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Effect of Sample Preparation, Length of Time, and Sample Size on Quantification of Total Lipids from Bovine Liver†

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The objective was to evaluate the effect of sample preparation (pulverization under liquid nitrogen, homogenization, or sonication), time length of sonication (0−60 s), shaking in chloroform/methanol solvent (0, 2, 4, or 12 h), incubation in chloroform (0 or 12 h), and drying of extracted lipids at 50 °C (2, 4, 6, or 24 h), and sample size (50−250 mg) on quantification of total lipids from bovine liver. Pulverization under liquid nitrogen yielded the lowest recovery. Sonication was least time-consuming for sample preparation. Precise estimates and the greatest recovery were obtained with 30 s of sonication, at least 2 h of shaking in chloroform/methanol solvent, 12 h of incubation in chloroform, and at least 6 h of drying. Sample sizes of at least 150 mg gave precise estimates. The results demonstrate that sample preparation, time length of different steps of the extraction procedure, and sample size affect quantification of total lipid from bovine liver.

KEYWORDS: Bovine; extraction; homogenization; liver; pulverization; quantification; sonication; total lipids

INTRODUCTION

Fatty liver (i.e., hepatic lipidosis) is a metabolic disorder that affects ca. 14% of the human population (*1*) and over 50% of all dairy cows during the peripartal and early postpartal period (*2*). Fatty liver is usually benign and reversible; however, it negatively affects the outcome of other metabolic and infectious diseases (*3*−*5*).

Total lipid (TL) concentrations of liver are determined usually by puncture biopsy followed by histological analysis or chemical analysis (*6*) using various organic solvents for TL extraction (*7*−*13*). The focus of most papers is to compare the efficiency of various organic solvents on lipid extraction from various tissues (*14*−*26*). Prior to extraction, animal tissues are disrupted by using various methods (*7*−*13*, *18*, *19*, *22*, *23*, *25*, *27*−*31*). Although the method of tissue disruption affects the concentrations of extracted lipids (*20*, *22*, *29*, *30*), different methods of tissue disruption rarely have been compared in publications (*20*, *29*, *30*). Therefore, a published comparison of the effectiveness of various methods of tissue disruption on TL extraction is warranted.

Other steps for TL quantification that, to our knowledge, have not been reported are the effectiveness of different time lengths of (a) sonication, (b) shaking in organic solvent, (c) incubation in chloroform, and (d) drying of extracted lipids on TL quantification. Often, the length of these procedures is not specified (*7*−*10*, *13*). Preliminary experiments showed that the time used for these procedures affects the efficiency of TL extraction and could partially explain incomplete TL extraction of some of the major methods for TL extraction (*8*, *11*, *12*, *15*, *19*, *21*, *25*) and differences in TL extraction between methods (*15*, *16*, *19*, *21*).

Current methods for quantification of TL are tested and developed using at least 1 g per determination, or the sample size is not reported (*7*, *8*, *10*−*12*). The amount of tissue obtained by liver biopsy is usually 1−2 g and becomes limiting when samples are determined in triplicates and other tissue components have to be measured. Therefore, the minimal sample size needed to precisely estimate TL concentrations in liver is another parameter that needs to be evaluated.

The objective of the current study was to compare the effect of various (a) methods of tissue disruption, (b) time lengths of sonication, (c) time lengths of shaking in chloroform/methanol solvent, (d) time lengths of incubation in chloroform, (e) time lengths of drying, and (f) sample sizes on TL quantification in bovine liver.

