Can Tho University Journal of Science
website: sj.ctu.edu.vn

DOI: 10.22144/ctu.jen.2019.030

A simple spectrophotometric method for quantifying total lipids in plants and animals

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Article info.

Received 03 Jan 2019
Revised 20 May 2019
Accepted 30 Jul 2019

Keywords

Measurement, sulfo-phospho-vanillin, total lipid

ABSTRACT

Lipids play important roles for the cell in storing energy, structuring cell membrane, and signaling pathway. Consequently, lipidis are analyzed routinely in various research fields. In the current research, a reliable, rapid and economical assay has been developed to quantify the total of lipid in various samples. The development of colorimetric sulfo-phospho-vanillin is for high throughput analysis of total lipids. In this method, a reaction mixture is performed in a 96-well microplate. The advantages provided from this new assay over other lipid measurement methods, included only small amount of sample requirement for fitting in the standard range (less than 100 µg/mL), less time requirement and labor in analyzing a large number of sample (about 1 hour), and the more consistent of color development between lipid content and reagent concentration.

Cited as: Men, T.T., Thanh, N.Q.C., Yen, N.D.H., Binh, T.D. and Trang, D.T.X., 2019. A simple spectrophotometric method for quantifying total lipids in plants and animals. Can Tho University Journal of Science. 11(2): 106-110.

1 INTRODUCTION

Lipids are considered as an important group of compounds providing several biological functions as energy storage, cell membrane structure and signaling (Wymann and Schneiter, 2008). This is the reason why the analysis of lipids is performed routinely in various research areas. For instance, in both research and industry settings, the screening of oleaginous living beings has broad application to recognize and produce food supplements and renewal biofuels (Ratledge, 2002; Ratledge and Cohen, 2008). There are several methods developed for quantifying total lipids. Among them, a macro-gravimetric is the basic technique in which lipids are separated from a sample, the extraction solvent is evaporated, and the remaining extract is estimated as the lipid content (Folch *et al*., 1957; Bligh and Dyer, 1959). It is necessary to have a relatively large quantity of sample in this traditional gravimetrical method. Time-consumption and labor-intension are also required in case the analysis of many samples is needed. Lipid spectrofluorometric analysis using Nile red fluorescent dye is the method that was originally developed by Greenspan *et al*. (Greenspan *et al*., 1985) and has also been modified to quantify the total of lipids (Fowler and Greenspan, 1985; Huang *et al*., 2009). This approach is high-throughput while the environmental factors and other components in the cell cytoplasm, including proteins and pigments, may affect the fluorescence intensity (Chabrol and Charonnet, 1937; Desvilettes *et al*., 1997). Because of this reason, the lipid quantification accuracy by

applying this approach requires a determination of the optimal spectra and reaction conditions for each type of specimen prior to fluorescent measurements (Johnson, *et al*., 1977).

Because of its fast response and relative ease in sample handling, the colorimetric sulfo-phospho-vanillin (SPV) method developed by Chabrol and Charonnet (1937) is considered as an attractive alternative for lipid measurement (Chabrol and Charonnet, 1937; Johnson *et al*., 1977). The adjustment of SPV method has been executed for various applications, for example, the examination of total lipids in serum, sustenance and biological examples (Nakamatsu and Tanaka, 2004; Haskins *et al*., 2010). A micro-scale modification of the SPV assay was developed by Van Handel (1985) to determine the total lipids in a single mosquito, and assessed by several investigators as an efficient approach in time and labor compared to the gravimetric method (Lasorsa and Casas, 1996; Lu *et al*., 2008). Because of the continuous development of color, it is important to handle the sample carefully and control the color development in using this micro-scale approach. From the results of previous works, it is necessary to have an adaptation of the SPV method in order to complete the lipid quantification in a 96-well microplate for higher throughput and reduced costs. This adapted method requires an assay in which the reagent mixture is confined to one microplate for the entire assay. This prompts the faster estimation of different examples with easy background correction and the more reliable checking of color development. For example, the application of this assay method on soybean oil and triolein as a standard has successfully measured the total lipids in extracts from fruit flies, which contain lipid in the 3rd instar larvae. The objective of this study is to establish the method for quantifying total lipid from samples.

