Comparative Study of Total Lipids in Beef Using Chlorinated Solvent and Low-Toxicity Solvent Methods

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ABSTRACT: Nine extraction methods (three using chlorinated and six nonchlorinated solvents) were compared for determining lipids in samples of low- (<5%) and high-fat (>20%) ground beef. The nine methods investigated were: Folch, Lees, and Sloane Stanley (FLS); Bligh and Dyer (BD); Bligh and Dyer modified by Undeland, Harrod, and Lingnert (BDU); Bligh and Dyer modified by Smedes (BDS); Hara and Radin (HR); Schmid, Bondzynski, and Ratzalaff (SBR); Roese-Gottlieb (RG); Burton, Webb, and Ingold (BWI); and Soxhlet (SE). The BDS and HR methods do not include solvents such as chloroform and methanol and can be recommended for meat samples that have both low and high fat content. If the use of organic solvents is not critical, the FLS and the BD extraction methods yield the highest results, but the FLS is more expensive owing to the high amount of solvent required. Without considering the toxicity of the solvents, the three BD extraction methods provided the best yields.

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KEY WORDS: Beef, chlorinated solvents, extraction methods, fat, total lipids.

The solvent extraction step is the most critical in the analysis of total fat, neutral and polar lipids, and FA composition of meats. Excessive sample manipulation can introduce error in the chemical analysis, such as contamination and/or inadequate extraction of the analyte. There is a consensus that the Soxhlet method, which uses petroleum ether, *n*-hexane, or diethyl ether as specified in the official methods of AOAC (1), extracts only the free lipids. Although several methods have been proposed for lipid extraction from biological tissues, only two (2,3), published over four decades ago, permit quantitative extraction of lipids. However, these methods use chloroform, a carcinogenic agent, as organic solvent, which from improper use and long-term exposure can lead to health problems in humans (4).

Considering the adverse effects of chlorinated solvents on the environment and human health, several researchers have proposed the replacement of the chloroform/methanol extraction methods by solvent mixtures such as cyclohexane/propan-2-ol (5), methylene chloride/methanol (6), *n*-hexane/propan-2-ol (7,8), and *n*-heptane/ethanol (9). A crude fat determination method for meat using supercritical carbon dioxide has been developed (10). This method does not use organic solvents, and the results are comparable to those obtained using the AOAC crude fat analysis methods just discussed. In this context, it should be noted that the most popular extraction method for total fat determination in meat and meat products is based on fat extraction with a mixture of diethyl ether and petroleum ether after an acidic hydrolysis step (11).

Although efforts have been made to substitute organic solvents in fat extraction methods, these different approaches lead to varying results for total lipid content in meat samples (12,13). The Bligh and Dyer methods presented high variability when interlaboratory comparisons were completed (14).

As there is a controversy about which extraction methods give accurate results for lipid determinations in meat samples, the aim of the present study was to examine nine extraction methods for lipids based on the type of solvent used (including three extraction methods based on chlorinated solvents) to verify which one(s) give results that represent the "true" lipid contents in meat samples.

EXPERIMENTAL PROCEDURES

Meat sample preparation. Two composite meat samples containing different levels of fat were prepared from the longissimus muscle (10 kg) purchased from a local supermarket. Five kilograms of lean beef sample was prepared by trimming off all visible fatty tissue and the other 5 kg was left as is. The samples were ground by using an electric meat grinder, passing the samples two times through a 0.48 cm i.d. plate and blended to give a composite sample. The samples were placed in polyethylene bags and stored at −18°C until analyzed. For each extraction method, the moisture was determined by official methods (1). Five replicates were made for each study.

Extraction methodologies. The solvents used in the extraction methods are presented in Table 1. All reagents and solvents were of analytical reagent quality and were purchased from Merck (Darmstadt, Germany).

