Crude Lipids from Cow Dung: A Rapid and Efficient Extraction Method

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ABSTRACT

Background: The traditional Soxhlet method, known for its lengthy 8 to 16-hours extraction process according to AOAC (2005), prompted the exploration for a faster alternative to extract crude lipids from cow dung.

Methods: In this study, we compared and standardized three methods-Soxhlet, Mojonnier acid digestion and Folch method-for crude lipid determination. The dung sample of the Sahiwal cow breed was collected from the Live Stock Research Centre, NDRI-Karnal.

Result: Different sample size, nature of sample, amount of HCL for digestion was used for standardization of the methods. The crude lipid content as determined by Folch method, Mojonnier acid digestion method and Soxhlet method was 3.37±0.50, 4.41±0.07 and 4.13±0.25 per cent, respectively. Results from Sahiwal breed dung samples showed significantly lower crude lipid content using the Folch method. Mojonnier method, particularly with dry samples, demonstrated comparable results to Soxhlet with an extraction time (1 to 1.3 hours), offering a viable alternative for rapid extraction of crude lipid.

Key words: Crude lipid, Dung, Extraction, Standardization, Validation.

INTRODUCTION

Cow dung, as per Ayurveda, is not merely waste but a natural purifier (Randhawa and Kullar, 2011). It comprises undigested remnants of herbivorous bovine animals' food, representing an abundant and economical bio-resource globally. In rural India, cows (*Bos indicus*) produce 9-15 kg dung/day (Brown, 2003), while buffalo yields 19.05 to 26.75 kg dung per day (Karttek *et al.*, 2014). Traditional applications of cow dung, such as fuel, mosquito repellent and cleansers, are well-established (Girija *et al.*, 2013; Gugalia, 2021). The Indigenous Indian cow dung is a rich source of important minerals like calcium, phosphorus, zinc and copper and is found in a higher amount compared to the dung of cross-bred cows (Garg and Mudgal, 2007; Randhawa and Kullar, 2011; Patra and Bharti, 2024). Cow dung contains a diverse group of microorganisms that, due to their ability to produce a variety of metabolites, may be beneficial to humans (Sagar *et al.*, 2024; Randhawa and Kullar, 2011; Somrug *et al.*, 2024). The relationship between the feed and the excreta in cattle has been well-studied by many researchers (Kissinger *et al.*, 2007; Kissinger *et al.*, 2006; Hao *et al.*, 2004; Hollmann *et al.*, 2008; Sweeten *et al.*, 1985). Extensive studies have been done on the conservation of nutrients in manure, but most of the time the attention has always been on nitrogen, phosphorous and potassium while other nutrients were not given adequate attention (Tanimu *et al.*, 2012). The current study predominantly aims to evaluate a specific component like crude lipid analysis in the dung samples. To check the proximate composition of feed, fodder, dung, urine *etc.*, the determination of crude lipids is one of the most important parameters. Moreover, to check the lipid digestibility of animals, crude lipid extraction in feed and excreta is carried out. Generally, laboratory evaluation of crude lipid content is based upon organic solvent extractions. There are a wide variety of methods available for the analysis of crude lipid or fat in different samples that may provide variable results. Most commonly, Soxhlet method (AOAC, 2005) is used for crude lipid extraction from feed, fodder, dung and urine samples. Other methods available for fat determination are the Gerber method, Mojonnier method, Soxhlet method, Folch method, Rose Gottlieb method, *etc*. Generally, the Gerber method [IS: 1224 (Part-1)-1977], Mojonnier method (IS: 10484-1983) and Rose Gottlieb method (IS: 10484-1983) are used for the estimation of fat in the milk and milk products. Folch method (Folch *et al.*, 1957) is used for the extraction of crude lipid from animal tissues. These methods rely on different solvents and extraction

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techniques to estimate the crude lipid content. The long reflux time (8-16 h) of the Soxhlet method makes it time-consuming for the extraction of crude lipid. Therefore, there is a need to standardize a method that can extract crude lipid efficiently from cow dung samples and within a short period of time. In the present study, three methods (Mojonnier acid digestion, Folch and Soxhlet method) were evaluated for the analysis of crude lipid from cow dung samples. These three methods were standardized and compared and a rapid method was finalized for the estimation of crude lipid from cow dung samples.