2 MATERIALS AND METHODS

2.1 Chemicals

All chemicals used were from Japan. Soybean oil and triolein are standard lipids as well as methanol and sulfuric acid were from Sigma; others at analytical grade including sodium sulfate (Na_2SO_4), chloroform, vanillin, and phosphoric acid were from Wako.

2.2 Preparation of phospho-vanillin reagent

Vanillin (0.6 gram) was dissolved in 100 mL of hot distilled water (vanillin reagent), then vanillin reagent was mixed with 400 mL phosphoric acid (85%). The phospho-vanillin reagent was stored in a brown bottle at room temperature (Kaufmann and Brown, 2008).

2.3 Preparation of lipid standard

Soybean oil (vegetable oil) and triolein were used as the standards for the colorimetric method in surveying the applicability of the assay. The content of total lipid in plants usually has a higher unsaturated proportion and is relatively close in composition to vegetable oil, concurrently triolein is a symmetrical triglyceride which is structured from three units of the unsaturated fatty acid oleic acid and glycerol. Most triglycerides are unsymmetrical and derived from mixtures of fatty acids. The main constituent of vegetable oil and animal fats is triglycerides. In this work, soybean oil was tested in the range of 0 – 100 µg/mL, and triolein was tested in the range of 0 – 125 µg/mL. There are two of these standards which were dissolved in chloroform and added in 96-well microplate. After the evaporation of chloroform at 90°C for 20 minutes, 50 µL concentrated sulfuric acid (98%) was added to each well, and then the microplate was incubated at 90°C for 20 minutes. A volume of 150 µL vanillin–phosphoric acid reagent was added to each well for color development. After 10 minutes, the absorbance at 530 nm was measured using a SH-1200 microplate reader (Corona Electric, Japan). This method is based on the reaction of lipids with concentrated sulfuric acid at high temperature to form carbonium ions, then these ions subsequently react with the vanillin phosphate ester to yield a pink-colored complex which is examined photometrically (Frings and Dunn, 1970). The ion formed is stable on cooling down at room temperature for at least several hours. The condition for a positive SPV reaction requires the presence of double bonds or free hydroxyl groups within the lipid analytes (Johnson *et al*., 1977).

2.4 Extraction of total lipid in animal tissue

The 3rd instar larvae *Drosophila melanogaster* (from 1 to 5 flies) was homogenized in 100 µL of 2% sodium sulfate, and then 900 µL of chloroform/methanol (1:1) was added. The supernatant was collected by centrifugation (10,000 rpm, 5 minutes), mixed with 300 µL of distilled water, and centrifuged again (10,000 rpm, 5 minutes). For lipid measurement, the chloroform layer was transferred into 96-well microplate and dried at 90°C to evaporate the chloroform (about 20 minutes), and then 50 µL of 98% sulfuric acid was added, and the solution was incubated for 20 minutes at 90°C. A volume of 150 µL vanillin–phosphoric acid reagent was added to each well for color development. After cooling down to room

temperature for 10 minutes, absorbance was measured at 530 nm.

2.5 Data analysis

Linearity was determined by plotting absorbance versus lipid amount in the assay and examining the R^2 value upon linear regression of the data. The error bars represent the standard deviation.

3 RESULT AND DISCUSSION

3.1 Relative absorbance and linearity of standard lipid samples

Depending on previous report by Ahlgren and Merino (1991), the selection of an appropriate standard was important to assess lipid content in different types of samples. Therefore, soybean oil and triolein are two types of standards which were tested using a new assay format (Fig. 1 and Fig. 2). This assay system shows that soybean oil has driven an increase in absorbance. The result from Fig.1 showed that this method is sensitive and adaptable to measure a small amount of lipid in the sample. The concentration of soybean oil used is from 0-100 µg/mL, and the absorbance dramatically increased in the well having a high concentration of soybean oil. In this assay, soybean oil was used as a standard for lipid from plants. This method is suitable to measure the total of lipids from plant samples according to the result.

