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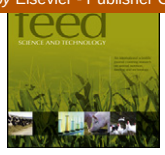

# Evaluation of total and non-fatty ether extract in feeds and cattle feces using two analytical methods

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ABSTRACT

The objective of this study was to evaluate ether extract (EE) concentrations, pigments, and wax in forages ($n = 14$), concentrates ($n = 23$), and cattle feces ($n = 100$) using extraction methods recommended by Association of Official Analytical Chemists (AOAC; method 920.39) and American Oil Chemist's Society (AOCS; method Am 5-04). The EE contents were compared by adjusting a linear regression model for each sample type. For the feces and forage samples, the EE contents produced by the AOCS method were greater ($P<0.05$) compared to those obtained using the AOAC method. No differences between methods were observed in EE content of concentrates ($P>0.05$). Concentration of vegetable pigments and wax were evaluated by using analysis for variance. Vegetable pigments were lower ($P<0.05$) in the post-extraction residues using the AOCS method, than the AOAC method, indicating greater participation of vegetable pigments in the EE. No differences were observed between the methods in wax concentration of the post-extraction residues ($P>0.05$). The quantification method of the EE content that is recommended by AOCS is not suggested for analyses of forage and feces of ruminants because it possibly increases the removal of non-fatty material, mostly pigments, in comparison to the method recommended by AOAC.



## 1. Introduction

Functional knowledge of the capacity of a food or feed to supply energy begins by measuring the components capable of providing energy to ruminant animals. Although found in lower concentrations than other components for the majority of food produced under tropical conditions, ether extract (EE) is able to produce energetic levels similar to other components due to its greater caloric concentration (Detmann et al., 2006, 2008).

Laboratory evaluation of EE content is based upon organic solvent extractions of nonpolar food components. The existing methods are highlighted, among other aspects, by their lengthy processes that can take between 3 h and 16 h for completion (Palmquist and Jenkins, 2003), which is a characteristic of the most widely used method recommended by the Association of Official Analytical Chemists (AOAC; method 920.39; Windham, 1998).

*Abbreviations:* AOAC, Association of Official Analytical Chemists; AOCS, American Oil Chemist's Society; DM, dry matter; EE, ether extract.

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Recently, analytical methods using filter bags were developed to quantify EE content in food, promoting practicality and speed in organic solvent extractions. This method is based on extraction with organic solvent at high temperature and pressure, which reduces the extraction time. This method was made official by the American Oil Chemist's Society (AOCS; method Am 5-04; AOCS, 2009).

However, utilization of the AOCS method has produced systematically negative apparent EE digestibility in experiments conducted by our research group, projecting distortions in the estimates of energy contents of feeds and diets. Despite this, no references know to the authors were found in the literature that demonstrate the efficiency of the AOCS method in quantifying EE content in samples obtained from ruminant digestion trials involving forages, concentrates and feces. Thus, the purpose of this study was to compare EE content in forages, concentrates, and cattle feces samples using the AOAC and AOCS recommended methods.

## 2. Materials and methods

### 2.1. Location and samples

The experiment took place at the Animal Nutrition Laboratory of the Animal Science Department of the Universidade Federal de Viçosa, Viçosa, MG, Brazil. Samples of cattle feces ($n = 100$), forages ($n = 14$) and concentrates ($n = 23$) were used. The fecal samples were collected from animals involved in grazing and feedlot experiments. The forage samples implemented were fresh grasses and legumes, sugar cane and corn silage, and the concentrate samples were composed of grains, meals and by-products from the agricultural and biodiesel industries. The goal was to extend the range of EE values in each sample type to have a greater representation and better comparative evaluation between the methods.

Samples with high moisture contents were oven dried (60 °C). All samples were processed in a knife mill using a 1-mm screen sieve. Subsequently, the dry matter (DM) content was quantified for all samples (AOAC method 934.01; Windham, 1998).

### 2.2. Evaluation of ether extract content

Following the AOAC method (920.39; Windham, 1998), quantification of the EE content in the samples was conducted using a Soxtherm 2000 extractor (Gerhardt®) using petroleum ether (Palmquist and Jenkins, 2003). Aliquots of approximately 2 g were packed in cartridges made of qualitative filter paper and underwent extraction for 3 h at a drip rate of 5–6 drops/s. After this period, the cups were dried (105 °C for 30 min) and weighed. The EE mass was quantified as weight gain of the cup after extraction and solvent recovery.

