alcohol would remove the interfering fluorescence from the hydrolysis mixture without affecting the fluorescence due to the Co-Ral hydrolysis products.

Recovery Experiments. To test the efficiency of the cleanup procedures described, known amounts of Co-Ral were added to samples which were subsequently analyzed. In the case of fat samples, the Co-Ral was dissolved in Skellysolve B. With the muscle and other tissues, the Co-Ral was dissolved in acetone. In both cases, the appropriate amounts of solution were added to the blender in the initial extraction steps. The results of these experiments are shown in Table II.

It is apparent from the data in Table II that Co-Ral in concentrations down to 0.04 p.p.m. can be recovered quantitatively by the method described.

Precision and Sensitivity. The data in Table III show the precision of results obtainable by this method. In the range up to 0.1 p.p.m., the average deviation from the mean is approximately 10% of the measured value. At higher concentrations, the precision is somewhat better. The precision is considered to be excellent for the low concentrations being measured.

The sensitivity of the procedure is determined, in this case, by the amount of fluorescence obtained when the method is applied to untreated samples. In the case of both fat and meat samples this value is equivalent to about 0.02 p.p.m. of Co-Ral. Thus, to contain a reportable residue, a sample must give a value twice that obtained for an untreated control. On this basis, the minimum sensitivity of the method would be set at 0.02 p.p.m. of Co-Ral, although this concentration is many times greater than the instrumental sensitivity limit.

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INSECT REPELLENT ANALYSIS

Colorimetric Determination of 2-Ethyl-1,3-hexanediol

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2-Ethyl-1,3-hexanediol gives a colored compound upon reaction with concentrated sulfuric acid and *p*-dimethylaminobenzaldehyde which can be estimated colorimetrically. The method can be used for the determination of the polyalcohol on glass, cloth, and human skin.

2-ETHYL-1,3-HEXANEDIOL is an effective repellent against various arthropod pests (*1*). An analytical method for this compound in aqueous or alcoholic solution was required in conjunction with studies on the evaporation and absorption of insect repellents. A method known to function for the higher alcohols in the presence of ethyl alcohol was selected for study.

In the presence of concentrated sulfuric acid, higher alcohols (*2, 3*) are dehydrated to unsaturated compounds, which then react with aromatic aldehydes to produce colors (known as the Komarowsky reaction). Snell (*3*) stated that this reaction is not given by polyalcohols. On the contrary, 2-ethyl-1,3-hexanediol reacts with *p*-dimethylaminobenzaldehyde in the presence of strong sulfuric acid to give a colored product. An analytical method for this repellent has been developed, based upon this reaction.

The colored product has an absorption maximum at 500 mμ (Figure 1). Beer's law is obeyed within the range of 5 to 105 γ when the reaction time, temperature, and the concentrations of sulfuric acid and *p*-dimethylaminobenzaldehyde are controlled.

The method has given satisfactory recoveries of 2-ethyl-1,3-hexanediol applied to glass, cloth, and human skin and functions in either aqueous or alcoholic medium.

Procedure

Chromogenic Reagent. Reagent grade *p* - dimethylaminobenzaldehyde (100 mg.) is mixed with 50 ml. of reagent grade concentrated sulfuric acid and dissolved by shaking. This reagent should be freshly prepared before each analysis.

Preparation of Standard Curve. 2-Ethyl-1,3-hexanediol (105 mg.) was diluted to 200 ml. with distilled water; 20 ml. of this solution was diluted to 200 ml. to give a standard solution containing 52.5 γ per ml.

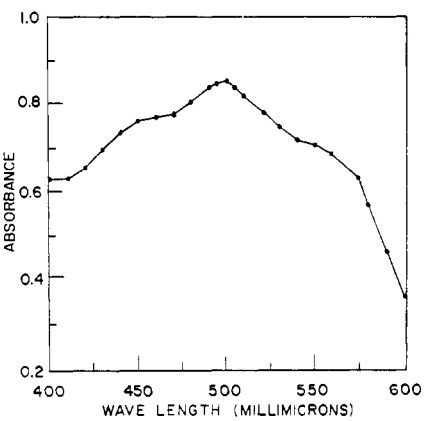

Figure 1. Absorption spectrum of color produced by reaction of 2-ethyl-1,3-hexanediol with p-dimethylaminobenzaldehyde in 66% sulfuric acid

Aliquots of the standard solution con-

taining 5.2, 10.5, 21.0, 42.0, 63.0, 84.0, and 105.0 γ of ethyl hexanediol were added from a microburet to 20 × 150 mm. glass-stoppered tubes, and the total volume of each solution was adjusted to 2 ml. with distilled water from a microburet. A reagent blank containing 2 ml. of distilled water was also prepared. The tubes were chilled in an ice bath and swirled gently, while 4 ml. of the chromogenic reagent were added to each from a volumetric pipet. The tubes were stoppered loosely at first and then tightly when warm, heated in boiling water for exactly 60 minutes, and then placed in an ice bath. After 5 minutes, they were removed and allowed to stand for 30 minutes at 25° C. in a constant-temperature cabinet. The absorbance of each solution was then determined in 1-cm. square cells with a Beckman DU spectrophotometer at 500 mμ by using distilled water in the reference cell.