MATERIALS AND METHODS

Materials. Liver tissue samples were obtained from the liver of a freshly slaughtered, healthy Angus x Hereford bull and then diced and stored at − 80 °C until analysis. Liver tissue was disrupted with a model 106 homogenizer (Talboys Instrument Corp., Emerson, NJ) or a model 350 sonifier (Branson Sonic Power Corp., Danbury, CT). Lipid extraction was done with reagent spectrograde chloroform and methanol (Fisher Scientific, Pittsburgh, PA), a model 75 wrist action shaker (Burrel Scientific, Pittsburgh, PA), 42.5 mm glass microfiber filters (Whatmann International Ltd., Maidstone, England), a Model K

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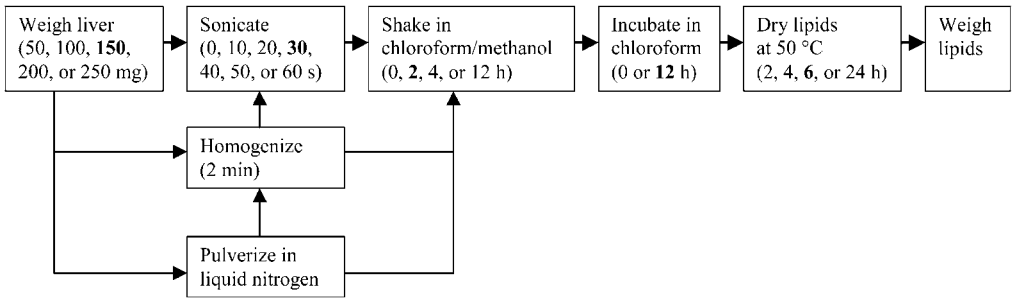

Figure 1. Outline of the various methods for determination of total lipids of bovine liver. Bold numbers indicate the method that is used most often.

centrifuge (International Equipment Co., Needham Heights, MA), and a SC/48R sample concentrator (Brinkmann Instruments Inc., Westbury, NY).

Methods. An outline of the various methods for quantification of TL is given in **Figure 1**. For comparison of various methods of tissue disruption, liver samples (250 mg) were weighed into a Pyrex culture tube (20 × 150 mm; Fisher Scientific, Pittsburgh, PA) that was closed with a Teflon−fluorocarbon−resin-faced rubber-lined cap. The sample was then pulverized under liquid nitrogen using a mortar and pestle (P), pulverized and homogenized for 2 min (PH), pulverized and sonicated for 30 s (PS), pulverized, homogenized, and sonicated (PHS), homogenized (H), sonicated (S), or homogenized and sonicated (HS). The sequence of steps, depending on the treatment group, was pulverizing, weighing, addition of 10 mL of chloroform/methanol (2:1, by volume), homogenization, and sonication. After homogenization, lipid extraction was done similarly to a commonly used method (*10*). After the samples were shaken for 12 h in a chloroform/methanol solution, 4 mL of demineralized and deionized H_2O was added, and then the samples were centrifuged for 20 min at 500*g*. After centrifugation, the methanol−water layer was removed by aspiration. After that, the contents of the test tubes were filtered and then rinsed and filtered three times with 2, 2, and 4 mL of chloroform, respectively, into a 20 mL borosilicate glass scintillation vial (28 × 57 mm; Kimble Glass Inc., Vineland, NJ) using a Buchner funnel with slight suction. The scintillation vials were closed with an aluminum foil-lined polypropylene cap (Kimble Glass), dried under air for 6 h at 50 °C, and then filled with nitrogen gas. The samples were stored for 12 h at 20 °C and then weighed. All treatments were done in quadruplet.

To compare different time lengths of shaking in the samples and various methods of tissue disruption (2 min of homogenization, 30 s of sonication, or homogenization and sonication), liver samples (250 mg) were shaken for 0, 2, 4, or 12 h in chloroform/methanol (2:1, by volume) before extraction. On the basis of the results, the shaking time was decreased from 12 to 2 h and sonication was chosen for sample homogenization for the following experiments. To compare the effect of different time lengths for sonication, liver samples (150 mg) were sonicated for 0, 10, 20, 30, 40, 50, or 60 s before shaking. To compare the effect of different time lengths for incubation in chloroform and for drying for extracted liver lipids, liver samples (250 mg) were filtered immediately or 12 h after removal of the water−methanol layer by aspiration (samples were stored at 4 °C). After that, extracted liver lipids were dried for 2, 4, 6, or 24 h at 50 °C under air. On the basis of the results, samples incubated at 4 °C in chloroform for 12 h and the extracted liver lipids were dried for 6 h for the following experiments. To determine the minimal amount of tissue needed to obtain a precise estimate of total lipid concentrations, 50, 100, 150, 200, or 250 mg of liver samples was used. All treatments were done in quadruplet. We did not add antioxidants to the samples, because peroxidation of lipids does not affect the precision of TL estimates and TL recovery (data not shown). We did not determine lipid and fatty acid profiles, because the objective of the study was to demonstrate that the above-mentioned factors affect quantification of TL in bovine liver.

