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9. ABSTRACT

A description of a simplified method of determining the total solvent extract of foods and feed samples and a further partitioning of the solvent extract into fractions called nutritive lipid and non-nutritive lipid. This method is intended as a replacement for the commonly used ether extract procedure. Conclusions drawn were that the solvent extract of all foods and feeds is greater than ether extract while nutritive lipid is less than ether extract, and that the non-nutritive lipid of the leaves and stems of plants usually exceeds the nutritive portion.

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DETERMINING SOLVENT EXTRACT AND NUTRITIVE LIPIDE

This report describes a method for extracting solvent soluble matter from food and feed samples and determining the amount of nutritive lipide (trigylcerides plus fatty acids). The solvent extract analysis involves (1) extraction with 2:1 chloroform-menthanol at room temperature, (2) washing extract with water to remove dissolved sugars, proteins, amino acids and minerals, (3) drying and weighing the solvent extract in a tared flask. To separate the solvent extract more nearly into nutritive and non-nutritive fractions the solvent extract is dissolved in 1:9 ethyl ether-hexane and chromatographed in a column of silicic acid with 25 ml 1:9 ethyl ether-hexane and 50 ml 1:3 ethyl ether-hexane. The extract contains the triglyderides, free fatty acids, carotenoids, tocopherols, waxes and sterol esters. The free sterols, chlorophyll and other plant pigments are retained in the silicic acid. The method is intended as a replacement for the commonly used ether extract procedure for estimating fat in foods and feeds. The combination of a polar solvent with the non-polar solvent is necessary to rupture the lipide-protein linkage and obtain the maximum solvent extract. A single extraction does not distinguish between nutritive and nonnutritive lipides. The nonnutritive extract of the leaves and stems of plants often exceeds the nutritive portion. More refinement is necessary to separate nonnutritive waxes and sterol esters from tryglycerides and fatty acids. Solvent extract and nutritive lipide are compared to ether extract for several feeds and foods.

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DETERMINING SOLVENT EXTRACT AND NUTRITIVE LIPID

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Lipids are a heterogeneous group of organic compounds found in living organisms. The compounds have the common chemical property of being more soluble in non-polar organic solvents than in water. The lipids, which encompass numerous compounds that differ widely in biochemical and physical properties, include triglycerides and fatty acids, particularly important sources of metabolizable energy for animals. These compounds are the major lipid components of plant seeds and animal tissue. Fat soluble vitamins, though present in much smaller amounts, would also be among the nutritive lipids. Of the numerous other solvent-soluble compounds that can be extracted from the leaves, stems, roots and fruits of plants, some are absorbed but are not metabolized to an appreciable extent to supply energy. More knowledge about the metabolism of specific lipid compounds is needed, but waxes, sterols and sterol esters, chlorophyll, anthocyamins, other plant pigments and essential oils can generally be categorized as nonnutritive lipids.

The lipid fraction of foods and feeds had been officially estimated by ether extraction, but ether extract greatly overestimates the triglyceride and fatty acid content of forages (Fraps and Rather, 1912). Experiments have shown that the ether extract from many plants is fairly high in apparent digestibility but for some plants, most of the ether extract is not metabolizable (Cook, Stoddart and Harris, 1952).

Ethyl ether does not extract all lipids of plants and animal tissue. Many lipids are bonded to protein with water molecules involved in the union. Lipids are released from the lipoprotein, only if a dehydrating agent such as acetone, methanol or ethanol ruptures the linkage (Hanahan, 1960).

Fonnesbeck and Harris (1973) have been attempting to develop chemical methods that more realistically partition the chemical components of feeds and foods as they are utilized by animals. This paper reports developments toward a procedure to determine total solvent extract and nutritive lipid content.

Experimental Methods

Eterman (1956) in a review article, compared various solvent mixtures for extracting lipids. For most purposes a chloroform-methanol (2:1, v/v) or an ethanol-diethyl ether (3:1, v/v) mixture was most efficient. Extraction at room temperatures (25-28°) is advisable to reduce alterations in lipid structure (Hanahan, 1960) because certain lipolytic enzymes are activated by elevated temperatures in the presence of diethyl ether and many other lipid solvents (Kates, 1957).

