Journal of Insect Physiology 57 (2011) 1602–1613

Contents lists available at SciVerse ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Triacylglyceride measurement in small quantities of homogenised insect tissue: Comparisons and caveats

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ARTICLE INFO

Article history:
Received 9 June 2011
Received in revised form 4 August 2011
Accepted 5 August 2011
Available online 22 August 2011

Keywords:
Storage lipid
Triacylglyceride
Sulfo-phospho-vanillin
Enzymatic
Gravimetric
TLC-FID

ABSTRACT

Triacylglycerides (TAGs) are the most important stored energy reserve in eukaryotes and are regularly measured in insects. Quantitative analysis of TAGs is complicated by their diversity of structure, and there are concerns with the quantitative accuracy of commonly used analytical methods. We used thin layer chromatography coupled to a flame ionisation detector (TLC-FID), an accurate method that is not sensitive to saturation or chain length of fatty acids, to quantify TAG content in small amounts of insect tissue, and used it to validate three high-throughput lipid assays (gravimetric, vanillin, and enzymatic). The performance of gravimetric assays depended on the solvent used. Folch reagent (chloroform: methanol 2:1 v/v) was a good index of TAG content, but overestimated lipid content due to the extraction of structural lipid and non-lipid components. Diethyl ether produced reasonable quantitative measurements but lacked precision and could not produce a repeatable rank-order of samples. The vanillin assay was accurate both as a quantitative method and as an index, preferably with a standard of mixed fatty acid composition. The enzymatic assay did not accurately or precisely quantify TAGs under our assay conditions. We conclude that the vanillin assay is suitable as a high-throughput method for quantifying TAG providing fatty acid composition does not change among treatment groups. However, if samples contain significant quantities of di- or mono-acylglycerides, or the fatty acid composition differs across treatment groups, TLC-FID is recommended.

1. Introduction

Storage lipids, or triacylglycerides (TAGs), are the dominant energy reserve in eukaryotic organisms (Bell and Coleman, 1980), and the storage, mobilization, and metabolism of fat stores in insects are key metabolic processes (Fast, 1964; Gilby, 1965; Fast, 1970; Downer and Matthews, 1976; Beenakkers et al., 1985; Arrese and Soulages, 2010). Insect lipid stores are key fitness determinants in overwintering insects, especially those which rely on lipid catabolism to meet metabolic demands over winter and to fuel metamorphosis and reproduction in the spring (Hahn and Denlinger, 2011), and are widely used as indirect measures of insect performance and fitness (e.g. Otronen, 1995; Ojeda-Avila et al., 2003; Hahn, 2005; Huho et al., 2007; Bosch et al., 2010).

Triacylglycerides are comprised of a trihydric alcohol glycerol esterified with three long chain fatty acids (acyl chains). Hydrocarbon chain length, number and position of double bonds, and relative molecular mass of the acyl chains will all influence the polarity and chemical reactivity of TAG molecules (Nikolova-Damyanova, 1999), which can lead to systematic biases in some methods of quantification. The presence of other neutral lipids with similar chemical properties but different functional significance (e.g. mono- and di-acylglycerides) makes the development of a specific assay difficult, particularly when multiple tissue types are present in a sample.

Small body size and a diffuse fat storage organ (the fat body) (Downer and Matthews, 1976; Arrese and Soulages, 2010) also complicate lipid analysis in insects and make techniques such as condition indices or fat scoring (Krementz and Pendelton, 1990), magnetic resonance imaging (McGuire and Guglielmo, 2010), or dual energy x-ray absorptiometry (Stevenson and van Tets, 2008) less accessible than they are to researchers working on larger

Abbreviations: BHT, butylated hydroxytoluene; CE, cholesterol ester; DAG, diacylglycerol; DM, dry mass; FFA, free fatty acid; FM, fresh mass; GC, gas chromatography; GLY, glycerol; HPLC, high-pressure liquid chromatography; LFDM, lipid-free dry mass; MAG, monoacylglycerol; TAG, triacylglycerol; TLC-FID, thin layer chromatography coupled to a flame ionisation detector; TLL, trilinolein; TOL, triolein; TPT, tripalmitin.