Statistical Analysis. The ANOVA procedure of SAS (version 8.2, SAS Institute Inc., Cary, NC) was used for statistical analysis. The statistical model contained the above-described treatments and possible interactions as fixed effects and the residuals as error terms. Contrasts were computed when treatment groups were compared, and the Bonferroni multiple comparison test was used when individual treatment groups were compared (version 8.2, SAS Institute). Statistical differences were judged significant at $P \leq 0.05$. Standard errors (SEs) and coefficients of variation (CVs) were computed separately for each treatment group.

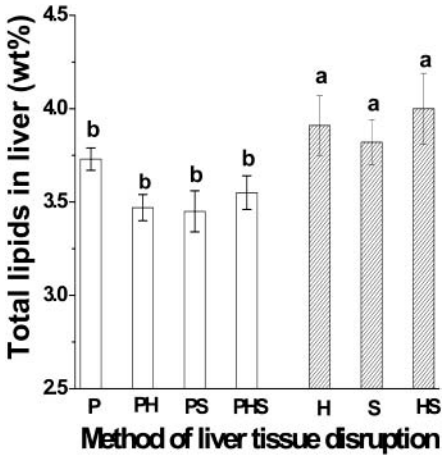

Figure 2. Effect of sample preparation on total lipid recovery from bovine liver. Bovine liver samples (250 mg) were pulverized (P), pulverized and homogenized for 2 min (PH), pulverized and sonicated for 30 s (PS), pulverized, homogenized, and sonicated (PHS), homogenized (H), sonicated (S), or homogenized and sonicated (HS) before lipid extraction. Columns and bars represent average total lipid concentrations ($n = 4$) and their standard errors, respectively. Clear and patterned columns represent pulverized and nonpulverized liver samples, respectively. Different letters above the columns indicate differences at $P \leq 0.05$. Effect of pulverization on total lipid concentrations: $P \leq 0.05$.

RESULTS

Sample preparation affected TL extraction from liver (**Figure 2**). Pulverization under liquid nitrogen yielded the lowest TL recovery (**Figure 2**; $P \leq 0.05$) with 6.0% (SE = 2.0%) lower TL concentrations because of condensation water (18−25 mg) and caused the greatest sample loss (10−47%), because some pulverized sample particles froze onto the mortar, pestle, and funnel when the samples were transferred to extraction vials. Homogenization, sonication, or combined homogenization and sonication had similar TL recovery (**Figure 3**; $P \leq 0.97$); however, sonication was the least time-consuming method (1 min vs 5 min).

The time length of shaking in chloroform/methanol solvent (2:1, by volume) affected TL recovery and the precision of extraction (**Figure 3**). Liver samples that were shaken for at least 2 h had 27.7% (SE = 3.23%) greater TL recovery and greater precision (CV = 5.9% vs CV = 12.3%) than did liver samples that were not shaken (**Figure 3**; $P \leq 0.0001$). There

