1971 by the Texas Agricultural Experiment Station, Weslaco, Tex. Fully red ripe samples were handpicked to avoid damaged and nonuniform fruit, and were carefully washed. Samples of approximately 600 g were selected, frozen solid with liquid nitrogen, finely crushed, sealed in cans, and stored at −20° until the alcohol-insoluble solids (AIS) were prepared. Quick freezing of the tomato tissue prevented the degradation of pectic substances observed during the crushing process.

The crushed, frozen samples were weighed and slowly added to boiling 95% ethanol (2 parts ethanol to 1 part tomatoes) in a Waring Blendor equipped with a stainless steel blendor vessel and heavy duty heating straps to keep the alcohol near boiling during constant blending. The