Based on the above knowledge, 2-gram samples of dry Kentucky bluegrass were covered with chloroform-methanol (2:1) in a stoppered flask and placed in the dark for 1-, 2-, and 3-day periods at room temperature. The extract was collected and washed with water in a separatory funnel to remove soluble sugars, amino acids, inorganic containments, etc. similar to the procedure of Folch, et. al., (1951). The solvent extract was dried in a tared flask in a vacuum oven at 50° with less than 250 mm Hg pressure. The solvent extract is partially protected from oxygen during extraction and vacuum oven drying by the displacement of air by heavier chloroform vapors. Recovery of solvent extract of various samples is compared in Table 1 with extractions over 1, 2 or 3 days. One day is adequate for extraction at room temperature for most plant tissue samples except possibly forage samples which may require two days.

We then separated the solvent and dissolved extract from the sample solids by filtering the liquid through a fritted glass emersion filter tube while collecting the solvent in a clean filter flask. Five successive 5-ml rinses of the sample particles with chloroform-methanol sufficiently removed residual colored pigments in the sample indicating lipids were also extracted.

TABLE 1. Solvent extract recovery with duration of extraction of various feeds and foods (dry basis).

Sample	Extract recovery with extraction time		
	24 hrs.	48 hrs.	72 hrs.
	%	%	%
Bluegrass, Kentucky, early vegetative	6.4	7.4	7.3
Broccoli, aerial parts	6.9	6.6	6.8
Peanut seeds, roasted	51.5	50.9	50.7
Soybean seeds, raw	20.6	20.8	20.8

In water washing of the chloroform-methanol extract of samples, containing substantial oil, a small part of the oil rises to the top of the water rather than remaining with the chloroform layer. Drying the chloroform-methanol extract and then washing the extract with water gave better recovery. The amount of water-soluble containants in the chloroform-methanol extract was greatest with samples of immature leaves and buds, which have a relatively high content of simple sugars, but washing with only two 200-ml portions of water gave good solvent extract recovery. There was no significant reduction in the amount of extract recovered with more than two washings, indicating that the water soluble containants had been adequately removed. Bligh and Dyer (1959) found an insignificant loss of lipids in water washing and an essentially complete removal of nonlipid contaminants by the water.

Silicic acid was used in column chromatography to separate the nutritive lipids from the nonnutritive portion of the solvent extract.

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2. Department of Animal Science. The authors acknowledge the technical assistance of Irma E. Fernandez during development of the chemical method.

Our goal was to collect the nutritive lipids in one fraction rather than to isolate and identify the various lipid components as in the procedure of Barron and Hanahan (1958). Therefore, we used a higher concentration of ether in hexane to elute the lipid with less solvent.

The recovery of pre-weighed amounts of various purified lipids (tryglycerides, fatty acids and tocopherol) from the silicic acid with measured volumes of the solvent mixtures was observed to determine the solvent concentration and volume necessary for efficient separation of nutritive lipids. The most successful combination tried was: (1) A 20 mm (inside diameter) chromatographic column was filled to a height of about 100 mm with about 15 g silicic acid. (2) The silicic acid was wetted with hexane, then the solvent extract was introduced in solution with 10 ml 25% ethyl ether in hexane (1:3, v/v). (3) This solvent was followed by 75 ml ether in hexane (1:3, v/v). (4) The solvent was collected in a tared flask and dried in a vacuum oven to determine lipid recovery.

The recovery (X) of various purified lipids versus the amount added to the column (Y) is shown in Figure 1. The amount of nutritive lipid in the sample can be estimated with the regression equation. The sample standard deviation from regression (Sy.x) was 13.6 mg. Experimental error this large suggests that a sample likely to contain over 50 mg of nutritive lipid ought to be used for each determination.

Free sterols and phospholipids are not recovered by the above procedure. Phospholipids should be included with the nutritive lipids, but sterols are not a source of energy for animals. By the time the measured amount of solvent passes the column, the various plant pigments are within 20-30 ml of the bottom of the silicic acid. Nutritive lipids in samples that do not contain these plant pigments are carried through the column with the same volume of solvent required to recover nutritive lipid from colored plant tissue samples.

Method

Principle. Solvent extract is removed from samples with a chloroform-methanol mixture, washed free of non-lipid contaminants with water, dried and weighed in a tared flask. The extract is then dissolved in ethyl ether-hexane and chromatographed through a silicic acid column. The nutritive lipid fraction is collected in a tared flask, dried and weighed.