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doi:10.1016/j.jinsphys.2011.08.008

animals such as birds or mammals (but see Bosch et al., 2010). In addition, storage lipids are often not the only analyte of interest, and the measurement of protein, RNA, DNA or other metabolites can involve the homogenisation and aliquoting of small tissue samples, prohibiting the dissection of specific tissues. Chromatographic methods such as gas chromatography (GC), high performance liquid chromatographthy (HPLC), or thin-layer chromatography (TLC) can be used for the quantification of insect lipids in mixed tissue samples (e.g. Cvačka et al., 2006), but these methods are not usually high-throughput and may be qualitative or semi-quantitative depending on the detector. Current high-throughput methods used for the quantification of storage lipids in insects include gravimetric determination after organic solvent extraction (e.g. Pullin, 1987; Blows and Hoffmann 1993; Ojeda-Avila et al., 2003; Colinet et al., 2007; Sinclair et al., 2011), glycerol-linked enzyme assays (e.g. Clark and Wang, 1994; Gefen et al., 2006; Pospisilik et al., 2010; Marshall and Sinclair, 2010; Marshall and Sinclair, 2011), or chemical methods such as the (sulfo-phospho-) vanillin assay (e.g. Otronen, 1995; Timmerman and Briegel, 1999; Lorenz, 2003; Hahn, 2005; Huho et al., 2007).

Here we will outline these methods, provide examples of their use in quantifying insect storage lipids, and detail the problems associated with them; however we do not intend this to be a comprehensive review of methods of lipid measurement or of lipid storage in insects.

1.1. Chromatography

Analytical chromatography, including GC, HPLC and TLC, can be used to quantify TAGs (D'Alonzo et al., 1982; Homan and Anderson, 1998; Fried, 2003). It involves separating substances based on their polarity by passing the substance of interest, dissolved in a mobile phase (solvent or gas), through a stationary phase (Fried, 2003) to which the components will adsorb differentially depending on their hydrophobicity. The resulting classes of each molecule are then quantified using one of a range of detectors (Scott, 1996). As the various lipid classes contained in a biological sample may be separated and thus quantified separately, these techniques are highly specific. Chromatographic techniques are often used in a qualitative or semi-quantitative manner, with gravimetric methods used to quantify total TAG content (e.g. Chirumamilla et al., 2010). HPLC can be used to quantify TAGs but requires the use of multiple methods such as reversed phase and silver ion HPLC and a range of detectors (e.g. Cvačka et al., 2006), which makes it inaccessible for high-throughput analysis. GC is the most commonly used chromatographic technique in the analysis of oils and fats. It offers superb resolution and reliability, and is often coupled to a flame ionisation detector which is one of the most quantitative detectors currently available (Scott, 1996). However, the high molecular weights of TAGs mean that the temperatures required to elute them from the columns typically approach the limits of thermal stability of the stationary phase and the column itself, and at these temperatures there is a risk of losses due to pyrolysis (Christie, 1989). Thus GC is usually performed on fatty acid methyl esters rather than on intact TAGs (but see Yocum et al., 2011), making it difficult to quantify TAGs without an additonal step to separate TAGs from other acylglycerols and free fatty acids. TLC is a powerful and simple method for separating lipid classes, although the perceived weakness of this method is in quantitation (Shantha and Napolitano, 1998). This can be overcome by combining TLC with densitometric analysis (e.g. Nikolova-Damyanova, 1999; Al-Anzi and Zinn, 2010; Santos et al., 2011) or flame ionisation detection (TLC-FID) (Mukherjee, 2003), although to our knowledge the latter has not been performed in insects. TLC-FID gives highly repeatable and accurate measurements of TAGs, requires only one solvent system, and utilises a highly quantitative detector (Mukherjee, 2003). Compared to GC, it is fast and inexpensive, and gives comparable results in the analysis of acylglycerides (Shantha and Napolitano, 1998). Chromatography is relatively low-throughput, and requires expertise and specialised equipment. These restrictions can be prohibitive and a host of alternative methods are used to quantify storage lipids without prior separation.