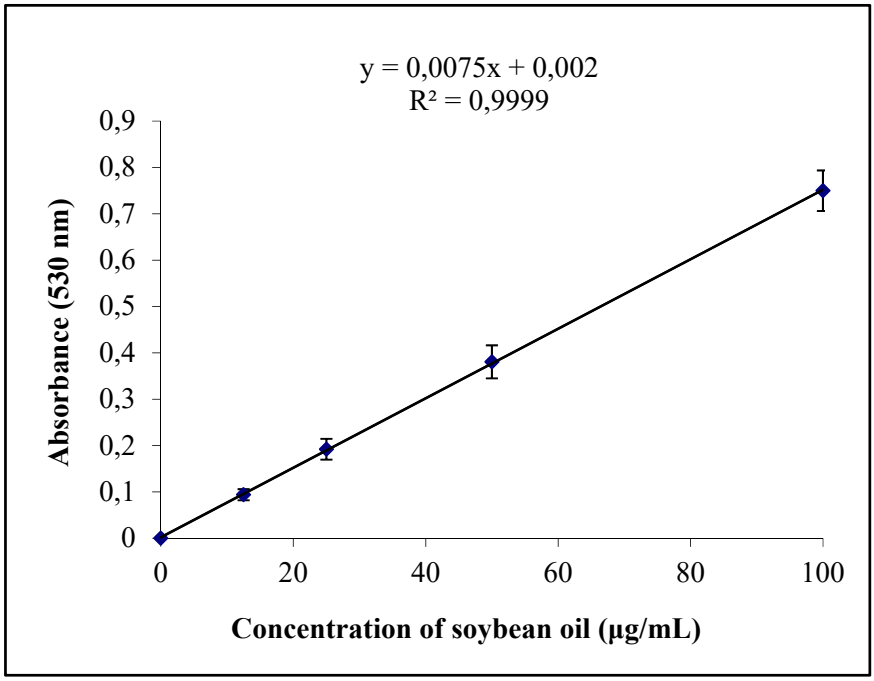

Fig. 1: Lipid measurement by the coupled colorimetric test

The linear relationship between the absorbance and soybean oil concentration (0 - 100 µg/mL). Soybean oil measured by the SPV method with a correlation coefficient of 0.9999 and regression equation of y = 0.0075x + 0.002. Each point in the regression represents the replicate measurement (n = 3).

In the current study, triolein was used as a standard. It is an unsaturated lipid which reacts with a good yield. The method easily manipulated and inexpensive reagents can be purchased in many chemical companies (Izard and Limberger, 2003). Fig.2 shows the correlation of lipid concentration (triolein) and absorbance using the SPV method. Depending on the spectrophotometric result in a linear increase (R^2 = 0.9924) of absorbance values, it indicates that the assay allows a reliable lipid measurement in this concentration range.