(i) Method 1. Folch, Lees, and Sloane Stanley (FLS) (2). For each analysis, sample (10 g) was homogenized (Waring blender) with a mixture of chloroform/methanol (2:1) for 2 min. Each homogenate was filtered using a Whatman number 1 paper filter in a Büchner funnel, and the filtrate was collected and transferred to a separation funnel containing 50 mL of NaCl 0.9% (wt/vol). After allowing the filtrate to separate into

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TABLE 1
Extraction Methods

Method	Abbreviation	Solvents
1. Folch *et al.* (1957) (2)	FLS	Chloroform/methanol
2. Bligh and Dyer (1959) (3)	BD	Chloroform/methanol
3. Bligh and Dyer (3) by Undeland *et al.* (1998) (15)	BDU	Chloroform/methanol
4. Bligh and Dyer (3) by Smedes (1999) (5)	BDS	Cyclohexane/propan-2-ol
5. Hara and Radin (1978) (7)	HR	*n*-Hexane/ propan-2-ol
6. Schmid *et al.* in Croon and Wallim, (1992) (11)	SBR	Hydrochloric acid/diethyl ether/petroleum ether
7. Roese-Gottlieb (1998) (1)	RG	Ammonia/methanol/diethyl ether/petroleum ether
8. Burton *et al.* (1985) (9)	BWI	*n*-Hexane/ethanol/*n*-heptane
9. Soxhlet (1998) (1)	SE	Petroleum ether

two layers, the lipid content was determined gravimetrically after evaporation of the chloroform phase.

(ii) Method 2. Bligh and Dyer (BD) (3). Sample (100 g) was homogenized (Waring blender) with a mixture of 100 mL chloroform and 200 mL of methanol for 2 min to obtain a monophasic system. To this monophasic ternary system, 100 mL of chloroform was added and the mixture blended for 30 s. Then 100 mL of water was added and the system blended again for 30 s. The homogenate was filtered using Whatman number 1 filter paper, and the filtrate was collected in a graduated cylinder. Mechanical force was used to press the liquid out of the tissue. After allowing the filtrate to separate into two layers, the volume of the chloroform layer was measured. The lipid content was then determined by weighing the sample after evaporation of the known aliquot of chloroform to dryness at 40–50°C in a water bath; the final chloroform residue was eliminated by flowing a stream of nitrogen through the sample.

(iii) Method 3. Bligh and Dyer modified by Undeland, Harrod, and Lingnert (BDU) (15). Sample (20 g) was homogenized (Waring blender) with mixtures of 50 mL of chloroform, 100 mL of methanol, and 40 mL of water for 1 min. To the ternary system, 50 mL of chloroform was added and the mixture blended for 30 s. Water (50 mL) was added and then blended for 30 s. Finally, the homogenate was centrifuged (19,600 × *g*) for 15 min. The lipid content was determined by weighing the residue after evaporation of the chloroform phase.

(iv) Method 4. Bligh and Dyer modified by Smedes (BDS) (5). A sample mass containing <1 g of lipid and <5 g of water was weighed in a 100-mL glass jar, and 16 mL of propan-2-ol and 20 mL cyclohexane were added and mixed for 2 min using an UltraTurrax mixer. Taking into account the amount of water in the original sample, more water was included to obtain 22 g of water and mixed for 1 min. The phases were separated by centrifugation (450 × *g*), and the organic phase was transferred to an evaporation flask using a glass pipette. In a second extraction process, 20 mL of cyclohexane and 2.6 mL of propan-2-ol were added and mixed for 1 min in an UltraTurrax mixer. The organic phase was combined with the first extract and the solvent was evaporated on a water bath at 85°C. The residue was transferred quantitatively to a wide-mouthed weighing flask, with one glass petri dish using a few milliliters of the cyclohexane/propan-2-ol mixture or diethyl ether. The solvent was allowed to evaporate to dryness in a water bath (*ca.* 50°C), and the resulting material was placed in an oven at 103°C for 1 h. The lipid content was than determined by difference.

(v) Method 5. Hara and Radin (HR) (7). To 1 g of meat sample, 18 mL of hexane/propan-2-ol (3:2; HIP) was added. The mixture was homogenized for 30 s, and the suspension was filtered by using a sintered glass Büchner funnel (with a filter paper of 14 μm) fitted with a ball joint for use under pressure. The homogenizer, funnel, and residue were washed three times with 2-mL portions of HIP; the residue was resuspended each time and allowed to soak for 2 min just before applying air pressure. The extract was removed by mixing the pooled filtrates for 1 min with 12 mL of aqueous sodium sulfate solution (prepared from 1 g of anhydrous salt and 15 mL of water). The two layers formed in the process represent a volume of 18 mL. After processing the sample as described above, the lipid was isolated in the upper phase (hexane-rich layer).

