Measuring Total Lipid Content in Rat Carcasses: A Comparison of Commonly Employed Extraction Methods†

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Intermethod differences between four common procedures for measuring carcass lipid content, lipid composition, and lipid energy value were determined: chloroform−methanol extraction, saponification plus hexane extraction, dichloromethane−methanol extraction (Soxhlet) and hexane extraction (Goldfisch). Samples were either freeze-dried or air-dried prior to Soxhlet or Goldfisch extraction. Carcass lipid content ranged from 7.8 (chloroform−methanol) to 8.8 (saponification) g/100 g carcass: the lowest and highest values were significantly different from each other but not from other procedures. The energy value of the extracted lipid ranged from 8804 (dichloromethane−methanol) to 9176 (saponification) kcal/g. Lipid class compositional analysis and fatty acid profile analysis showed significant differences between all methods showing that these are best determined after extraction with chloroform−methanol. The results also illustrated that small discrepancies may exist between methods for measuring total carcasses lipid content and carcass lipid energy but showed that no single, best method exists for measuring these parameters.

Keywords: *Lipid extraction; methods; rat carcass*

INTRODUCTION

Total body energy measurements are a necessary component of energy balance experiments and are required in many areas of nutritional biochemistry; body energy is, however, rarely measured directly. More commonly, total body lipid (free fatty acids + phospholipids + mono-, di-, and triacylglycerols + sterols + sterol esters) as well as protein content are measured and used to calculate body energy using specific factors (Pullar and Webster, 1977). Several different methods are currently used to determine the total lipid content of animal carcasses. These methods can be either chemical in nature (extraction of the carcass) or noninvasive (differential buoyancy, bioelectrical impedance, electromagnetic scanning, densitometry, etc.). Noninvasive methodologies offer several advantages when exact body composition is not needed since animal sacrifice is not required in the majority of cases. However, these methods usually rely on calibration curves to establish a relationship between body lipid content and a measured parameter. The relationship between lipid and instrument response is not universal, and it is necessary to calibrate the response for each animal model/subject (Muscaritoli et al. 1993; Segal et al., 1988; Tobin and Finegood, 1995).

For total lipid determination in animal carcasses, ether (or hexane) extraction using a Soxhlet apparatus is the most popular method, although other, less toxic solvent systems (Hara and Radin, 1978) and other, more rapid methodologies are also available (Lee et al., 1996). Choice of an optimal system is not clear-cut and depends, at least partly, on the nature of the material (Lee et al., 1996; Smedes and Thomasen, 1996), the number of samples that need to be processed, and the ease of use (to reduce potential sources of error; Roose and Smedes, 1996). Methods for extracting lipids from foods (Carpenter et al., 1993; House et al., 1994; Lee et al., 1996), biological fluids (Christie, 1992), and animal tissues (Broekhuyse, 1974) have all been published demonstrating the scope of available techniques.

When using total lipid content to calculate body energy, one must also consider the chemical composition of the extract. For example, mono-, di-, and triacylglycerols have acyl moieties in addition to a glycerol backbone. Phospholipids contain specific headgroups in addition to acyl groups. Cholesterol, which is commonly extracted by chemical methods, is an alcohol, although it may be esterifed to a fatty acid. The efficiency of extraction of these components may be method dependent (Carpenter et al., 1993) and will affect the final calculated energy value. There is general agreement that chloroform−methanol mixtures are optimal for extracting total lipid (Bligh and Dyer, 1959; Carpenter et al., 1993; Christie, 1982; Folch et al., 1957; Roose and Smedes, 1996; Smedes and Thomasen, 1996), but, as indicated above, fatty acids, which are the primary contributors to the total energy, form only a part of the total lipid extract. The inclusion of components other than fatty acids may lead to an overestimation of the calculated energy content of tissues. This will also be true for the ether-extraction (Soxhlet or Goldfisch) methods since they extract mono-, di-, and triacylglycerols, most sterols, and glycolipids (Carpenter et al., 1993).