MATERIALS AND METHODS

Sample collection and preparation for method standardization

Pooled cow dung samples of lactating Sahiwal cows were collected from the Live-stock Research Centre (LRC), NDRI in the morning hours. Different sample preparation approaches were used for the extraction of crude lipid from cow dung in different methods, as described below.

Raw (native) sample

Raw cow dung was thoroughly homogenized using a spatula and then used in varying quantities for analysis.

Slurry sample

Raw cow dung was mixed with water in a ratio of 5:1 and mixed thoroughly to obtain a slurry.

Oven-dried sample

Raw dung samples were oven-dried at 70°C for 24 h to get a constant weight and processed in a Willey mill using a 1-mm screen sieve to get a constant weight (AOAC, 2005).

Cellulose dried sample

The cellulose dried sample was prepared by mixing cellulose powder with the dung slurry untill it dries.

The crude lipid content extracted was expressed as percentage on dry matter (DM) basis.

Methods of extraction

Crude lipid extraction by soxhlet method

Crude lipid content from cow dung was extracted by the Soxhlet method as described in AOAC, 2005 official method (920.39), which is mainly used for feed samples. In the present study, five-gram oven-dried and cellulose-dried dung sample was used for extraction of crude lipid using petroleum ether (60-80°C, LR) as a solvent. The sample was placed in a cellulose thimble which was kept in a solvent extractor (Jain Scientific Glass Works, Ambala, India). The entire Soxhlet assembly was placed on a heating mantle (PERFIT, India) and the lipid extraction was carried out for 16 hrs. After the complete extraction, the solvent was evaporated using a water bath (LABCO, India) followed by a hot air oven (100±2°C, Tempo Industrial corporation, India). The crude lipid content was then calculated gravimetrically.

Crude lipid extraction by mojonnier acid digestion method

Crude lipid content in cow dung was extracted by the Mojonnier acid digestion method (IS: 2785-1979). Using this method, in the present study, three different sample preparations *viz*., raw (native), slurry and oven-dried dung samples with different sample sizes *viz*. 1 g, 2 g and 3 g were evaluated. A varied amount of hydrochloric acid (HCl) *i.e* 10 ml, 15 ml and 20 ml was also evaluated for the extraction of crude lipid from the cow dung sample. In this method, the sample was first digested with HCl (AR grade) in a beaker. After that the mixture was transferred to Mojonnier flask and. After that 10 ml of ethyl alcohol (LR grade) was added followed by 25 ml of each petroleum ether (40-60°C, AR grade) and di ethyl ether (EMPLURA, Merck) and kept undisturbed for 30 min. The clear ether layer, separated at the top was collected from the mix and this extraction procedure was followed in the same way two times more with 15 ml of each ether. All the ether extracts were collected and evaporated using a water bath followed by a hot air oven (100±2°C). After that, the crude lipid content was calculated gravimetrically.

Crude lipid extraction by Folch method

Crude lipid content from cow dung was extracted as per the method used by Folch *et al*. (1957). Oven-dried samples with different sample sizes (1 g, 2 g and 3 g) were evaluated in this method. The sample was mixed with chloroform-methanol mixture (AR grade) in a ratio of 2:1 and agitated at room temperature for 1 hour. After mixing, the sample was filtrated and washed with NaCl solution. Thereafter, the sample was vortexed using a vortex mixer (Spinix, Tarsons Product Pvt. Ltd., India) and the lower layer containing lipid was separated and evaporated using a hot air oven (100±2°C). The extracted crude lipid was determined gravimetrically.