(vi) Method 6. Schmid, Bondzynski, and Ratzalaff (SBR) (11). Approximately 3–5 g of homogenized meat sample was weighed on aluminum foil and transferred to an extraction tube; then 10 mL of hydrochloric acid (8 mol/L) was added and the tube was placed in a boiling water bath for 1 h. After the sample had cooled to approximately 30°C, 10 mL of 95% (vol/vol) ethanol was added and the sample was mixed. Diethyl ether (25 mL) was added and mixed, followed by addition of another 25 mL of petroleum ether and mixed. The tube was allowed to stand overnight to effect phase separation. The ether phase was siphoned off into a flat-bottomed flask, and the sample was re-extracted with 30 mL of a diethyl ether/petroleum ether (50:50, vol/vol) mixture. After phase separation, the organic phase was siphoned into the same flask as noted previously. This extraction was repeated a third time, and the organic phases were collected in the same flask. The solvent in the flask was then evaporated, and the flask was placed in a drying oven for 2 h at 102–105°C. Finally, the fat in the flask was weighed.

(vii) Method 7. Roese-Gottlieb (RG) (AOAC Official Method

920.177, 1997) (1). Sample (4 g) or an amount of uniform solution equivalent to this weight in dry substance was introduced into a Mojonnier fat extraction tube or similar apparatus. The sample volume was made up to 10 mL with water; then 1.25 mL of 25% (wt/vol) ammonia solution was added and the system mixed. Alcohol (10 mL) was then added and mixed; 25 mL of ether was added, and the system was shaken vigorously for *ca*. 30 s. Finally, 25 mL of petroleum ether (b.p. <60°C) was added and the mixture was shaken again for 30 s. The system was allowed to stand for 20 min or until separation of liquid phases was completed. The ether–fat solution was then drawn off (usually 0.5–0.8 mL) into a weighed flask. The liquid remaining in the tube was extracted with 15 mL of ether and with 15 mL of petroleum ether; the liquid was shaken vigorously for *ca.* 30 s with each solvent and allowed to settle. The mouth of the tubing and the filter were washed with a few milliliters of a mixture of equal parts of the two solvents (previously mixed and free of water), and the extraction step was repeated. If the first two solvent–fat solutions had been drawn off accurately, a third extraction would usually yield less than 1 mg fat, or *ca*. 0.02% for a 4-g sample. The solvent was then slowly evaporated on a steam bath and dried in an oven at 100°C to a constant weight. To test the purity of fat, it was dissolved in a small amount of petroleum ether. If a residue remained, the fat was washed again with petroleum ether and the dry residue weighed.

(viii) Method 8. Burton, Webb, and Ingold (BWI) (9). Sample (20 g) was homogenized (Waring blender) with 80 mL of water for 30 s. SDS (80 mL; 0.1 mol/L) was added to the system and the mixture blended for 30 s. Then 160 mL of ethanol (99.5% vol/vol) was added and blended for 30 s; 160 mL of *n*-heptane was added and blending was continued for 1 min. The homogenate was centrifuged (19,600 × *g*) for 15 min. The lipid content was then determined by weighing the residue after the evaporation of the *n*-heptane phase.

(ix) Method 9. Soxhlet extraction (SE) [AOAC Official Method 991.36, Fat (Crude) and in Meat Products] (1). Sample (3 g) was weighed into a thimble. Sand was added to the sample and mixed with a glass rod. The sample was dried for 1 h in an oven at 125°C and then cooled. The sample/sand mixture was loosened with a glass rod and then transferred to the Soxhlet extractor. The extraction cup containing a few glass beads was weighed and the sample was initially extracted with 40 mL of petroleum ether at its b.p. for 25 min, followed by rinsing for 30 min. The temperature of the system was adjusted to ensure a condensation rate of ≥5 drops/s. When the extraction process was completed, the condenser valves were closed and the ether recovered. The sample was dried in an oven at 125°C for 30 min, cooled, and weighed.

Fractionation of lipid classes. Lipid (1 g) was extracted by each extraction method and separated into three fractions on a column packed with 28 g of silicic acid as described by Sahasrabudhe and Smallbone (16). Fraction I was eluted with 300 mL of benzene and contained TG; fraction II was eluted with 300 mL of diethyl ether and contained the FFA, MG, DG, and sterols; and fraction III was eluted with 300 mL of chloroform/methanol (1:4) and contained polar lipids.

Statistics. The mean values were statistically compared by the Tukey test at a 5% confidence level with one-way ANOVA. Data were processed using Statistica 5.1 software (17).