The present study compared different commonly utilized extraction methodologies to determine if they significantly impact on the measured body lipid content and on the body energy associated with this lipid. To this end, we measured the carcass lipid content, the energy value of the extracted lipid, the overall lipid

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profile, and the fatty acid profile from a single, whole body homogenate. The homogenate was extracted by four different methods to determine the effect of saponification, types of solvents, extraction time, and extraction apparatus on measured lipid composition.

MATERIALS AND METHODS

Animal and Sample Preparation. One male, 191 g Sprague−Dawley rat was used as the source of all material. The animal was sacrificed by CO_2 asphyxiation, and the carcass was processed by homogenizing 1:1 (w:w) in water after autoclaving (Hartsook and Hershberger, 1963). Before extracting using the Soxhlet or Goldfisch apparatuses, the samples were either freeze-dried or air-dried for 5 h at 84 °C.

Procedures. Four different extraction procedures were followed. (1) **Saponification:** A 1 mL aliquot of 50% (w/v) aqueous KOH and a 2 mL aliquot of 95% ethanol were added to approximately 1.5 g of the homogenized carcass. The tubes were incubated at 92 °C for 2 h with repeated, vigorous vortexing. Samples were cooled to room temperature and extracted 3 times with 3 mL of hexane to remove the nonsaponified material. The remaining aqueous sample was then acidified to pH ≤2 by addition of 1.0 mL of 90% (v/v) concentrated HCl. The fatty acids were then extracted three times with 3 mL of hexane. The hexane was washed with 1 mL of H_2O and dried over Na_2SO_4. The hexane solvent was evaporated under N_2 at approximately 40 °C. (2) **Chloroform−Methanol:** This procedure was performed according to Bligh and Dyer (1959) as follows. A 10.6 mL aliquot of a 1.6:3:6 (v:v:v) mixture of water:chloroform:methanol was added to a 1.0 g sample of homogenate. The final ratio of water:chloroform:methanol was 0.8:1:2 at this stage. The mixture was homogenized (with ice cooling to prevent solvent evaporation) for 1 min using a Brinkman Polytron. Another 3 mL of chloroform was added, and the sample was homogenized again for 1 min with cooling. Finally, 3 mL of water was added, and the sample was homogenized for 15 s with cooling. The final water:chloroform:methanol ratio was 1.8:2:2. The sample was centrifuged to separate the organic from the aqueous layers. The chloroform layer was collected and dried over Na_2SO_4. The dried extract was filtered, and the solvent evaporated under N_2 at approximately 40 °C. (3) **Soxhlet:** Either freeze-dried or air-dried samples were extracted for 90 min with 9:1 (v:v) dichloromethane:methanol. (4) **Goldfisch:** Either freeze-dried or air-dried samples were extracted for 6 h with hexane. The amount of lipid in the tissue was determined gravimetrically for all procedures.

Other Methods. The energy of the extracted lipids was determined by bomb calorimetry (Parr Model 1261, Isoperibol bomb calorimeter, Parr Instrument Co., Moline, IL). Approximately 100 mg of sample was spiked with a known quantity of mineral oil to obtain a total heat production within the range of the bomb. This method gives an error of approximately 1% in the final energy value of the sample (data not shown). Analysis of lipid class composition was performed by Iatroscan TLC/FID (Ackman and Ratnayake, 1989). Different lipid classes were identified against standard runs containing corn oil (triacylglycerols; Mazola, Best Foods Canada, Inc., Etobicoke, ON, Canada), cholesterol oleate (Sigma Chemical Co., St. Louis, MO), stearic acid (Applied Science Laboratories Inc., State College, PA), phosphatidyl choline (Sigma Chemical Co.), and cholesterol (Sigma Chemical Co.). The identification of the Iatroscan peaks was confirmed using silica gel TLC analysis in hexane:diethyl ether:acetic acid (85:15:1) with standards containing stearic acid, tripalmitin (Sigma Chemical Co.), 2,3-dipalmitin (Sigma Chemical Co.), monopalmitin (Sigma Chemical Co.), cholesterol, and phosphatidyl choline. Fatty acid analysis was performed by GC analysis after converting the extracted fatty acids to the fatty acid methyl esters using a 7% borontrifluoride−methanol methylating reagent (Morrison and Smith, 1964). The moisture content of the carcass homogenate was analyzed by drying to constant weight at 90 °C in an oven. Statistical analyses were performed using Statistica for Windows (StatSoft, Inc. 1998, Tulsa, OK). Data were analyzed by ANOVA followed by Tukey's LSD test for unequal N when indicated by the F value. Percentages were transformed by square root arc sin prior to analysis.