1.2. Gravimetric measurement of lipid

In gravimetric methods, the animal is weighed to give fresh mass (FM), dried to give dry mass (DM) from which water content can be calculated, then the lipids are extracted by a solvent (sometimes in conjunction with a Soxhlet apparatus that recycles heated solvent through a thimble containing the tissue) and the tissue re-weighed to give lipid-free dry mass (LFDM). Lipid can be calculated from the difference between DM and LFDM. Alternatively, the lipid extract may be weighed directly or used for further chromatographic or chemical analysis. Folch reagent (2:1 v/v chloroform: methanol; Folch et al., 1957) has been widely used for gravimetric lipid quantification in insects (e.g. Pullin, 1987; Ziegler, 1991; Ziegler and Ibrahim, 2001; Colinet et al., 2007; Sinclair et al., 2011). Extraction with Folch reagent removes glycerol, carbohydrates, amino acids, and phospholipids, as well as the non-polar neutral lipids (Newman et al., 1972), thus lipid mass will be overestimated by this method. Non-polar solvents such as chloroform (e.g. Hahn, 2005), petroleum ether; a mixture of light hydrocarbons formed from petroleum (e.g. Djawdan et al., 1997; Ojeda-Avila et al., 2003; Lease and Wolf, 2011), or diethyl ether (Blows and Hoffmann, 1993; Ballard et al., 2008) can be used to extract neutral lipids. Chloroform also extracts small amounts of non-lipid material (c. 6%), whereas petroleum ether and diethyl ether extract only neutral lipids (Dobush et al., 1985).

1.3. Vanillin Assay

The vanillin assay (Zöllner and Kirsh, 1962) is widely used to estimate insect storage lipids (e.g. Mwangi and Goldsworthy, 1977; Lorenz, 2003; Hahn, 2005; Huho et al., 2007; Geister et al., 2008). In this assay, TAGs are hydrolysed by sulfuric acid producing fatty acids, whose double bonds or hydroxyl groups react with the sulfuric acid to form an alkenyl cation (which is the chromogen). The alkenyl cation then reacts with the vanillin reagent (an aromatic hydrocarbon) to form a chromophore with maximum absorbance at 530 nm (Johnson et al., 1977). This absorbance is compared to a standard calibration curve, often cholesterol (e.g. Lorenz, 2003; Geister et al., 2008) or extracted insect fat (e.g. Otronen, 1995). The choice of standard is important, as saturated fatty acids do not form a chromogen and are not detected. In addition the reactivity of polyunsaturated fatty acids decreases with increasing unsaturation (Knight et al., 1972). Since insect TAGs have a high proportion of saturated and polyunsaturated fatty acids (Fast, 1970), we expect that a standard which shows 100% reactivity on a molecular basis (such as monounsaturated TAGs or cholesterol) will under-estimate lipid reserves.

1.4. Enzymatic Assays

Enzyme assays are specific, high-throughput, generally avoid the use of strong acids or toxic solvents, and are commercially available as kits, e.g. Serum Triglyceride Determination Kit, Sigma-Aldrich, St Louis, MO, USA; Infinity triglyceride assay, Thermo Electron, Arlington, TX, USA; StanBio Triglyceride Liquicolor Kit, StanBio, Boerne, TX, USA; Genzyme Triglyceride-SL, BioPacific Diagonostics Inc., Bellevue, WA, USA; Triglycerides - GPO reagent set, Pointe Scientific, Canton, MI, USA; and many other similar