RESULTS AND DISCUSSION

The chlorinated solvent methods (Table 1) use chloroform, methanol, and water following the BD (3) method, but with different solvent/mass ratios. The BD is the most widely used method for total lipids determination and is accepted as giving the most accurate results (12).

The results obtained for total lipids, TG, polar lipids, and other lipid components in low- and high-fat ground beef from longissimus dorsi muscle are presented in Tables 2 and 3, respectively. Table 2 shows that the BD method yielded the highest extraction of total lipids for meat samples with low fat content. However, the highest level was not statistically different from most of the other values. Only the BWI and SE methods showed significantly different ($P < 0.05$) extraction yields, 2.14 and 2.2 g/100 g fresh weight, respectively, corresponding to 71.3 and 73.3%, from that obtained by the BD method (3.0 g/100 g fresh weight). For meat samples with high fat contents

TABLE 2-
Total, Neutral, and Polar Lipids from Lean Beef (g/100 g fresh weight)[a]

	Fractions			
Method	Total lipids	TG	Other[b]	Polar lipids
FLS	2.9 ± 0.2a	2.2 ± 0.2a	0.21 ± 0.01a,b	0.56 ± 0.02a
BD	3.0 ± 0.1a	2.2 ± 0.1a	0.23 ± 0.01a	0.55 ± 0.03a
BDU	2.82 ± 0.04a	2.11 ± 0.06a	0.18 ± 0.02b,f	0.52 ± 0.03a,b
BDS	2.68 ± 0.03a	2.01 ± 0.03a	0.19 ± 0.02ab	0.47 ± 0.02b
HR	2.86 ± 0.04a	2.12 ± 0.02a	0.23 ± 0.02a	0.50 ± 0.02a,b
SBR	2.78 ± 0.03a	2.08 ± 0.05a	0.17 ± 0.02b,d,f	0.51 ± 0.02a,b
RG	2.75 ± 0.02a	2.06 ± 0.04a	0.18 ± 0.01b,c,f	0.50 ± 0.03a,b
BWI	2.14 ± 0.03b	1.65 ± 0.02b	0.14 ± 0.01c,d,e	0.33 ± 0.04c
SE	2.2 ± 0.4b	2.0 ± 0.3a	0.15 ± 0.02e,f	0.05± 0.02d

[a]All values are means ± SD of five determinations.
[b]Includes FFA, MG, DG, and sterols. Averages followed by different letters in the same column are significantly different ($P < 0.05$) by the Tukey test.

TABLE 3
Total, Neutral, and Polar Lipids from High-Fat Beef (g/100 g fresh weight)[a]

	Fractions			
Method	Total lipids	TG	Other[b]	Polar lipids
FLS	20.8 ± 0.9a	18.68 ± 0.04a	1.53 ± 0.04a	0.53 ± 0.03a
BD	20.1 ± 0.4a	18.17 ± 0.09b	1.39 ± 0.03b	0.52 ± 0.03a
BDU	19.73 ± 0.02a,b	18.00 ± 0.03c	1.22 ± 0.02c	0.51 ± 0.02a,b
BDS	18.4 ± 0.5c,d	16.73 ± 0.02f	1.22 ± 0.03g	0.42 ± 0.02c,e
HR	18.54 ± 0.05b,c	16.93 ± 0.03d	1.14 ± 0.04d,g	0.46 ± 0.02b,c,d
SBR	18.5 ± 0.3b,d	16.68 ± 0.03f	1.34 ± 0.04b,e	0.48 ± 0.02a,d
RG	18.22 ± 0.02c,d	16.51 ± 0.02e	1.28 ± 0.01c,e	0.42 ± 0.03c,e
BWI	16.54 ± 0.02e	15.10 ± 0.02g	1.08 ± 0.01d	0.36 ± 0.03e
SE	16.1 ± 0.7e	15.14 ± 0.02g	0.71 ± 0.02f	0.24 ± 0.02d,f

[a]All values are means ± SD of five determinations.
[b]Includes FFA, MG, DG, and sterols. Averages followed by different letters in same column are significantly different ($P < 0.05$) by the Tukey test.

(Table 3), the FLS extraction method gave higher extraction yields, and the result was not significantly different from the yields obtained by the BD and BDU extraction methods. It should be noted that total lipid contents from the other six extraction methods were significantly different from those obtained by the FLS, BD, and BDU methods.