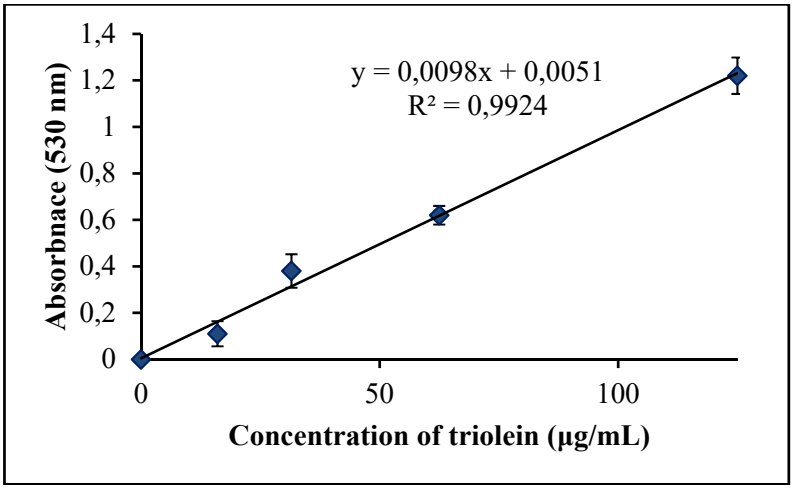

Fig. 2: Reliable lipid determination by the coupled colorimetric assay

Linear absorbance increase of triolein (0 - 125 µg/mL) and measured by SPV method with a correlation coefficient of 0.9924 and regression equation of y = 0.0098x + 0.0051. Data points and error bars represent the mean and standard deviation of four replicate samples.

3.2 Total lipid in animal sample

DNA, RNA, and proteins did not detectably react or interfere with the SPV, the reagents used for lipid extraction as described in this report also did not affect the assay (Izard and Limberger, 2003). The SPV reaction detected microgram level of lipids (as shown in Fig. 1 and Fig. 2). The wavelength was referenced to measure the absorbance of the sample is 530 nm. It was selected based on the wavelength of maximal absorption on tested lipid standards and total lipid extracted from fruit flies (data not shown), and this wavelength was also used for the determination of lipid in some previous reports (Folch *et al*., 1957; Izard and Limberger, 2003). In this assay, chloroform/methanol was used to extract total lipid from the 3rd instar larvae of *Drosophila melanogaster*. Lipids exist in form of unsaturated compounds (*or move to introduction of lipids*) that do not dissolve in polar solvents like water but are highly soluble in the non-polar or weakly polar organic solvents, including chloroform, ether, benzene, and acetone (Reis *et al*., 2013). Fig. 3 shows that there is a relationship between the lipid extracted from fruit flies and the absorbance at 530 nm (R^2 = 0.9924). This result explained that the SPV method is suitable for measuring total lipid from animal samples.

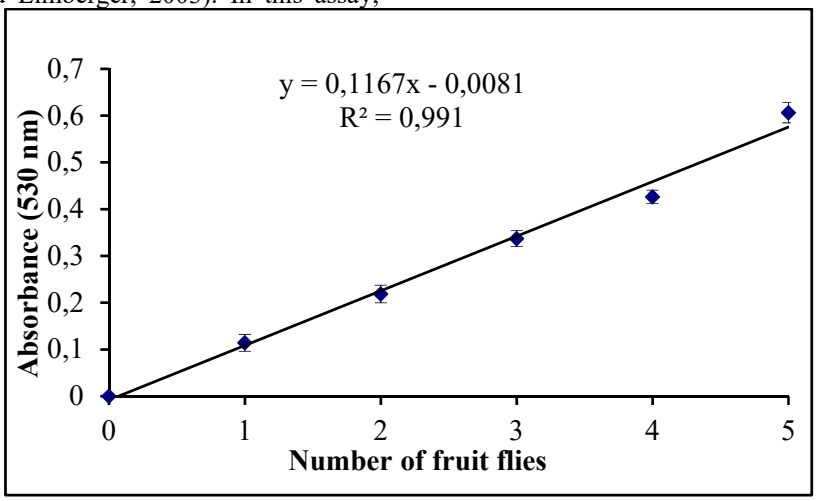

Fig. 3: SPV measurement accuracy depends on the number of flies per assay

Data showed total lipid measurements of three replicates each of group from 1 to 5 fruit flies (yellow white flies). Absorbance measurements were made after 10 minutes of color development. Data points represent the mean of three replicate samples.

In various studies, the total lipid quantification was frequently performed with different kinds of samples. The present work reports a modified colorimetric method for quantitative analysis of total lipid using a high throughput microplate format, where extracted and purified lipid from samples was used. The extraction procedure limited the interferences associated with other components in the sample and allowed different samples from various research areas to be analyzed in the same conditions.

