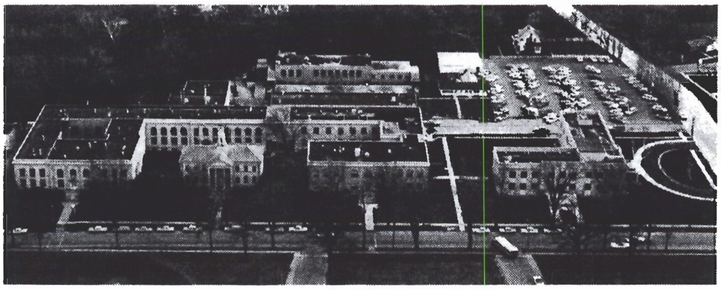

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DETERMINATION OF TRIGLYCERIDES IN PLANT TISSUES

J. H. CONKEY AND R. P. FEIRER

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J. H. Conkey and R. P. Feirer

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DETERMINATION OF TRIGLYCERIDES IN PLANT TISSUES

J.H. Conkey and R.P. Feirer
Forest Biology Division
Institute of Paper Chemistry

INTRODUCTION

Recent work carried-out as part of Project 3223, the conifer cloning project, has suggested that lipids are a major constituent of zygotic conifer seeds and embryos. Somatic embryos and other conifer tissues grown in vitro have been found to be deficient in these storage lipids. Continued studies on the occurrence, accumulation and metabolism of lipids in conifer tissues are currently underway. During the course of this work, the necessity of a convenient, reliable assay for the measurement of storage lipids (triglycerides) became apparent. Since an assay suitable for small amounts of plant tissues was not found in the literature, a technique designed for the clinical determination of triglycerides in blood was adapted for use. The triglyceride (TGC) portion of the assay is based on a diagnostic kit procedure available from Sigma Chemical Company (1) for the measurement of TGC in serum or plasma. The method, described in this report, has been modified to better suit plant tissues.

METHOD

General

Initially the tissue must be homogenized and the TGC extracted with isopropanol. The insoluble fraction is saved for later use in the protein assay. The supernatant is treated to remove interfering substances such as glucose, endogenous glycerol and phospholipids. The purified TGC extract is moved stepwise through the following reactions:

1. Triglycerides + KOH ---> Glycerol + Fatty Acids
2. Glycerol + Periodate ---> Formaldehyde
3. Formaldehyde + NH_4^+ + Acetylacetone ---> Diacetyldihydrolutidine

The colored product diacetyldihydrolutidine is proportional to TGC concentration and is quantified photometrically.

The protein assay includes the extraction of the insoluble fraction followed by the color development procedure of Bradford (2). This too is quantified photometrically. Reagents and components used are described in Appendix A.

Tissue amount and handling

Due to the sensitivity of both the TGC and protein assays, only small amounts of tissue are required. Conifer embryo and gametophyte tissues were found to contain 5-10% TGC or protein per unit of fresh weight during early stages of development. As seeds matured, the TGC level increased to 30-50% and the protein to 15-30%. When using mature seed, as little as 5 mg of tissue is required. Somatic tissues, however, are not as rich in TGC and required 50-200 mg.

The tissue is collected in tared 1.5 mL microfuge tubes and the fresh weight determined. The tubes may be stored at -70 °C until needed.

The assay reagents are conveniently available from Sigma in kit form but the volumes used have been altered resulting in some kit components being consumed more rapidly than others. The kit purchase remains the most economical form, however, until the imbalance in components becomes quite marked.

Triglyceride extraction

A 100 µL volume of isopropanol is added per microfuge tube and the tissue homogenized using a pestle fitted to the microfuge tube (Kontes Inc). Homogenization is carried out for a period of 1-2 minutes; adding a small amount (50 mg) of aluminum oxide purifier improved the grinding. The tube volume is brought to one mL with isopropanol using the added volume to rinse adhering material on the pestle into the tube. The extraction is continued by placing the tubes on a shaker for fifteen minutes. Finally, the insolubles are pelleted by centrifuging the tubes for five minutes in an Eppendorf microfuge.