Table 1. Carcass Lipid Content and Energy of Extracted Lipid Determined by Six Different Methodologies[a]

method	N	carcass lipid (g/100 g carcass)	energy (kcal/g)
chloroform−methanol	12	7.84 ± 0.13^{a}	9126
saponification	5	8.81 ± 0.23^{b}	9176
Soxhlet, air-dried	4	8.47 ± 0.06^{ab}	9054
Goldfisch, air-dried	4	8.72 ± 0.44^{ab}	9122
Soxhlet, freeze-dried	3	8.66 ± 0.07^{ab}	8804
Goldfisch, freeze-dried	3	8.56 ± 0.31^{ab}	9036

[a] Values for carcass lipid content represent means ± SEM for the indicated number of determinations. The energy value per g extracted lipid was determined one time only ($N = 1$) on the pooled, extracted lipid. Values in columns with different superscripts are statistically different at the $P < 0.05$ level as determined after ANOVA analysis followed by Tukey's LSD test for unequal N.

RESULTS AND DISCUSSION

All methods were compared to the chloroform−methanol extraction procedure of Bligh and Dyer (1959). Several reasons underlie this choice. The Bligh and Dyer method represents the method of choice for lipid class compositional analysis and fatty acid profile analysis of animal tissue (Christie, 1992) and a variety of foodstuffs, especially meat (Carpenter et al., 1993). It is a highly reproducible method (Roose and Smedes, 1996), and it is frequently used for measuring body lipid composition. It should be noted that no standard method currently exists for determining carcass lipid content in laboratory animals.

Extraction by the saponification method gave a carcass lipid content that was slightly higher than the other methods, whereas extraction by the chloroform−methanol method gave a lipid content that was slightly lower than the other methods. The saponification and chloroform−methanol methods were significantly different from each other but not from any other method (Table 1). The slightly higher lipid content associated with the saponification method could have (at least partly) been the result of lipid oxidation. Incorporation of extra oxygen atoms into unsaturated fatty acids may increase the overall weight. Evidence for increased oxidation with the saponification method is presented in Table 2 (see below). Saponification may also give higher values because of its superior ability to break down the structural matrix of the sample which could result in higher lipid extractability. Initially, we expected the chloroform−methanol method to give a higher lipid content of the tissues, as compared to the saponification method, since the chloroform−methanol extract is comprised of many components including sterols and glycerol (in the form of mono-, di-, and triacylglycerols) as well as phospholipid headgroups (Table 3). It was thought that inclusion of these components would increase the weight of the final lipid extract and, consequently, decrease the energy value of the extracted lipid since they have a lower energy value (per g) than the fatty acid component. This clearly was not the case (Table 1). The lower lipid content observed after chloroform−methanol extraction may have been the result of methodological differences in extraction temperatures. Although a significantly higher lipid content

Table 2. Fatty Acid Distribution in Rat Carcass after Various Methods of Extraction[a]

	percentage of total fraction					
fraction	chloroform−methanol	saponification	Soxhlet, air-dried	Goldfisch, air-dried	Soxhlet, freeze-dried	Goldfisch, freeze-dried
saturates	31.61	40.08	35.00	52.40	32.93	33.77
monounsaturates	34.84	30.50	33.09	26.31	33.93	34.89
n-6 fatty acids	28.20	24.92	26.94	18.16	27.96	26.84
n-3 fatty acids	3.77	3.18	3.61	1.85	3.71	3.15
other fatty acids	1.58	1.33	1.36	1.28	1.47	1.36

[a] Samples represent a single determination.