RESULTS AND DISCUSSION

The methodology for extraction of crude lipid from cow dung sample was standardized by using different sample preparation approach and sample size. The detailed discussions of each method are as follows.

Standardization of method for crude lipid extraction by mojonnier acid digestion method

To standardize the Mojonnier acid digestion method for extracting crude lipid content from cow dung, various sample preparation approaches were tested, including raw (native) dung, dung slurry and oven-dried dung samples. Additionally, different volumes of HCl (10 ml, 15 ml and 20 ml) were evaluated to determine their effect on lipid extraction. The results (Table 1) indicated that the raw (native) cow dung samples yielded the highest crude lipid content, followed by the slurry and oven-dried samples. The higher crude lipid content in raw (native) cow dung samples could be attributed to the uneven mixing of the

sample. The data (Table 1) also revealed a high standard deviation in measurements when using the native wet sample, indicating inconsistent results across different trials. To address this issue, slurry samples were prepared by mixing the dung with water in a 5:1 ratio, ensuring even mixing and uniformity of the sample. The lower standard deviation value for the slurry samples indicates more repeatable results and consistent sample mixing (Table 1). Although the extracted crude lipid content was higher compared to literature values, this elevation in wet samples (both raw and slurry) could be due to the co-extraction of water-soluble components such as carbohydrates, urea and glycerol. These components may be extracted along with the lipids. The Association of American Feed Control Officials (AAFCO) has noted that many organic (non-polar) solvents can extract water along with lipid compounds, which leads to errors in measurement (AAFCO, 2014).

For the oven-dried sample approach, repeatable results were observed across different sample sizes, with the standard deviation being minimal (Table 1). The crude lipid content extracted by this method is comparable to reported values in the literature, such as 4.0% (Chinwendu *et al.*, 2013) and 4.0±0.30% (Chibueze *et al.*, 2017). Literature also suggests that samples must be dried before lipid extraction, as polar solvents cannot effectively permeate samples with more than 8% moisture. Samples with higher moisture content often result in elevated (and false) crude fat content (AAFCO, 2014).

After comparing the crude lipid content by three different sample preparation approaches, it was observed that there was no significant ($p>0.05$) difference (Table 1.1). The calculated t value is less than the t statistic value which implies a non-significant difference between the crude lipid content extracted by three different sample preparation approaches. Though there was a non-significant difference, the oven-dried sample was found suitable for the extraction of crude lipid as the results were repeatable and the values were comparable with the previous results. Also, in wet samples, water-soluble components may be extracted together with nonpolar components and can show a false elevated crude lipid content (Pomeranz and Meloan, 1978; Palmquist and Jenquins, 2003) whereas there was no such problem in the case of the oven-dried sample.

Therefore, it was concluded that samples must be dried before lipid extraction. The oven-dried sample approach was selected for crude lipid extraction in dung.

Table 1: Effect of type and size of cow dung sample on estimation crude lipids content using mojonnier digestion method.

Sample size (g)	Wet (native) cow dung sample	Cow dung slurry (dung: water @ 5:1)	Oven dried cowdung
		Crude lipid (%)	
1.0	6.31 ± 2.03^{aA}	5.78 ± 0.52^{aB}	4.53 ± 0.21^{aC}
2.0	6.11 ± 2.28^{bA}	5.67 ± 0.47^{bB}	4.63 ± 0.13^{bC}
3.0	7.67 ± 2.97^{cA}	5.57 ± 0.67^{dB}	4.50 ± 0.18^{dC}

Data are represented as Mean ± SD, n= 6; $^{a-d}$- different superscripts within the row and $^{A-C}$- different superscripts within the column shows non-significant difference between each other ($p>0.05$).

Table 1.1: Evaluation of the interaction between different sample types on crude lipid extraction by mojonnier acid digestion method.

