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Short communication

Evaluation of micro-colorimetric lipid determination method with samples prepared using sonication and accelerated solvent extraction methods

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ABSTRACT

Two common laboratory extraction techniques were evaluated for routine use with the micro-colorimetric lipid determination method developed by Van Handel (1985) [2] and recently validated for small samples by Inouye and Lotufo (2006) [1]. With the accelerated solvent extraction method using chloroform: methanol solvent and the colorimetric lipid determination method, 28 of 30 samples had significant proportional bias ($\alpha=1\%$, determined using standard additions) and 1 of 30 samples had significant constant bias ($\alpha=1\%$, determined using Youden Blank measurements). With sonic extraction, 0 of 6 samples had significant proportional bias ($\alpha=1\%$) and 1 of 6 samples had significant constant bias ($\alpha=1\%$). These demonstrate that the accelerated solvent extraction method with chloroform:methanol solvent system creates an interference with the colorimetric assay method, and without accounting for the bias in the analysis, inaccurate measurements would be obtained.

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1. Introduction

Recently, Inouye and Lotufo [1] demonstrated the utility of a micro-colorimetric assay method for measuring lipid contents of small tissue samples. The micro-colorimetric assay method permits the measurement of lipid contents in tissue samples of 10 mg and less where traditional gravimetric methods are very difficult to perform due to small quantities of lipid in the sample. Additionally, because of the sensitivity of the assay, only a small portion of the total extract is required for the assay, and thus, a large portion of the total extract is left for other analyses. With the gravimetric method, the entire extract would be used in the measurement and no further measurements could be performed.

In the report by Inouye and Lotufo [1], 10–50 mg of tissue was extracted using 4 mL of a chloroform:methanol mixture (1:1 v-v) with a handheld ground glass homogenizing tube [1]. The homogenate after grinding was centrifuged, and the supernatant carried forward to the micro-colorimetric assay developed by Van Handel [2]. In this study, the suitability of accelerated solvent extraction (ASE) and sonic extraction, two commonly used laboratory extraction procedures, were evaluated for use with the micro-colorimetric method.

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2. Experimental

2.1. Reagents

All solvents, 20–30 mesh Ottawa sand, and phosphoric acid (>85%) were from Fisher Scientific (Hampton, NH, USA). Concentrated sulfuric acid and silica gel 60–200 mesh were from J.T. Baker (Phillipsburg, NJ, USA). Other reagents were soybean oil – neat (Supelco, Bellefonte, PA, USA), diatomaceous earth (Varian Hydromatrix, Agilent, Santa Clara, CA, USA), and vanillin (Eastman, Rochester, NY, USA). Diatomaceous earth and Ottawa sand were pre-cleaned using a muffle furnace at 450 °C for 8 h.

The phosphovanillin reagent was prepared by placing 3.0 g of vanillin into 0.5 L of hot water. After dissolution of the vanillin, the vanillin solution was added to 2 L of 85% phosphoric acid. The reagent was stored at room temperature in darkness in an amber color bottle.

Soybean standard solution (10 mg/mL) was prepared by adding 50 mg of neat soybean oil to a 5 mL volumetric flask and diluted to volume using methanol/chloroform (1:1 v/v).

2.2. Biological tissue samples

Lumbriculus variegatus, a fresh water oligochaete (worm), was obtained from in-house culture facility.

2.3. Sample extraction

The extraction of lipid from the tissues was done using an accelerated solvent extraction system (ASE 300, Dionex, Sunnyvale, CA, USA) equipped with 33 mL stainless steel extraction cells. Tissue samples were dehydrated and homogenized using approximately 2 g of diatomaceous earth in a beaker, and then transferred to the extraction cell having a cellulose filter (Dionex, Sunnyvale, CA, USA) or glass fiber filter (Ahlstrom, Mt. Holly Springs, PA, USA) placed at the bottom of the cell. Surrogate standard was spiked into the cell and remaining volume in the cell filled with precleaned Ottawa sand. The extractions were performed using a chloroform:methanol (1:1 v/v) solvent mixture with extraction conditions of pressure of 1500 psi, temperature at 125 °C, heat time of 6 min, static time of 10 min, static cycles of 1, purge % of 60, and purge time of 60 s. Alternative extraction temperatures of room temperature (approximately 20 °C) and 50 °C were also used. The final volume of the extract was approximately 55 mL.