A standard curve was prepared by plotting concentration against the absorbance corrected for the reagent blank.

Determination of 2 - Ethyl - 1,3-hexanediol from Glass, Cloth, and Human Skin. Because of the low solubility of 2-ethyl-1,3-hexanediol in water, a solvent such as 95% ethyl alcohol was desirable for the quantitative removal of the repellent from various surfaces or from a stream of air during evaporation. Redistilled 95% ethyl alcohol was used in quantities up to 5% by volume without appreciable interference, but at higher concentrations the interference increased (Table I) and the absorbance readings were erratic for replicated samples. As dilutions are necessary to bring the concentration of the repellent within the range of the method, it is convenient to use a final solution containing 5% of ethyl alcohol.

For the analysis of ethyl hexanediol on glass plates, 1 ml. of ethyl alcohol containing 241 mg. of the repellent was applied by volumetric pipet to a 6 × 6 inch glass plate that had been previously washed with ethyl alcohol and dried. After the sample had spread over the plate, it was immediately washed in a funnel over a 250-ml. volumetric flask with approximately 230 ml. of ethyl alcohol. The solution was made to volume, and a 25-ml. aliquot was diluted to 500 ml. with distilled water. Two milliliters of the solution, containing 5% of ethyl alcohol and approximately 96.5 γ of repellent, and a reagent blank consisting of 2 ml. of 5% ethyl alcohol were treated with chromogenic reagent and analyzed as described in the preparation of the standard curve. The corrected absorbance was related to micrograms on the standard curve, and the dilution factor was applied to give the quantity of ethyl hexanediol recovered.

New cotton sheeting was laundered with soap and air-dried. Pieces of the laundered cloth were treated with ethyl alcohol for 6 hours in a Soxhlet extractor and air-dried. Five milliliters of solvent was required for saturation, and this volume containing 248 mg. of the repellent was applied to 6 × 6 inch pieces from each batch of cloth. The treated cloth was placed in a funnel and washed with ethyl alcohol, and the solution was analyzed as described.

Table I. Variation of Reagent Blank Due to Ethyl Alcohol

Ethyl Alcohol, Volume %	*Absorbance, Av.*
5	0.009
10	0.065
20	0.297
30	0.593

Analyses of ethyl hexanediol from the forearm of the same human subject were made on three separate days. Two milliliters of ethyl alcohol containing approximately 477 mg. of the repellent was applied to a measured area of one forearm. The arm was immediately placed over a funnel, and the treated area was washed with approximately 480 ml. of ethyl alcohol. The washings were collected in a 500-ml. volumetric flask and made up to volume with ethyl alcohol. Likewise, a blank solution was obtained by washing a similar area of the untreated arm. Twenty-five-milliliter aliquots of the solutions were diluted with water and treated as described in the determination of the repellent from glass.

Results and Discussion

The absorbance of the reagent blank varies with each preparation and usually is 0.040 to 0.050. The absorbance increased sharply as the percentage of sulfuric acid in the reaction mixture exceeded 55 (Figure 2). By increasing the quantity of *p*-dimethylaminobenzaldehyde above 2.5 mg., only small changes in the absorbance were obtained (Figure 3). Therefore, the concentration of acid and the amount of *p*-dimethylaminobenzaldehyde per analysis were chosen arbitrarily to give a practical ratio of absorbance to concentration within the range of 5 to 105 γ of 2-ethyl-1,3-hexanediol.

Variation in the quantity of chromogenic reagent added to the solution of repellent can cause serious error, because of the large change in absorbance with change in concentration of acid. This error can be minimized by allowing the pipet to drain against the wall of the container. Rapid dissipation of the heat is necessary to prevent premature development of color, during the addition of the chromogenic reagent. This was accomplished by swirling the chilled tubes in the ice bath.

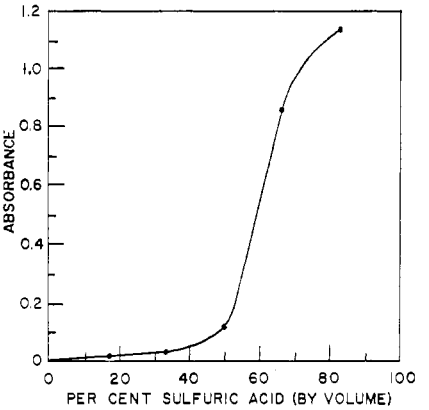

Figure 2. Absorbance related to different concentrations of sulfuric acid containing 8 mg. of *p*-dimethylaminobenzaldehyde and 105 γ of 2-ethyl-1,3-hexanediol