According to the AOCS method (Am 5-04; AOCS, 2009), evaluation of the EE content was performed with an XT15 extractor (Ankom®) using petroleum ether. Aliquots of 1.2 g were packed in XT4 bags (Ankom®) that were sealed by heat. The bags were dried (105 °C) for 3 h to reduce the moisture content and were then weighed. After this procedure, the extraction was performed for 50 min at 90 °C. After that, bags were oven dried (105 °C for 30 min) and weighed. The EE mass was quantified by the weight difference of the bag with the sample before and after extraction.

### 2.3. Evaluation of pigments and wax

Subsequently, five fecal and five forage samples were randomly chosen to quantify the pigment levels. Aliquots of the non-extracted and post-extraction samples from both methods were analyzed for a total of 30 aliquots. The analyses were performed according to the method described by Lichthenthaler (1987).

Aliquots of approximately 100 mg of DM were placed in porcelain crucibles, and approximately 50 mg calcium carbonate and 5 mL acetone solution (800 mL/L) were added. The mixture was then homogenized with a glass rod, and the material was transferred to a 10 mL volumetric flask. Qualitative filter paper (*Qualy*, J. Prolab Co., 80 g/m$^2$, average pore size 14 μm) was used for the transfer. Acetone solution (800 mL/L) was used to wash material and to complete the flask volume (25 mL). The solutions were kept in the dark by wrapping the flask in aluminum foil.

The absorbance of the solution was evaluated at the following wavelengths: 470 nm, 646.8 nm and 663.2 nm. Acetone solution (800 mL/L) was used to blank the spectrophotometer.

The pigment concentrations in the solutions were obtained according to Lichthenthaler (1987), and the equations used are as follows:

$$\text{ChlorA} = 12.25 \times A_{663.2} - 2.79 \times A_{646.8}, \quad (1)$$

$$\text{ChlorB} = 21.50 \times A_{646.8} - 5.10 \times A_{663.2}, \quad (2)$$

$$\text{ChlorT} = 7.15 \times A_{663.2} + 18.71 \times A_{646.8}, \quad (3)$$

$$\text{Carot} = \frac{1000 \times A_{470} - 1.82 \times A_{663.2} - 85.02 \times A_{646.8}}{198}. \quad (4)$$

In these above equations, ClorA, ClorB, ClorT, and Carot are the concentrations of chlorophyll A, chlorophyll B, total chlorophyll, and carotenoids (xanthophylls and carotenes), respectively, with mg/L as the units, and $A_{470}$, $A_{646.8}$, and $A_{663.2}$ are the absorbencies of the respective wavelengths.

Chlorophyll is essentially indigestible. However, during its passage through the gastrointestinal tract, the magnesium ion could be removed, disrupting its porphyrin ring. In addition, carotenoids are destroyed by anaerobic bacterium (Van Soest, 1994). Pigment degradation also occurs because of sample processing, usually by heat drying. Thus, the pigment concentrations were determined based on the solution concentration and not based upon the DM of the samples, due to the biases caused by the events described above.

Due to the differences in the weights of the aliquots, the concentrations obtained through the equations were corrected with a multiplicative correction factor as follows:

$$f = \frac{100}{M}, \tag{5}$$

where $f$ is the multiplicative correction factor, and $M$ is the aliquot mass (mg of DM).

Five forage samples were randomly chosen to evaluate the wax content according to the method described by Ebercon et al. (1977). Aliquots of the non-extracted and post-extraction samples were analyzed using both methods, for a total of 15 aliquots. Approximately 300 mg of DM was added to a glass tube, and 15 mL of redistilled chloroform was added. After 15 s, the material was filtered, and the extract was incubated in a water bath at 100 °C until the chloroform odor was undetectable. Subsequently, 5 mL of the coloring reagent was added while incubating the extract in water bath (100 °C) for 30 min. After cooling, 12 mL of distilled water was added to the tubes, and several minutes were allowed for color development and cooling. The optical density was then read at 590 nm.

The coloring reagent was prepared by mixing 40 mL of distilled water with 20 g potassium dichromate. The resulting slurry was mixed vigorously with 1 L of concentrated sulfuric acid and heated below boiling until a clear solution was obtained (Ebercon et al., 1977). Carbowax-3000 (polyethylene glycol 3000) was used to produce standard solutions.

### 2.4. Statistical analysis

The EE contents of the samples obtained using both methods were compared by a simple linear regression adjustment using simultaneous hypothesis testing (Mayer et al., 1994):

$$H_0 : \beta_0 = 0 \quad \text{and} \quad \beta_1 = 1 \tag{6}$$

The EE values obtained by the AOAC and AOCS methods were considered to be similar when the null hypothesis was not rejected ($\alpha = 0.05$).