Table 3. Relative Distribution of Extracted Lipid between Cholesterol Ester, Free Fatty Acid, Triacylglycerol, Cholesterol, Diacylglycerol, and Monoacylglycerol + Phospholipid Fractions as Determined by Iatroscan TLC/FID[a]

		relative area percentage of component in each fraction				
method	*N*	free fatty acids	triacylglycerols	cholesterol	diacylglycerols	monoacylglycerols + phospholipids
chloroform−methanol	6	45.0 ± 0.6^{a}	8.19 ± 0.45^{a}	0.83 ± 0.11^{a}	16.2 ± 0.1^{a}	29.8 ± 1.6^{a}
saponification	18	99.1 ± 0.1^{b}	ND[b]	ND	0.95 ± 0.10^{b}	ND
Soxhlet, air-dried	6	46.2 ± 0.9^{a}	2.58 ± 0.07^{b}	0.49 ± 0.06^{b}	9.58 ± 0.37^{c}	41.1 ± 1.2^{b}
Goldfisch, air-dried	6	46.1 ± 1.0^{a}	6.52 ± 0.21^{c}	0.85 ± 0.08^{a}	16.8 ± 0.7^{a}	29.8 ± 1.9^{a}
Soxhlet, freeze-dried	6	29.9 ± 0.7^{c}	12.0 ± 0.4^{d}	0.60 ± 0.05^{ab}	18.9 ± 0.8^{d}	38.6 ± 2.0^{b}
Goldfisch, freeze-dried	4	45.7 ± 0.9^{a}	30.1 ± 0.7^{e}	0.90 ± 0.05^{a}	15.8 ± 0.6^{a}	7.55 ± 0.20^{c}

[a] Values represent means ± SEM for the indicated number of runs. The values represent the area of the individual peak divided by the total Iatroscan response. They were not corrected for TLC/FID response. Values in columns with different superscripts are statistically different at the $P < 0.05$ level as determined after ANOVA analysis followed by Tukey's LSD test for unequal *N*. Values were transformed by arcsin($\sqrt{y}$) prior to analysis. [b] Not detected.

was observed when chloroform−methanol mixtures were compared to other solvents in Goldfisch or Soxhlet apparatuses (Dobush et al., 1985), our chloroform−methanol extraction was carried out at room temperature (using ice to cool the sample). In addition, problems with lipid adsorption and incomplete solvent recovery are known to occur with the chloroform−methanol extraction procedure (Smedes and Thomasen, 1996). These are apparently still observed at the relatively high 10:1 chloroform to "original sample" ratio used in the present extraction and may be magnified somewhat by the single extraction procedure used in the present study (Smedes and Thomasen, 1996). Neither of these problems would have been apparent with the saponification procedure and may explain much of the discrepancy between the two methodologies.

In contrast to previous results (Williams et al., 1995), freeze-drying or air-drying the samples had a minimal effect on the total lipid content as measured after Soxhlet or Goldfisch extraction. It was expected that these procedures may have reduced the lipid extractability by altering the physical structure of the carcass sample. This was apparently not the case as the lipid carcass content was similar to that determined by the saponification and chloroform−methanol methods.

All methods gave similar energy values when the pooled extracts were measured by bomb calorimetry ($N = 1$, pooled samples). The samples were pooled to obtain more accurate readings since the extraction methodologies gave small yields of lipid extract (50−80 mg). Under the procedure adopted in our laboratory, the final energy value has an error of ≤1%. This suggests that, with the exception of the Soxhlet, freeze-dried sample, the energy values are not statistically different.