Regarding the fractionation of TG for low-fat meat (Table 2), the SE method yielded an extraction efficiency for total lipids of 90.9% (2.0 and 2.2 g/100 g fresh weight for TG and total lipids, respectively), which was the highest observed extraction yield. For the BWI method, the percentage of extraction was 77.1% from total lipids (1.65 and 2.14 g/100 g fresh weight for TG and total lipids, respectively), and the result was significantly different ($P < 0.05$) from those obtained by using the other extraction methods (Table 2).

With respect to the polar lipid fraction in low-fat meat (Table 2), the results of the BWI and SE methods were significantly different from the others. The highest observed value for this fraction was 0.56 g/100 g fresh weight, obtained by the FLS method, and corresponding to 19.3% of the total lipids (2.9 g/100 g fresh weight). The lowest value for polar lipids was obtained by the SE method (0.05 g/100 g fresh weight) corresponding to only 2.3% of the total lipids (2.2 g/100 g fresh weight) and 8.9% of the polar lipids (0.56 g/100 g fresh weight) determined by the FLS method.

For lipid content of high-fat meat samples, the FLS, BD, BDU, SBR, and HR methods yielded the highest values (0.53, 0.52, 0.51, 0.48, and 0.46 g/100 g fresh weight, respectively). The FLS, BD, and BDU methods did not yield significantly different results ($P < 0.05$) (Table 3). Again, the less effective methods for extracting total lipids were BWI and SE (16.54 and 16.1 g/100 g fresh weight), as the results for both corresponded to *ca.* 78% of that determined by the FLS method (20.8 g/100 g fresh weight).

Significantly different results were observed for all the TG extraction methods for high-fat meat (Table 3), except those of BDS and SBR, BWI, and SE. The SE method yielded high extraction values (15.14 g/100 g fresh weight) that corresponded to 94% of the total lipids (16.1 g/100 g fresh weight).

In meat samples with high fat levels, the TG content was greater when compared with those meat samples having low fat amounts. The polar lipids content in samples with high and low fat presented similar values (g/100 g). It should be noted that the amount of polar lipids extracted from low-fat meat when compared with total lipids (Table 2) was higher than those extracted from high-fat meat (Table 3).

For meat samples with a high fat content, the TG/total lipids ratio was almost unaffected by the extraction method (Tables 2 and 3). This observation contrasted with the lipid results, which were shown to depend on the extraction method (15). Results for total lipids content probably are dependent on the polarity of the solvent system. The chloroform/methanol/water extracts are more polar than those of alkane/alcohol/water (15).

The low extraction efficiency of the alkane/alcohol/water mixture with respect to phospholipids occurs for two reasons. First, ethanol and isopropanol are less polar than methanol in the BD method [as observed by Zief and Kiser (18)]; second, the solubility of polar lipids is low in hydrocarbon solvents, such as hexane, when compared with their solubility in chloroform (15). When the results obtained using the chloroform/methanol/water solvent system were compared with that of hexane/propan-2-ol/water (19), the former had a higher extraction yield.

The alkane/propan-2-ol mixture has been used to obtain quantitative results for neutral lipids, and it has been proposed as an alternative to the chloroform/methanol extraction (7), mainly because the former is less toxic than the latter.

Sahasrabudhe and Samallbone (16) compared the amount of neutral and polar lipids that were extracted from meat with low, medium, and high fat contents using seven extraction methods. Among the methods studied were BD and HR, and the HR method was found to extract the smallest amount of polar lipids from meat samples with medium and high fat contents (19).

The SE method can be easily carried out without chlorinated solvents, but the results obtained are frequently lower than those obtained by the BD method. Thus, the SE results cannot be considered as total lipids but as extractable lipids. According to de Boer (20), the fraction that is not extracted by the SE

method can be considered as bound lipids. Furthermore, the extraction yield in the SE method is determined by the solvent composition and by the extraction time, i.e., by the number of extraction cycles (5).

If the analyst does not intend to use solvents such as chloroform and methanol for analysis, the BDS and HR extraction methods can be recommended for meat samples that have either a low or high fat content. On the other hand, if the use of organic solvents is not critical, the FLS and the BD extraction methods yield the highest results. It is important to note that the FLS method is more expensive because of the high amount of solvent required to carry out the extraction process. Finally, without considering chloroform and methanol toxicity, the three BD (unmodified and modified) extraction methods provided the best yields.

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