Sample type	Sample size (g)	T statistic	T table value	Summary
Wet vs Slurry	1	0.6459	2.015	Non-significant
	2	0.7750	2.015	Non-significant
	3	2.547	2.015	Significant
Wet vs Dry	1	2.156	2.570	Non-significant
	2	2.039	2.570	Non-significant
	3	3.839	2.570	Significant
Slurry vs Dry	1	1.510	2.015	Non-significant
	2	1.263	2.015	Non significant
	3	1.292	2.015	Non significant

Table 2: Effect of sample size and amount of HCl on the crude lipid determined by mojonnier acid digestion method.

Amount of HCl	Weight of oven-dried cow dung sample (g)		
	1 g	2 g	3 g
		Crude lipid (%)	
10 ml	4.47 ± 0.22^{a}	4.56 ± 0.18^{a}	4.46 ± 0.19^{a}
15 ml	4.46 ± 0.22^{a}	4.56 ± 0.19^{a}	4.46 ± 0.18^{a}
20 ml	4.47 ± 0.23^{a}	4.55 ± 0.19^{a}	4.45 ± 0.18^{a}

Data are represented as Mean ± SD, n= 6; ^{a} superscript within column shows non-significant difference ($p>0.05$).

Moreover, the weight of the sample taken for crude lipids extraction did not show any significant effect on the crude lipid extraction; hence, 1.0 g sample weight was finalized to be used for further analysis.

A study was also carried out by varying the amount of HCl, required for the digestion of the dried sample. It was evident from the data (Table 2) that the amount of HCl did not improve or deteriorate the efficiency of crude lipid extraction. The difference in the extracted lipid content was non-significant ($p>0.05$) on account of the amount of acid as well as the amount of sample. Therefore, the minimum amount of HCl *i.e.*, 10 ml was finalized for the digestion of the dried cow dung samples.

Standardization of method for crude lipid extraction by Soxhlet method

The Soxhlet method is the widely used method for crude lipid extraction from dried cow dung samples (Hewavitharana *et al.*, 2020). In the present study, to shorten the drying time for the sample, the mixing of cellulose powder approach was evaluated wherein, cellulose powder was mixed with the dung to dry the sample. The cellulose-dried sample so prepared was then used for crude lipid extraction and the results were compared with the oven-dried dung sample.

Table 3: Crude lipid content of oven-dried and cellulose dried cow dung sample by soxhlet method.

Cow dung sample	Sample size (g)	Crude lipid (%)
Oven-dried	5.0	4.13±0.25[a]
Cellulose dried	5.0	10.95±1.80[b]

Data are represented as Mean ± SD, n= 4; [a], [b] different superscripts within the column differ significantly ($p<0.05$).

Table 4: Crude lipid content of oven-dried cow dung sample determined by folch method.

Sample size (g)	Crude lipid (%)
1.0	3.37±0.50[a]
2.0	3.34±0.57[a]
3.0	3.36±0.49[a]

Data are represented as Mean ± SD, n= 4; [a]- superscript within column shows non-significant difference between each other ($p>0.05$).

Table 5: Crude lipid content of oven-dried cow dung sample determined by mojonnier acid digestion method, soxhlet method and folch method.

Method name	Sample size (g)	Crude lipid (%)
Mojonnier acid digestion method	1.0	4.41±0.07[a]
Soxhlet method	5.0	4.13±0.25[a]
Folch method	1.0	3.37±0.50[b]

Data represented as Mean ± SD; n=4 [a], [b] – different letters represent significant difference between each method ($p<0.05$).