4 CONCLUSIONS

There are advantages of the new assay method, including (1) it just uses a small amount sample and the sample volume can be adjusted to fit in the standard range, (2) it requires less time (<2 hours) and less labor when a large number of samples is analyzed, and (3) the color development is more consistent between lipid contents and reagent concentrations. Moreover, the reagents used in this assay are inexpensive and easy for the handle. In the final procedure it is recommended that the volume of the sample should be less than 100 µL in order to ensure a complete reaction with the sulfuric acid. In addition, uniform heating and cooling are important for consistency in the reaction. When soybean oil and triolein were used as standards, this method can be used to measure the total lipid in different samples including cell cultures, plants, and animal tissues.

REFERENCES

Ahlgren, G. and Merino, L., 1991. Lipid analysis of freshwater microalgae: a method study. Archiv Für Hydrobiologie, 121(3): 295-306.

Bligh, E. G. and Dyer, W. J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Physiology and Pharmacology, 37(8): 911-917.

Chabrol, E. and Charonnet, R., 1937. Une novelle reaction pour´l letude des lipides. Presse Med, 45: 1713.

Desvilettes, C., Bourdier, G., Amblard, C. and Barth, B., 1997. Use of fatty acids for the assessment of zooplankton grazing on bacteria, protozoans and microalgae. Freshwater Biology, 38(3): 629-637.

Folch, J., Lees, M. and Sloane Stanley, G. H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. The Journal of Biological Chemistry, 226(1): 497-509.

Fowler, S. D. and Greenspan, P., 1985. Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: Comparison with oil red O. Journal of Histochemistry and Cytochemistry, 33(8): 833-836.

Frings, C. S. and Dunn, R. T., 1970. A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. American Journal of Clinical Pathology, 53(1): 89-91.

Greenspan, P., Mayer, E. P. and Fowler, S. D., 1985. Nile red: A selective fluorescent stain for intracellular lipid droplets. Journal of Cell Biology, 100(3): 965-973.

Huang, G. H., Chen, G. and Chen, F., 2009. Rapid screening method for lipid production in alga based on Nile red fluorescence. Biomass and Bioenergy, 33(10): 1386-1392.

Izard, J. and Limberger, R. J., 2003. Rapid screening method for quantitation of bacterial cell lipids from whole cells. Journal of Microbiological Methods, 55(2): 411-418.

Johnson, K. R., Ellis, G. and Toothill, C., 1977. The sulfophosphovanillin reaction for serum lipids: a reappraisal. Clinical Chemistry, 23(9): 1669-1678.

Kaufmann, C. and Brown, M. R., 2008. Regulation of carbohydrate metabolism and flight performance by a hypertrehalosaemic hormone in the mosquito Anopheles gambiae. Journal of Insect Physiology, 54(2): 367–377.

Lasorsa, B. and Casas, A., 1996. A comparison of sample handling and analytical methods for determination of acid volatile sulfides in sediment. Marine Chemistry, 52(3-4): 211-220.

Lu, Y., Ludsin, S. A., Fanslow, D. L. and Pothoven, S. A., 2008. Comparison of three microquantity techniques for measuring total lipids in fish. Canadian Journal of Fisheries and Aquatic Sciences, 65(10): 2233-2241.

Ratledge, C., 2002. Regulation of lipid accumulation in oleaginous micro-organisms. Biochemical Society Transactions, 30(Pt 6): 1047-1050.

Ratledge, C. and Cohen, Z., 2008. Microbial and algal oils: Do they have a future for biodiesel or as commodity oils? Lipid Technology, 20: 155–160.

Reis, A., Rudnitskaya, A., Blackburn, G. J., Fauzi, N. M., Pitt, A. R. and Spickett, C. M., 2013. A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. Journal of Lipid Research, 54(7): 1812-1824.

Van Handel, E., 1985. Rapid determination of total lipids in mosquitoes. J. Aru. Mosq. CoNrnol Assoc., 1(3): 302-304.

Wymann, M. P. and Schneiter, R., 2008. Lipid signaling in disease. Nature Reviews Molecular Cell Biology, 9(2): 162-176.