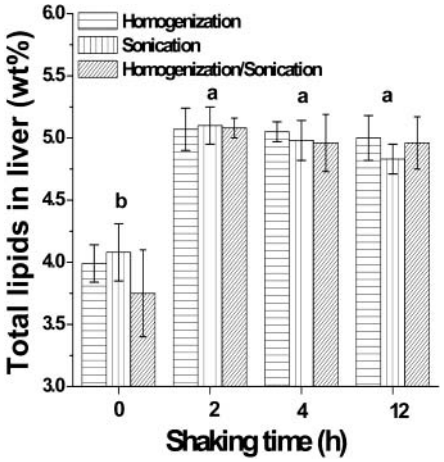

Figure 3. Effect of sample preparation and the time length of shaking on total lipid recovery from bovine liver. After treatment with various methods of tissue disruption [2 min of homogenization (H), horizontal lines in columns; 30 s of sonication (S), vertical lines in columns; homogenization and sonication (HS), ascending lines in columns], bovine liver samples (250 mg) were shaken for 0, 2, 4, or 12 h in chloroform/methanol (2:1, by volume) before extraction. Columns and bars represent average total lipid concentrations ($n = 4$) and their standard errors, respectively. Different letters above the columns indicate differences at $P \leq 0.05$. Effect of the method of tissue disruption: $P \leq 0.97$. Effect of the time length of shaking for 0 h vs ≥2 h: $P \leq 0.0001$; CV = 12.3% vs CV = 5.9%. Interaction between the method of tissue disruption and time length of shaking: $P \leq 0.62$.

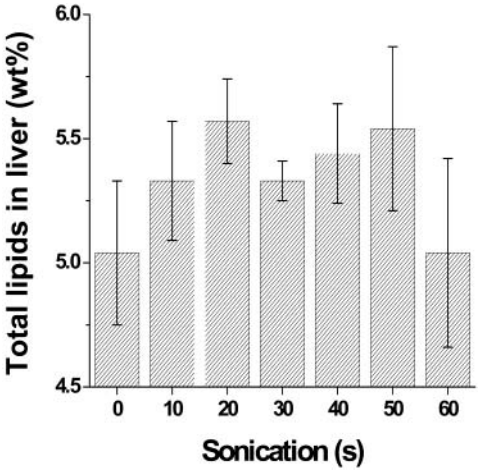

Figure 4. Effect of the time length of sonication on total lipid recovery from bovine liver. Liver samples (150 mg) were sonicated for 0, 10, 20, 30, 40, 50, or 50 s (*x*-axis) in chloroform/methanol (2:1, by volume) before shaking. Columns and bars represent average total lipid concentrations ($n = 4$) and their standard errors, respectively. Effect of the time length of sonication: $P \leq 0.68$. The coefficients of variation of total lipid concentrations for liver samples that were sonicated for 0, 10, 20, 30, 40, 50, and 60 s were 10.0%, 9.1%, 6.0%, 3.0%, 7.3%, 11.7%, and 15.0%, respectively.

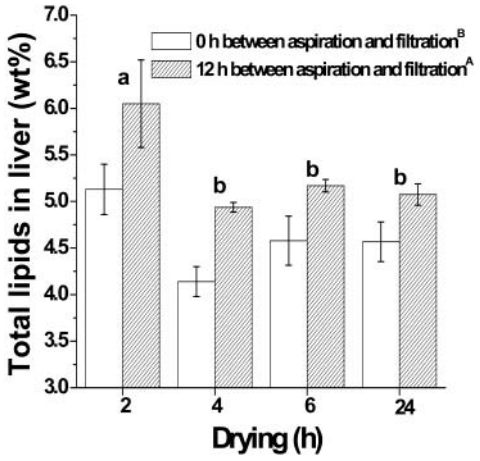

Figure 5. Effect of the time length of incubation in chloroform and time length of drying extracted liver lipids on total lipid recovery from bovine liver. Liver samples (250 mg) were filtered immediately (empty columns) or after 12 h of incubation in chloroform (patterned columns; samples were stored at 4 °C). After that, extracted liver lipids were dried for 2, 4, 6, or 24 h at 50 °C under air (*x*-axis). Columns and bars represent average total lipid concentrations ($n = 4$) and their standard errors, respectively. Different uppercase letters as superscripts after the legend labels indicate differences at $P \leq 0.05$ for the time length of incubation in chloroform. Different lowercase letters above the columns indicate differences at $P \leq 0.05$ for the time length of drying extracted lipids. Effect of the time length of incubation in chloroform: $P \leq 0.001$. Effect of the time length of drying extracted lipids for 2 h vs ≥4 h: $P \leq 0.001$. Interaction between the time length of incubation in chloroform and time length of drying extracted lipids: $P \leq 0.62$.