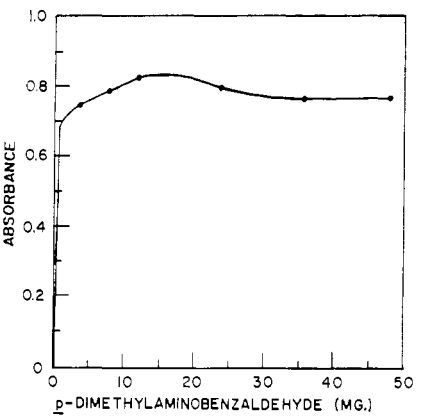

Figure 3. Absorbance developed by 105 γ of 2-ethyl-1,3-hexanediol with different quantities of *p*-dimethylaminobenzaldehyde in 66% sulfuric acid

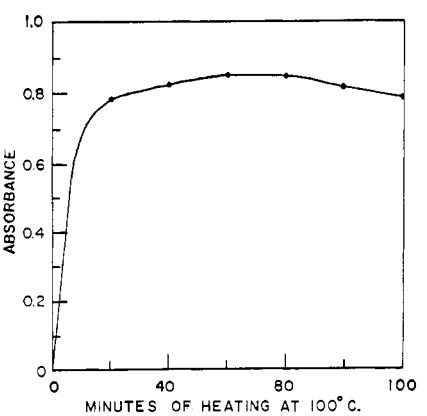

Figure 4. Effect of heating time on absorbance of reaction product

Table II. Stability of Color at 25° C.

Hours after First Reading	Absorbance Loss, % In dark	Absorbance Loss, % In artificial light
4	0.4	0.9
21	1.5	5.6
28	2.1	8.5
52	3.2	16.0

Table III. Average Milligrams of 2-Ethyl-1,3-hexanediol Recovered from Various Surfaces

Surface	Applied	Recovered
Glass plates	241	242
Unextracted cloth	248	249
Extracted cloth	248	245
Human skin	477	465

Figure 4 shows that a reaction time of 60 minutes is required to develop maximum absorbance. The absorbances failed to check well when duplicate samples were heated for shorter periods.

The data presented in Table II show that the color fades after development at the rates of 0.1% and about 0.25% per hour in the dark and in artificial light, respectively. Therefore, error due to fading is insignificant.

The results of the analyses for 2-ethyl-1,3-hexanediol recovered from glass, cloth, and human skin are shown in Table III. Quantitative recoveries were obtained from glass and cloth. The recovery from skin was 97.5%. Failure to obtain quantitative recovery from skin may have been due to absorption, a phenomenon which is being investigated. The absorbance due to material washed from an untreated arm varies somewhat with individuals and usually is less than 0.010.

Acknowledgment

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FORAGE NITRATE

Improved Microbiological Method for Nitrate Determination

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Nitrate is quantitatively reduced to nitrite by nitrate reductase, an enzyme produced by certain bacteria. *P. oleovorans* is ideal for such use, because this enzyme is always present, although the organism does not have a nutritive requirement for nitrate. Nitrite reductase, an enzyme which frequently interferes with this method, is not formed. Prior isolation of nitrate is not required and the usual biological tissues and fluids contain no apparent inhibitor for this enzyme. The method as described covers 2 to 200 γ, in nitrate content at any one sample dilution.

Consumption of forages and silages containing as little as 1.5% potassium nitrate, on a dry basis, frequently causes death of sheep and cattle (*5*). Any rapid method for the determination of nitrate capable of giving dependable results on a variety of materials, without requiring numerous modifications or separation procedures, would be of great value. The numerous simple chemical or colorimetric methods adaptable to relatively pure systems are usually not applicable to complex mixtures such as plant or animal tissues, the recoveries of added nitrate differing greatly depending on the particular method and plant species involved (*6, 7*).

The use of a partially purified adaptive enzyme, nitrate reductase, as suggested by Nason and Evans (*8*) had little to offer, because the isolation of the enzyme, which was rather unstable, required considerable time. Garner and coworkers (*4*) contributed greatly to the usefulness of the enzymatic method by demonstrating that this enzyme which reduces nitrate to nitrite did not require isolation, prior to use, but could be produced by growing a suitable microorganism directly in the test tube containing the solution to be investigated. These authors employed an unidentified bacillus obtained from sheep rumen to produce the desired enzyme. The nitrite formed was then determined colorimetrically. This microbiological method, although accurate and precise, entails considerable preparation. The stock culture must be subcultured on nitrate-containing agar, but excess subculturing on such a medium allows the formation of an undesirable adaptive enzyme, nitrite reductase. After culturing, the organism must be washed repeatedly to remove all nitrate and nitrite. In addition to the possible presence of nitrite reductase, the time of incubation of the nitrate solution with the bacteria is very critical, the greatest effect being noticed at low nitrate concentrations.

Pseudomonas oleovorans (Lee and Chandler) (American Type Culture Catalog)

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