Sonic extractions were performed by placing tissue into 7 mL muffled (4 h, 450 °C) scintillation vial with 4 mL chloroform: methanol (1:1 v/v) solvent mixture. The contents were sonicated (Branson Sonifier 450 and Micro Adapter tip, Branson Ultrasonics, Danbury, CT, USA) for 2 min on power level 3 duty cycle 60%. Subsequently, the sample and extract were transferred with rinsing into a 15 mL centrifuge tube. The extracts were centrifuged at 3140 rpm for 15 min at 5 °C. After decanting into a 60 mL centrifuge tube, 10 mL of chloroform:methanol (1:1 v/v) solvent mixture was added to the 15 mL centrifuge tube, and the tube was sonicated, centrifuged, and decanted. This step was repeated one more time.

The sample extracts were concentrated to 2.5 mL using a gentle flow of N_2 gas and a 40 °C water bath.

2.4. Lipid analysis—phosphovanillin method

With the soybean standard solution, a standard curve consisting of 50, 100, 200, 300, 400, and 600 μg of lipids was prepared by pipetting 5, 10, 20, 30, 40, and 60 μL of the standard into 13 mm culture tubes, respectively. For each tissue extract, 50 μL of the chloroform:methanol extract was transferred into a 13 mm culture tube. All the culture tubes were then placed on the heated aluminum block at 100 °C (temperature range 100 °C –110 °C) for 30 min to evaporate all solvent from the tubes. The culture tubes were allowed to cool to room temperature, and 250 μL of concentrated sulfuric acid was added to each tube. The tubes were again placed on the heating block for 20 min at 100 °C. Maintaining the temperature above 100 °C is critical in this step for the hydrolysis of lipid esters. The tubes were then allowed to cool to room temperature and 5 ml of phosphovanillin reagent was added to each tube while vortexing. After a minimum of 30 min for color development, the absorbance at 525 nm was measured on UV–vis spectrophotometer (Cary 300 Bio, Varian, Walnut Creek, CA, USA or BioTek Synergy 4, BioTek, Winooski, VT, USA). Using the standard curve, amounts of lipid in each extract were computed.

Standard additions and Youden blanks were performed on the individual extracts as described by Gonzalez and Herrador [3] and Cardone [4–6]. The ratio of the slope of the standard additions method line (b_{SAM}) (absorbance vs. mass added) to the calibration line (b_{Cal}) (absorbance vs. mass) is the recovery (R) and in equation form:

$$R = b_{SAM}/b_{Cal} \tag{1}$$

The absence of proportional bias corresponds to a recovery of one, R=1. The Youden blank (θ) is determined by dividing the difference of intercepts of the Youden blank line (a_{YB})(absorbance vs. amount of sample) and calibration line (a_{Cal}) by the slope of the calibration line (b_{Cal}):

$$\theta = (a_{YB} - a_{Cal})/b_{Cal} \tag{2}$$

The absence of constant bias occurs when the intercepts of the Youden blank and calibration lines are equal, i.e., θ=0. The variances and statistical significances of the R and θ were computed as described by Gonzalez and Herrador [3].

3. Results

3.1. Accelerated solvent extraction

A series of *Lumbriculus variegatus* tissue samples and procedural blanks were prepared and extracted by using the ASE method. After concentration, the extracts were analyzed using the colorimetric lipid determination method. Standard additions and Youden blank analyses [3–6] were performed simultaneously on the extracts to examine for proportional and constant biases with the micro-colorimetric method (Table 1). Recoveries were significantly different from 1.00 (α=1%) indicating the presence

Table 1
Recoveries and Youden blanks for sample extracts prepared using accelerated solvent extraction and sonic extraction.