The data (Table 3) revealed that the crude lipid content determined in the oven-dried sample and cellulose powder-dried sample was 4.13±0.25 and 10.95±1.80 per cent, respectively. It was observed that the crude lipid content in the cellulose powder dried sample was significantly ($p<0.05$) higher than in the oven-dried sample. The observed higher crude lipid content in the cellulose powder dried sample can be attributed to the sampling error due to the uneven mixing of the dung sample with cellulose powder. It was observed that the cellulose-dried sample particles, due to the improper drying, were not finely ground which caused a non-homogeneous mixture. For effective extraction, the sample should be finely ground and homogeneous which results in an increased surface area of the lipids exposed to the solvent (Akoh and Min, 2008). Also, the presence of some water-soluble components (AAFCO, 2014) in the cellulose admixed dung mixture, wherein moisture remained in the sample due to inefficient drying can be a reason for the elevated crude lipid content. Therefore, this cellulose dried sample preparation approach failed to meet the criteria of effective extraction of crude lipid from cow dung samples and the approach was dropped for further studies.

The crude lipid content determined in the oven-dried sample was repeatable and comparable with the results of previous studies (Chinwendu *et al.*, 2013; Chibueze *et al.*, 2017). Silva *et al.* (2011) reported 0.7 to 7.3% crude lipid in cow dung when using Soxtherm 2000 extractor for the extraction.

Standardization of method for crude lipid extraction by folch method

Folch *et al.* (1957) used chloroform/methanol/water to isolate total lipids. The use of chloroform is not ideal, but the method is generally used in wet foods, especially meat and fish. Hence, the attempt was made to standardize the conditions of crude lipid extraction by the Folch method with variation in the amount of sample used. In this method, the oven-dried sample was used for the extraction of crude lipid from the dung sample. It was evident from the data (Table 4) that there was no significant difference ($p>0.05$) in per cent crude lipid content on account of the sample weight. The crude lipid extracted from oven-dried cow dung sample by Folch method was 3.37±0.50% in 1.0 g sample weight.

Comparative evaluation of crude lipid content by the three methods

A comparison between the three methods showed that the crude lipid extracted by the Folch method was significantly lower than the previous two methods *i.e.* Soxhlet and Mojonnier acid digestion method (Table 5). This could be attributed to the sample matrix or the solvents which were not able to extract the total crude lipid efficiently from dried dung samples. Also, the Folch method is generally used in wet foods, especially meat and fish. Hence, the Folch

method was not used in further experiments. It is clearer from the table (Table 5) that there was no significant difference between the crude lipids content extracted from oven-dried dung samples by the Mojonnier acid digestion method (4.41±0.07) and the Soxhlet method (4.13±0.25). The crude lipid content obtained by these two methods is comparable. On the contrary, the Folch method resulted in considerably lower values (3.37±0.50).

Acid digestion is necessary for the total extraction and measurement of lipids in order to liberate the bound lipids prior to their extraction into a desired solvent (Hewavitharana *et al.*, 2020). In a previous study conducted on cooked meat, it has been reported that the acid hydrolysis (Mojonnier acid digestion) method was a better choice than the chloroform-methanol method and Soxhlet method for extraction of total fat (Habeck *et al.*, 2013). Moreover, the Mojonnier method led to the extraction of crude lipids within 1.3 to 2 hr. compared to the Soxhlet method (8-16 hr.) and the standard deviation was also very small. Hence, the Mojonnier method was found to be the best choice to determine the crude lipid content in dried dung samples.

CONCLUSION

To minimize the extraction time of traditionally used Soxhlet method, different approaches were evaluated in the present investigation. Three methods *viz.*, Soxhlet, Mojonnier acid digestion and Folch method were assessed and standardized for the extraction of crude lipid from cow dung samples. Results showed a significantly lower amount of crude lipid extracted by the Folch method whereas a non-significant ($p>0.05$) difference was observed in the crude lipid content extracted using the Soxhlet method and Mojonnier method. The Mojonnier acid digestion method was found superior to the Soxhlet method as it takes less extraction time (1.3 to 2 hr.) than the Soxhlet method (8-16 hr.). Also, the results of the Mojonnier acid digestion method were comparable to the previously reported value of crude lipid content extracted from cow dung. Therefore, the Mojonnier acid digestion method can be used as an alternative and relatively rapid method for the extraction of crude lipid from cow dung.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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