Triglyceride purification

An 800 µL volume of the supernatant is transferred to a vial containing 0.8 g of activated alumina purifier and 1.8 mL of isopropanol. The vials are shaken for a minimum of five minutes before the alumina is sedimented by a brief centrifugation in a table top centrifuge.

Color development

An 800 µL volume of the "purified" supernatant is transferred to a small polypropylene test tube with a snap cap. It is important that this sample and the reagents are well mixed after each of the following additions. First, a 200 µL volume of a 1N KOH solution is added and the tubes held in a 60 °C water bath for five minutes. After cooling to room temperature, a 200 µL volume of a sodium m-periodate solution is added to each tube. After exactly ten minutes, a 1.2 mL volume of color reagent is added and the tubes returned to the 60 °C water bath and held for 30 minutes. The resulting color is quite stable but Sigma recommends that the absorbance be measured within 20 minutes.

Triglyceride quantification

The contents of each tube is transferred to a 4.5 mL cuvette and the absorbance measured at a wavelength of 410 nM. A series of standards prepared from triolein is introduced into the procedure at the extraction stage and handled in the same manner as the tissue extract from that point on. One mL volumes of isopropanol containing 0, 126, 375 and 750 µg of triolein provide a linear standard curve suited to the TGC levels encountered in our tissues. A linear regression based on the standards will result in a correlation coefficient (r) greater than 0.99 and is used to convert the unknown absorbance to g/mL TGC. If the absorbance of an unknown exceeds that of the highest standard, it may be diluted; however, a precipitate will result if isopropanol alone is used as the diluent. An "Absorbance diluent" is given in the appendix that avoids this problem.

Protein measurement

The insoluble pellet resulting from the initial centrifugation step of the TGC extraction serves as the protein sample. Any remaining supernatant is decanted and the pellet resuspended in 1 mL of 0.1 N NaOH. The tubes are agitated for several hours before a final centrifugation for five minutes in the microfuge. A volume of 10-50 µL of the supernatent is added to a 1.5 mL cuvette. If less than 50 µL of the sample is required for adequate color development, the volume is brought to 50 µL with 0.1 N NaOH. A 50 µL volume of HEPES buffer (50 mM, pH 7.5) and 1.0 mL of the Bradford Coomassie-blue Reagent are added to the cuvette. The samples are well mixed and the absorbance measured at a wavelength of 595 nM. The concentration of protein is determined on the basis of a standard curve prepared using bovine serum albumin.

LITERATURE CITED

1. Triglycerides Procedure No. 405. Sigma Diagnostics, St. Louis, MO (1984).

2. Bradford, M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254 (1976).

APPENDIX A. COMPONENTS AND REAGENTS

Item	Catalog Source
Complete kit for determining TGCs	Sigma 405 A
Triglyceride purifier (aluminum oxide)	Sigma 405-8
Isopropanol (anhydrous)	Sigma 405-7
Triolein (3 mg/mL isopropanol)	Sigma 405-10
Potassium hydroxide (1N)	Sigma 405-1
Sodium m-periodate	Sigma 405-9
Acetic acid (2N)	Sigma 405-12
Ammonium acetate (2M)	Sigma 405-2
Acetylacetone	Sigma 405-4
Microfuge tubes (1.5 mL) w/ pestles	Kontes K 749 520-0000
Polypropylene test tubes	Cole-Parmer 60819-740
Coomassie protein assay reagent	Pierce 23200
Albumin standard	Pierce 23210

Sodium m-periodate solution

$NaIO_4$	125 mg
Acetic acid (2N)	50 mL

Color Reagent

Isopropanol	80 mL
NH_4Ac (2M)	40 mL
Acetylacetone	0.15 mL

Absorbance diluent

Isopropanol	160 mL
NH_4Ac (2M)	40 mL
KOH (1N)	20 mL
$NaIO_4$ sol.	20 mL
Acetylacetone	0.3 mL