Analysis of the fatty acid profiles (Table 2) and lipid classes (Table 3) highlighted differences between the extraction methodologies. The saponified sample contained mainly free fatty acids with a small amount of diacylglycerols (the other components were below the detection limit of the instrumentation). The elimination of most of the nonfatty acid materials was accomplished by an ether extraction of the nonsaponified material prior to sample acidification and ether extraction of the saponified material. In contrast to the lipid class composition of the saponified sample, the other extracts contained measurable cholesterol and tri- and diacylglycerols as well as a combined monoacylglycerol + phospholipid peak (Table 3). A comparison of these extracts shows that the Soxhlet method (dichloromethane−methanol as solvent) gave a relatively higher percentage of diacylglycerols and monoacylglycerols + phospholipids in the final sample. When the samples were air-dried, the higher monoacylglycerol + phospholipid fraction increased at the expense of the di- and triacylglycerol fraction. This may have been the result of phospholipase action (to disrupt fatty acid−glycerol ester bonds) during the drying process, but our original sample was autoclaved to inactivate endogenous lipolytic enzymes and minimize any enzymatically linked triacylglycerol lipolysis (Williams et al., 1995). That this procedure was not totally successful is shown by the appreciable free fatty acid content of the lipid extracts: phospholipases may have been active during carcass processing prior to autoclaving. However, autoclaving should have prevented lipase activity during further processing (such as air-drying). This implies that the differences between air-dried and freeze-dried samples may have resulted from the air-drying process itself. A comparison of the lipid profile from the air-dried Goldfisch method (hexane as solvent) and the Soxhlet method (dichloromethane−methanol as solvent) highlights the inherent problems in controlling the air-drying process. Both methods should have given similar results but contained significantly different classes of lipids (Table 3) attesting to the difficulty in controlling the air-drying process.

Evidence for an effect of dichloromethane−ether extraction on the lipid distribution was supported by an altered lipid class distribution in freeze-dried dichloromethane−methanol extracts (Soxhlet): the area attributable to free fatty acids was reduced and areas of

the di- and triacylglycerols as well as the area of the monoacylglycerol + phospholipid band were higher (Table 3). The altered lipid distribution pattern is best explained by a differential extraction of lipid classes by dichloromethane−methanol. Lipid class analysis of freeze-dried, hexane-extracted material (Goldfisch) showed a larger proportion of triacylglycerols and a small proportion of monoacylglycerols + phospholipids in the final sample. This was probably due to a more efficient extraction of nonpolar material by a nonpolar solvent (hexane), but the physical manipulations (either freeze-drying or air-drying) were also important in determining the final fatty acid class distribution. Specifically, the drying procedure probably toughened up the matrix proteins to reduce access by the solvent to phospholipid-rich areas.

Examination of the fatty acid distribution in the various extracts showed a different effect of solvent and apparatus (Table 2). The saponified sample had a high percentage of saturates and a lower percentage of monounsaturates and n-6 fatty acids suggesting that our saponification conditions led to the destruction of some of the unsaturated fatty acids. If the destructive mechanism included the introduction of oxygen into the products, this may explain the increased weight of the final lipid extract and would account for the higher lipid content observed with this method. The air-dried samples also contained a large percentage of saturates with the Goldfisch (air-dried) procedure giving the highest values: the percentage of unsaturated fatty acid destruction depended on the extraction methodology as well as the sample treatment prior to extraction. The results, therefore, suggest that intermethod variability is in large part due to the air-drying process since the freeze-dried samples had ratios of saturates to unsaturates that were similar to that found in the chloroform−methanol extract. The freeze-dried samples also had similar percentages of n-6 and n-3 fatty acids showing that the freeze-drying procedure is superior to the air-drying method when fatty acid distribution is used as the measurement criterion. Air-drying is well-known to oxidize exposed unsaturated fatty acids (Williams et al., 1995) and is a difficult process to regulate.

In summary, it appears that the various methods are all adequate for measuring total lipid content gravimetrically in rat carcasses although a slight variation exists between methods with the chloroform−methanol method giving the lowest lipid content and the saponification method giving the highest value. The high value obtained from the saponification method may have been due to oxidation during the saponification process. Analysis of lipid classes and of lipid composition showed that these measurements should be performed on chloroform−methanol extracted samples only.

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