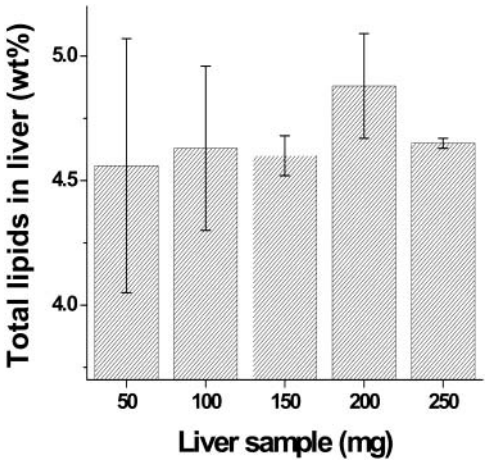

Figure 6. Effect of sample size on total lipid recovery from bovine liver. Liver samples of 50, 100, 150, 200, or 250 mg (*x*-axis) were used for lipid extraction. Columns and bars represent average total lipid concentrations ($n = 4$) and their standard errors, respectively. Effect of sample size: $P \leq 0.93$. The coefficients of variation of total lipid concentrations for liver samples of 50, 100, 150, 200, and 250 mg were 22.3%, 12.5%, 3.7%, 8.5%, and 0.96%, respectively.

was no interaction between the method of tissue disruption and duration of shaking (**Figure 3**; $P \leq 0.62$). The time length of sonication in chloroform/methanol solvent (2:1, by volume) affected the precision of TL extraction but not TL recovery (**Figure 4**; $P \leq 0.68$), with the most precise estimates at 30 s of sonication (CV = 3.0%).

The time length of incubation in chloroform affected TL recovery and the precision of extraction (**Figure 5**). Liver samples that incubated at 4 °C in chloroform for 12 h yielded 14.3% (SE = 3.7%) greater TL recovery and greater precision (CV = 3.5% vs CV = 9.9% in samples that were dried for at least 4 h) than did samples that were not incubated ($P \leq 0.001$; **Figure 5**). Drying of liver lipid extracts for 2 h under air at 50 °C was insufficient to completely dry liver lipid extracts (**Figure 6**; $P \leq 0.001$). Interactions between the time lengths for incubation in chloroform and for drying of extracted lipids were not significant ($P \leq 0.78$; **Figure 5**). The time length between drying and weighing and the temperature at which the samples

were stored before they were weighed affected TL concentrations. The most consistent results were obtained when empty and filled sample vials were kept before weighing for ca. 12 h at 20 °C (results not shown). Keeping empty and filled sample vials in a desiccator did not improve the results (results not shown).

Sample size affected the precision but not TL recovery ($P \leq 0.93$; **Figure 6**). Increasing the sample size improved the precision, as the CV decreased from 22.3% to 0.95% at sample sizes from 50 to 250 mg, respectively (**Figure 6**). The minimal sample size needed to precisely estimate TL concentrations in bovine liver is 150 mg with a CV of triplicates at nine different days of 3.6% and 8.3%, respectively.

DISCUSSION

It has been unequivocally demonstrated in many publications that the choice of organic solvent or solvent mixture, the sample-to-solvent ratio, and the choice of extraction system (dry column, Soxhlet, or wet extraction) affect TL recovery (*12*−*26*). Even standard procedures for TL extraction differ significantly in their TL recovery (*12*−*26*). The objective of the current study was to demonstrate that other parameters, which often are not even specified in the publications, also affect TL recovery and the precision of TL extraction.