The pigment levels were evaluated according to the following model:

$$Y_{ijk} = \mu + F_i + e_{(i)j} + M_k + FM_{ik} + \varepsilon_{ijk}, \tag{7}$$

where $\mu$ is the general constant, $F_i$ is the fixed effect of the material $i$ (feces or forage), $e_{(i)j}$ is the effect of sample $j$ of the material $i$ or the residual effect of plot (random effect), $M_k$ is the fixed effects of the extraction method (non-extracted material; AOAC and AOCS), $FM_{ik}$ is the interaction among factors (fixed effect), and $\varepsilon_{ijk}$ is the random error. The $e_{(i)j}$ term was used to test material effect and the random error was used to test extraction method and interaction effects.

Additionally, the wax levels were evaluated according to the model:

$$Y_{ij} = \mu + S_i + M_j + \varepsilon_{ij}, \tag{8}$$

where $\mu$ is the general constant, $S_i$ is the random effect of the sample, $M_j$ is the fixed effects of the extraction method (non-extracted material; AOAC and AOCS), and $\varepsilon_{ij}$ is the random error.

All statistical procedures were performed using the SAS program (PROC REG and PROC MIXED), adopting $\alpha = 0.05$. When necessary, the means were compared by using Tukey test.

## 3. Results

On average, the EE contents obtained by the AOCS method were 62% and 46% greater than obtained by the AOAC method in the forage and feces samples, respectively. These difference among methods were verified when regression analysis was performed on forage and feces samples ($P<0.01$; Table 2). The behavior of the ordered pairs indicates greater EE values in the feces and forage samples with the AOCS method (Fig. 1). However, these methods had similar means values when concentrate samples were compared (Table 1). In this case, there was no difference between methods ($P>0.05$; Table 2).

No differences ($P>0.05$) were detected in pigment concentrations in the solutions produced from forage and feces samples. However, the concentrations of all analyzed pigments were lower ($P<0.05$) after extraction by the AOCS method compared to the non-extracted material. Furthermore, the pigment concentration in solutions produced from the non-extracted material and post-extraction residue by the AOAC method were similar ($P>0.05$) (Table 3). The wax concentration was higher in the non-extracted material ($P<0.05$) than the post-extraction material when measured by either methods, which did not differ ($P>0.05$).

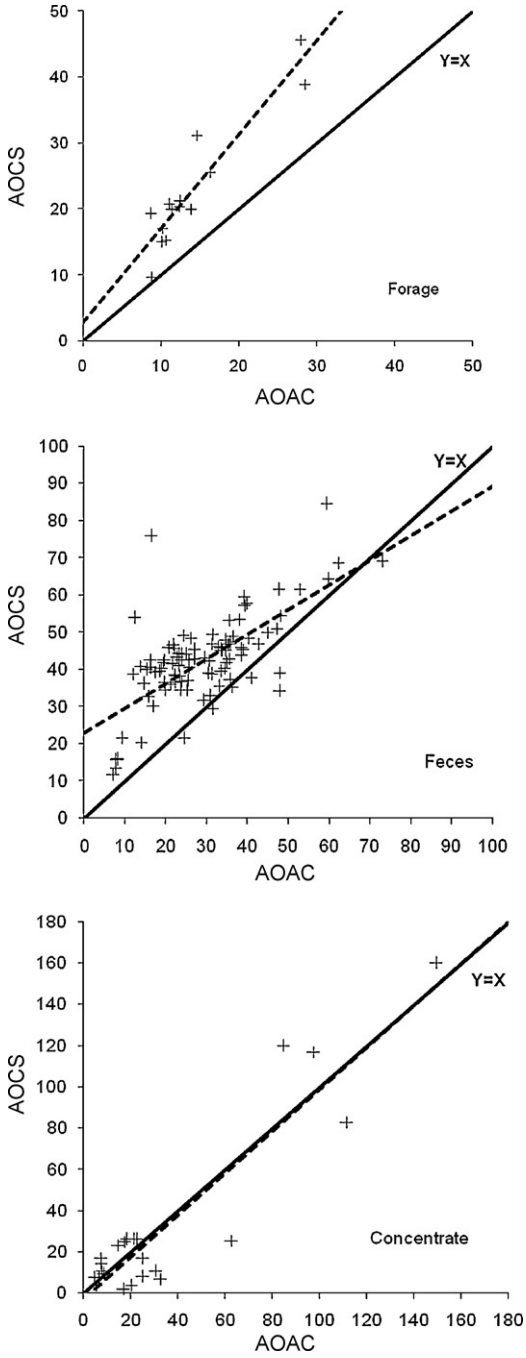


**Fig. 1.** Relationship between EE contents (g/kg DM) obtained using the AOAC and AOCS methods in forage, feces and concentrate samples. The dashed line represents the least squares straight line.