Extraction method / Sample type	Extraction temperature	Recovery		Youden blank	
		R	*t*-value	θ	*t*-value
ASE[a]: Cellulose Filter					
Blank	RT[b]	0.66	18.6*	−10.7	1.05
Blank	RT	0.57	16.2*	−11.5	1.11
Blank	RT	0.50	5.12*	−18.6	3.52^
Blank	50	0.70	9.01*	0.06	0.01
Blank	50	0.63	16.2*	−1.76	0.51
Blank	50	0.63	23.3*	−3.95	0.88
Blank	125	0.67	19.1*	−6.89	0.48
Blank	125	0.69	17.6*	−11.9	0.82
Blank	125	0.71	16.9*	−1.24	0.17
L. variegatus[c]	RT	0.83	3.24*	−22.1	1.78
L. variegatus	RT	0.67	3.26*	−16.6	1.01
L. variegatus	RT	0.53	13.1*	−14.1	1.11
L. variegatus	50	0.52	16.7*	−3.70	0.19
L. variegatus	50	0.51	12.6*	−13.8	1.06
L. variegatus	50	0.60	36.3*	2.80	0.49
L. variegatus	125	0.25	29.5*	33.0	1.38
L. variegatus	125	0.37	16.6*	−13.6	0.94
L. variegatus	125	0.39	8.27*	−1.42	0.18
Soybean oil	125	1.12	2.43	9.84	1.18
Soybean oil	125	0.71	14.7*	9.91	0.79
Soybean oil	125	0.76	4.12*	11.5	2.32
ASE: SA Glass Filter					
Blank	50	0.67	18.5*	−1.04	0.24
Blank	50	0.61	7.85*	−1.23	0.28
Blank	50	0.80	2.35	−2.01	0.45
Blank	125	0.67	5.15*	8.64	0.70
Blank	125	0.73	21.6*	−14.0	1.99
Blank	125	0.72	13.2*	−10.0	0.87
L. variegatus	125	0.50	15.8*	−2.37	0.18
L. variegatus	125	0.50	14.6*	−11.6	0.67
L. variegatus	125	0.47	31.5*	−13.5	0.76
Sonic					
Blank	RT	1.02	0.19	31.4	2.27
Blank	RT	1.15	0.29	32.5	2.36
Blank	RT	1.05	0.47	31.0	2.24
L. variegatus	RT	1.14	1.79	7.72	0.19
L. variegatus	RT	0.98	0.19	4.88	0.14
L. variegatus	RT	0.99	0.32	60.5	3.35^

[a] ASE=accelerated solvent extraction.
[b] RT=room temperature of approximately 20 °C.
[c] *Lumbriculus variegatus*.
* Significantly different from 1.0, α=1.0%.
^ Significantly different from 0.0, α=1.0%.

of a proportional bias in 28 of 30 extracts. Or, equivalently, the x-intercept of the standard additions lines were significantly different from the amount measured directly. Youden blank analyses yielded small total Youden blank values and the values were not significantly different from 0.00 ($\alpha = 1\%$) (Table 1). Included in the analyses were a series of extractions at lower extraction temperatures (room temperature and 50 °C), and these results were the same as that observed at the typical extraction temperature of 125 °C (Table 1).

The ASE method typically uses cellulose filters at the bottom of the extraction cell. To evaluate the possibility that the filters were the source of the interference, procedural blanks using the ASE method were prepared using ashed glass fibers at the bottom of the extract cell. The glass fiber filters had the same level of proportional bias (Table 1).

3.2. Sonic extraction

A second series of *Lumbriculus variegatus* tissue samples and blanks were prepared and extracted by using the sonic extraction method. Standard additions and Youden blank analyses [3–6] were simultaneously performed on the extracts to examine for proportional and constant biases with the micro-colorimetric method (Table 1). Recoveries (R) were not significantly different from 1.0 ($\alpha = 1\%$), indicating the absence of a proportional bias in the extracts (Table 1). One of the six samples had Youden blanks significantly different from 0.0 ($\alpha = 1\%$) indicating the presence of a constant bias in the extracts (Table 1).