Sample Preparation. Prior to solvent extraction, animal tissue samples are disrupted (a) with a blender or homogenizer (*8*, *9*, *11*, *12*, *25*), (b) by passage through a pressure cell (*22*), (c) by pulverization of frozen samples under liquid nitrogen with a mortar and pestle (*7*, *10*, *18*), (d) by sonication (*19*, *23*, *27*, *28*), (e) by pulverization and homogenization (*11*, *13*), or (f) by pulverization and sonication (*29*). Tissue disruption by pulverization or grinding is more effective in cell and cell membrane disruption than is tissue disruption by homogenization (*20*, *22*) because more physical pressure is applied to the cells, but sample losses are significant, as shown in the current study. Effective cell and cell membrane disruption is especially important in conditions that decrease TL extraction efficiency, such as extraction (a) by solvents with insufficient extraction efficiency, (b) at high temperatures, (c) of dried samples, and (d) of samples with low triacylglycerol concentrations (*20*, *22*, *24*, *29*). Drying the samples before analysis and pulverization under dry ice has the advantage that it decreases lipid hydrolysis probably by inactivating lipolytic enzymes (*24*, *30*); however, it also decreases extraction of TL, in particular of triacylglycerols, by decreasing their accessibility for solubilization by conformational changes (*20*, *24*, *29*, *30*). Furthermore, drying with heat further decreases extraction of TL and additionally increases lipid oxidation, which both can be prevented only partly by using antioxidants (*17*, *25*). Even freeze-drying decreased extraction of TL (*29*). Therefore, drying of the sample before TL extraction cannot be recommended. In the current study, pulverization in liquid nitrogen had the lowest TL recovery, which can be attributed to the sample dilution by condensation water, and had the greatest sample losses (**Figure 2**; see the Results). Because sample size is limited for TL determination of bovine liver, pulverization in liquid nitrogen cannot be recommended. In comparison to homogenization and pulverization, sonication is the least time-consuming method for tissue disruption, because cleaning of the apparatus between samples is the least tedious. At the same time, sonication does not decrease TL recovery (**Figures 2** and **3**). Sonication destroys membranes by liquid shear and cavitation and is used for bacteria lysis, but it can also be used for disruption of animal tissue membranes (*19*, *23*, *27*, *28*, *31*, *32*). The time length of sonication is either not mentioned (*23*, *28*, *29*) or varies in published reports (*19*, *27*, *31*). The current study demonstrates that sonication of liver tissue for less than 30 s is insufficient to consistently disrupt liver cells and that sonication for longer than 30 s also decreases the precision of TL extraction (**Figure 4**). The latter can be explained by conformational changes of lipids induced by heat from prolonged sonication (*29*, *32*). The heat-induced conformational changes might also explain the lower TL recovery in samples that were sonicated for 15 min (*19*). In summary, the current study clearly demonstrates that the method and time length of tissue disruption affects TL quantification and can explain differences in TL estimates of different publications.

Time Length. The time length for shaking and time length between shaking and filtration of tissue samples in organic solvents increased from a few minutes in the original TL extraction method papers (*7*−*9*, *11*−*13*) to 15 min or up to 18 h for each procedure in more recent papers (*10*, *18*, *22*, *25*, *29*, *31*). This observation indicates that the time lengths for shaking and incubation used in the original Folch and Bligh and Dyer methods (*8*, *11*) are insufficient for complete TL extraction of some tissues. It has been demonstrated that the efficiency of TL extraction can also be improved by repeated washing steps (*29*), but repeated washing steps can also either decrease the TL extraction efficiency or increase nonlipid extraction (*8*, *11*, *12*, *14*, *15*, *21*, *27*) and are, therefore, not recommended. The minimal time length for shaking depends on the organic solvent mixture and can extend to 6 h (*22*). The maximal time length for extraction depends on the organic solvent mixture (*22*). In the current study, it was demonstrated for chloroform/methanol solutions that shaking for at least 2 h improved TL recovery and that shaking for up to 12 h had no negative effect on TL recovery (**Figure 3**). Shaking improves TL extraction because shaking creates a physical shear on cells and lets all tissue cells come in contact with each organic solvent (*22*). Extended periods of shaking would improve dissolving the very hydrophobic triacylglycerols. Incubation of samples in chloroform for 12 h at 4 °C further improved the TL recovery and improved the precision of extraction (**Figure 5**). Chloroform has a high ionic strength and, therefore, is very effective in consistently dissolving nonpolar compounds (*21*, *31*), in particular the hydrophobic triacylglycerols. In the current study, incubation of samples in chloroform was chosen as an overnight step, and therefore, shorter incubation time lengths between 1 and 12 h were not tested, but it is very likely that shorter incubation time lengths would be as effective in improving TL recovery. The current study clearly demonstrates that sufficient time for interaction between the sample and solvent mixture is needed for complete TL extraction and that shortening the time length to speed up the extraction procedure decreases TL recovery. The time length needed for interaction between the sample and solvent mixture could also explain the underestimates in samples with high triacylglycerol concentrations with the traditional Bligh and Dyer method (*8*) and the improved TL recovery with repeated washing steps with other TL extraction procedures (*11*, *29*, *31*).