## 4. Discussion

The similarity of the EE values obtained by the AOAC and AOCS methods in concentrate samples (Fig. 1) supports the collaborative study results described by AOCS (2009). That study was conducted with food samples for humans and animals with the method using filter bags (method Am 5-04). In this study, however, only two forage samples were used (alfalfa and corn silage), and fecal samples were not evaluated (AOCS, 2009).

Utilization of the AOCS method has produced systematically negative apparent EE digestibility in experiments conducted by our research group (*e.g.* Fig. 2), projecting distortions in the estimates of energy contents of feeds and diets. According to

**Table 1**
Descriptive statistics of EE contents (g/kg DM) in forages, concentrates, and feces using the AOAC and AOCS methods.

| Item | Forage | Feces | Concentrate |
|---|---|---|---|
| | | AOAC | |
| Mean | 14.1 | 28.8 | 34.7 |
| Median | 12.0 | 26.6 | 20.0 |
| Standard deviation | 6.3 | 13.0 | 39.2 |
| Maximum | 28.5 | 73.1 | 149.6 |
| Minimum | 8.7 | 7.1 | 4.5 |
| | | AOCS | |
| Mean | 22.8 | 42.0 | 32.6 |
| Median | 20.2 | 42.5 | 16.9 |
| Standard deviation | 9.7 | 12.3 | 43.3 |
| Maximum | 45.7 | 84.4 | 159.9 |
| Minimum | 9.7 | 11.6 | 1.8 |
| *n* | 14 | 100 | 23 |
| AOCS/AOAC | 1.62 | 1.46 | 0.94 |

**Table 2**
Regression statistics for the relationship between EE contents using the AOAC ($X$) and AOCS ($Y$) methods (g/kg DM).

| | Parameter estimates [a] | | $R^2$ [b] | $S_{xy}$ [c] | P-value[d] |
|---|---|---|---|---|---|
| Material | Intercept | Slope | | | |
| Forage | 2.7781 ± 2.4467 | 1.4245 ± 0.1593 | 0.8695 | 3.65 | <0.0001 |
| Feces | 22.8395 ± 2.2173 | 0.6649 ± 0.07303 | 0.4769 | 8.95 | <0.0001 |
| Concentrate | −2.8090 ± 4.8079 | 1.0184 ± 0.0930 | 0.8511 | 17.08 | 0.8162 |

[a] Estimate ± standard error.
[b] Coefficient of determination.
[c] Residual standard deviation of relationship.
[d] $H_0$: $\beta_0 = 0$ and $\beta_1 = 1$.

**Table 3**
Concentrations of pigments (mg/L) in the solutions produced from feces and forage samples (mg/L), and wax contents in forage samples (mg/g dry matter) with or without extraction using the AOAC or AOCS methods.

| Method | Pigments | | | | Wax |
|---|---|---|---|---|---|
| | Chlorophyll A | Chlorophyll B | Total chlorophyll | Carotenoids | |
| No extraction | 7.93a | 3.38a | 11.30a | 2.11a | 40.8a |
| AOAC | 6.06a | 4.12a | 10.18a | 1.17ab | 14.6b |
| AOCS | 1.64b | 1.21b | 2.85b | 0.48b | 20.9b |
| SEM | 0.95 | 0.53 | 1.44 | 0.27 | 4.1 |
| AOCS/AOAC | 0.27 | 0.29 | 0.28 | 0.41 | – |

a, b: means in same column followed by different letters differ at P<0.05.

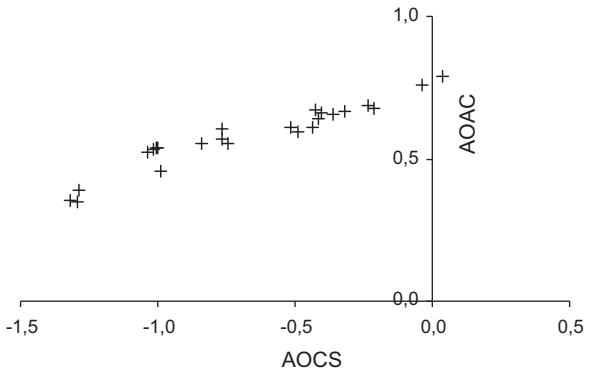


**Fig. 2.** Relationship between the apparent digestibility coefficient of EE (g/g) using the AOAC and AOCS methods in an experiment with grazing cattle. Data from V.A.C. Costa (not published).