4. Discussion

Dodd et al. [7] and Macnaughton et al. [8] reported that ASE combined with gravimetric analyses yielded quantitative measurements of lipid contents in fish and plants, and thus, indicating that extraction with ASE technique is quantitative. Inouye and Lotufo [1] and other [9–11] have reported no significant differences in lipid contents between the colorimetric and gravimetric methods with aquatic invertebrate, fish, and plant tissues where the samples were extracted using homogenization techniques, and thus, indicating that the colorimetric method is quantitative.

Based upon the measured recoveries and Youden blanks with ASE method, we conclude that the method, when using a chloroform:methanol extraction solvent, provides extracts that have a proportional bias and no constant bias with the micro-colorimetric lipid assay method. As a result, if analyses for lipid are done using ASE method with the micro-colorimetric assay method, corrections for proportional bias will need to be made in order to obtain accurate lipid measurements. We suspect that chloroform and methanol react during the extraction with the elevated pressure conditions in the stainless steel ASE cell. Temperature had little influence on the proportional bias suggesting that the elevated pressure conditions (1500 psi) were enough for the reaction creating the bias. In evaluating the ASE method for extraction completeness with extraction temperature of 125 °C, the insides of the extraction cells became discolored (brownish color) after three consecutive extractions with chloroform:methanol, and the discoloration strongly suggests that the chloroform and methanol were reacting in the extraction.

Based upon the measured recoveries and Youden blanks with the sonic extraction method, we conclude that neither a proportional nor a constant bias are present in the extracts created using the chloroform:methanol solvent system.

Based upon our findings of proportional bias with ASE combined with colorimetric lipid assay method, and consistent with the recommendations of Gonzalez and Herrador [3] and others [12,13], analytical methods should always be evaluated for precision, bias, and accuracy prior to their use in routine analysis. If the micro-colorimetric lipid assay method is to be used with ASE method, the proportional bias will need to be determined for the specific method, conditions, and reagents. With the determined proportional bias, corrected concentration amounts can be determined [3]. In our laboratory, we have changed to the sonic extraction method because of the absence of proportional and constant biases with the micro-colorimetric assay method.

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The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

References

[1] L.S. Inouye, G.R. Lotufo, Talanta 70 (2006) 584–587.
[2] E. Van Handel, J. Am. Mosq. Control Assoc. 1 (1985) 302–304.
[3] A. Gustavo González, M. Ángeles Herrador, TrAC Trends Anal. Chem. 26 (2007) 227–238.
[4] M.J. Cardone, Anal. Chem. 58 (1986) 433–438.
[5] M.J. Cardone, Anal. Chem. 59 (1987) 2818–2822.
[6] M.J. Cardone, Anal. Chem. 58 (1986) 438–445.
[7] E. Dodds, M. McCoy, A. Geldenhuys, L. Rea, J. Kennish, J. Am. Oil Chem. Soc. 81 (2004) 835–840.
[8] S.J. Macnaughton, T.L. Jenkins, M.H. Wimpee, M.R. Cormiér, D.C. White, J. Microbiolo. Methods 31 (1997) 19–27.
[9] H. Barnes, J. Blackstock, J. Exp. Mar. Biol. Ecol. 12 (1973) 103–118.
[10] Y.-S. Cheng, Y. Zheng, J.S. VanderGheynst, Lipids 46 (2011) 95–103.
[11] Y. Lu, S.A. Ludsin, D.L. Fanslow, S.A. Pothoven, Can. J. Fish. Aquat. Sci. 65 (2008) 2233–2241.
[12] L.S. Clesceri, A.E. Greenberg, R.R. Trussell, Standard Methods for the Examination of Water and Wastewater, Nineteeth Ed. American Public Health Association, American Water Works Association, and Water Polution Control Federation, Washington DC, USA, 1995.
[13] ASTM, ASTM D2777-13 Standard Practice for Determination of Precision and Bias of Applicable Test Methods of Committee D19 on Water, Annual Book of ASTM Standards, ASTM International, West Conshohocken, PA, USA, 2013.