In papers that specify the drying procedure, extracted lipids are dried by (a) concentration under nitrogen at 40−50 °C (*8*, *9*), (b) rotary evaporation at 40−50 °C under nitrogen and reduced pressure (*21*, *24*, *26*), (c) rotary evaporation at 40−50 °C under vacuum (*19*, *25*, *28*, *29*), or (d) an oven for 30 min at 100 °C (*16*, *23*), when no further analysis is required. To our knowledge, comparisons of the effects of different drying procedures have not been published. The results of the current

study demonstrate that sufficient drying time is important to get reliable estimates for TL extraction (**Figure 5** and the Results). We were concerned that the extended drying time in this study might decrease TL extraction through hydrolysis or oxidation (*16*, *30*), which could be prevented by the addition of an antioxidant (*17*, *25*), but we did not find decreased TL recovery caused by prolonged drying (**Figure 5**). It cannot be excluded from the current study that highly unsaturated fatty acids are partly oxidized by prolonged drying, because the fatty acid profile was not determined. On the basis of previously reported experiments, it can be safely assumed that prolonged drying results in greater TL recovery and less fatty acid modification in comparison with procedures that use higher temperatures (Soxhlet procedures), strong acidic or basic conditions (acid or basic hydrolysis of lipids), or pressure changes (*16*−*18*, *20*, *22*, *24*). Another point of importance is to keep empty and filled sample vials before weighing for ca. 12 h at 20 °C, because the weights of the glass vials and the accompanying lids are temperature and humidity sensitive.

Sample Size. Sample size is an important factor for quantification, in particular when the tissue amount is small, as for liver biopsies. The sample size varies between 1 and 200 g in the original and most other TL extraction papers (*7*, *8*, *11*, *12*, *16*, *21*−*24*, *26*, *29*) and between 250 and 500 mg in some more recent papers (*7*, *17*, *18*, *31*) or is not specified (*10*, *33*). A possible problem for decreasing the sample size is that fatty infiltration progresses concentrically from the blood supply through the tissue (*33*). Decreasing sample size does not affect TL recovery but decreases the precision of TL extraction (*31*). Our results support those findings and suggest that the minimal amount of sample to obtain a precise TL estimate must be 150 mg (**Figure 6**). The upper limit for sample size depends on the lipid profile of the sample. Iverson et al. (*21*) demonstrated that, in samples with high triacylglycerol and low water concentrations, the amount of solvent used in the Bligh and Dyer method (*8*) is insufficient for complete extraction of TL because triacylglycerol globules cannot be dissolved, which results in underestimation of TL concentrations.

In conclusion, the current study showed that TL recovery and the precision of TL extraction depend on the (a) method of tissue disruption, (b) time length of sonication, (c) time length of shaking in chloroform/methanol solvent, (d) time length of incubation in chloroform, (e) time length of drying, and (f) sample size. Therefore, it is important (a) to specify exactly the method and any modifications for quantification of TL, (b) to test whether the modifications of the procedure affect the quantification of TL, and (c) to consider differences in procedures when comparing results for TL concentrations from different papers. In the current study, precise estimates and the greatest recovery for bovine liver samples were obtained with samples sizes of at least 150 mg, 30 s of sonication, at least 2 h of shaking in chloroform/methanol solvent, 12 h of incubation in chloroform, and at least 6 h of drying.

ABBREVIATIONS USED

CV, coefficient of variation; SE, standard error; TL, total lipid.

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