Detmann et al. (2006, 2008), negative EE digestibility coefficients in tropical conditions should not be considered credible even at the relatively lower dietary levels than those found in non-tropical conditions.

The EE fraction, mainly derived from vegetable material, is considered nutritionally non-uniform because it has several non-nutritive substances (Palmquist and Jenkins, 2003). Therefore, EE is considered a purely analytical concept because it is exclusively based on the solubility of nonpolar compounds in organic solvents. Despite the divergence between the EE and the biochemical concept of lipids, the method recommended by the AOAC has been historically used to evaluate EE in food and feces to support the nutritional inference of this food fraction. This problem places nutritional results obtained by the AOCS method under questionable levels for evaluation of forages and feces (Fig. 2).

The principal physiochemical difference between the methods is observed in how the solvents react with the samples. In the AOAC method, the solvent mass is heated to boiling without direct contact with the sample. The solvent in its vapor form is placed in a condensation chamber, causing it to return to its liquid form, and it is then cooled for extraction. However, in the AOCS method, the solvent is heated to elevated temperatures with direct contact with the sample. It is maintained in its liquid form even at temperatures above boiling by the internal pressure of the containers containing the sample. Therefore, in the AOCS method, extraction of nonpolar compounds is accelerated by put forward the solvent contact with the sample at elevated temperatures. This higher temperature may be the cause for the elevated EE levels found with the AOCS method than the AOAC method.

The EE mass of any analyzed material can be subdivided into two fractions. The first, called fatty EE, is composed of the lipid fraction (triglycerides and galactolipids) and has direct nutritional interest. The second fraction, called non-fatty EE, is composed of the remaining nonpolar components of the samples, including waxes, vegetable pigments, and other unsaponifiable components (Van Soest, 1994). The quantity of the non-fatty fraction defines the difference between the analytic concept (EE) and biochemical concept (lipids) of the fatty fraction of food.

Therefore, the greater EE values obtained by the AOCS method (Table 1) could be attributable to a greater fatty fraction extraction efficiency of the EE. However, for this argument to be true, greater EE levels should also be observed in concentrates, which was not verified in this study (Tables 1 and 2; Fig. 1).

A part of the fatty acids found in feces may be found in the form of calcium soap, which is not completely soluble in ether. For the fatty acids to be consistently extracted, they should be hydrolyzed prior to the extraction (Sukhija and Palmquist, 1988). Thus, the superiority of the EE values from the AOCS method could be due to an increase of soap extraction from feces by the higher temperatures. However, if this was the cause of the difference among methods, these differences should have been greater between fecal samples than forage samples that do not contain calcium soaps. However, the results present here did not support this hypothesis (Table 1), because greater divergence between methods was verified in forage samples.

Discarding the hypotheses associated with greater extraction of fatty material, divergence among EE concentrations seems to be associated with the higher extraction of non-fatty material by the AOCS method. Considering that the principal components of the non-fatty EE in vegetables are not digestible (Van Soest, 1994), its greater content in food and fecal EE could result in lower estimates of apparent EE digestibility, which supports the data shown in Fig. 2.

Forages are naturally rich in pigments, principally chlorophyll and carotenoids, compared to concentrates. Specifically, chlorophyll is almost entirely indigestible in the ruminant's gastrointestinal tract (Van Soest, 1994). Therefore, this behavior implies a high concentration of pigments in the feces of these animals, supporting similarity between forage and feces samples in regard to the non-fatty fraction of EE.

The similarity of pigment concentrations in the solutions from the forage and fecal samples ($P>0.05$) supports the conclusion that these components are not digestible (Van Soest, 1994). Notably, for chlorophylls, the pigment concentrations of the solutions produced from the post-extraction residue by the AOCS method were approximately 0.28 of those observed in the material extracted by the AOAC method (Table 3). Possibly, this indicates that a greater proportion of non-fatty material was extracted by the AOCS method from feces and forages. Therefore, less bias was observed with AOAC method than the AOCS method.

Due to the physiochemical differences between the methods, it may be speculated that the higher solvent temperatures in the AOCS method increase the wax removal, which may also contribute to the greater EE values obtained with this method. However, the results obtained in this study did not support this hypothesis (Table 3).

## 5. Conclusions

The quantification method of the EE content that is recommended by AOCS is not suggested for analyses of forage and feces of ruminants because it possibly increases the removal of non-fatty material, mostly pigments, in comparison to the method recommended by AOAC.

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