Equipment. (a) Separatory funnels, 250 to 500 ml with teflon stopcocks.
(b) Immersion filter tube with fritted disc, medium porosity.
(c) Vacuum oven and vacuum pump. Water aspirator pump is satisfactory.
(d) Chromatographic columns (over 300 mm long with 20 mm I.D.)

Reagents. (a) Chloroform-methanol (2:1 v/v). Use a grade of chloroform and methanol that leaves no significant residue upon evaporation.
(b) Diethyl ether, anhydrus reagent grade.
(c) Hexane. Redistilled Skelley Oil Company solvent B is satisfactory.
(d) Diethyl ether-hexane (1:3, v/v).
(e) Silicic acid. Column Chromatography grade 200-325 mesh, pH 4 (Mallinckrodt No. 7082).

Procedure. (a) Weigh a sample large enough to contain about 100 mg nutritive lipid into a 250 ml Erlenmeyer flask and add 25 ml chloroform-methanol solution. Cover the flask with a plastic stopper and store in the dark at room temperature for 48 hours.
(b) Draw off the solvent through a glass immersion filtering tube and collect the solvent in a 250 ml filtering flask. Rinse the sample residue with 5 ml portions of chloroform-methanol until the filtrate is colorless (5-7 rinses may be necessary with forage samples). Transfer the extract to a 125 ml flask. Dry off solvent in vacuum oven at 50°C and pressure less than 300 mm Hg.
(c) After drying, dissolve solvent extract in 10 ml chloroform. Pour the chloroform-extract solution into a 250 ml separatory funnel and rinse the flask with chloroform.
(d) Carefully fill the separatory funnel with water and mix the liquids by gentle swirling action. (Avoid shaking the liquids together because this promotes emulsion.) Let the funnel stand for 15 minutes. Decant the water layer with a glass tube connected to a water aspirator filter pump. Repeat the water washing one more time.
(e) Drain the chloroform layer into a tared 125 ml flask leaving the emulsion layer in the separatory funnel. Dry off the chloroform in a vacuum oven at 50°C with less than 300 mm Hg of pressure for at least 1 hour after liquid has evaporated. Cool in a desiccator and weigh the flask plus solvent extract.
(f) Immediately after weighing, add 10 ml 1:3 ether-hexane. (The flask may be covered with a plastic stopper and stored in the dark to complete the procedure the following day.)
(g) Pour 15 g anhydrous silicic acid into clean dry chromatographic column (store a ready supply in an open beaker in a 105° oven). Wet the silicic acid with 25 ml hexane, and draw the hexane through the column with vacuum.
(h) As soon as all the hexane passes into the silicic acid, add the solvent extract and rinse the residue in the flask and on sides of column with 1:3 ether-hexane. Allow the solvent to pass through without suction. Collect the solvent in a tared 125 ml flask.
(i) When the solvent containing the extract has passed into the silicic acid, rinse down the inside of the column with 25 ml 1:3 ether-hexane. As soon as this solvent passes into the packing, add 50 ml 1:3 ether-hexane. Continue collecting the solvent in the same flask until the column stops draining.
(j) Place the flask in a vacuum oven and dry at 50°C with less than 300 mm Hg pressure for at least one hour after all liquid has evaporated. Cool in a desiccator and weigh the flask plus nutritive lipids.

Calculations.

(a) Solvent extract, % = $\frac{\text{(wt of flask and extract)} - \text{(wt of flask X 100)}}{\text{wt of sample}}$

(b) Nutritive lipid recovered =
wt of flask and nutritive lipid − wt of flask

(c) Nutritive lipid in sample, % =

$$\frac{[1.03\ \text{(Nutritive lipid recovered)} - .0037]\ \text{X 100 in grams}}{\text{Sample wt in grams}}$$

Results and Discussion

Solvent extract and nutritive lipid were determined by the above procedure on various plant tissue samples and compared to ether extract (Table 2). Except for the soybean seeds, the nutritive lipid was considerably less than solvent extract. Ether extract was generally less than solvent extract but greater than nutritive lipid. The procedure for determining total solvent extract is similar to those of Bligh and Dyer (1959) for animal tissue and Tsens, Levi and Hlynka (1962) for cereal grains.

The term nutritive lipid used in this paper is based on our present knowledge of the animal metabolism of various solvent-soluble substances of plant and animal tissue. More knowledge is needed about the metabolism of specific organic compounds. One problem with the chemical method presented herein is its exclusion of phospholipids

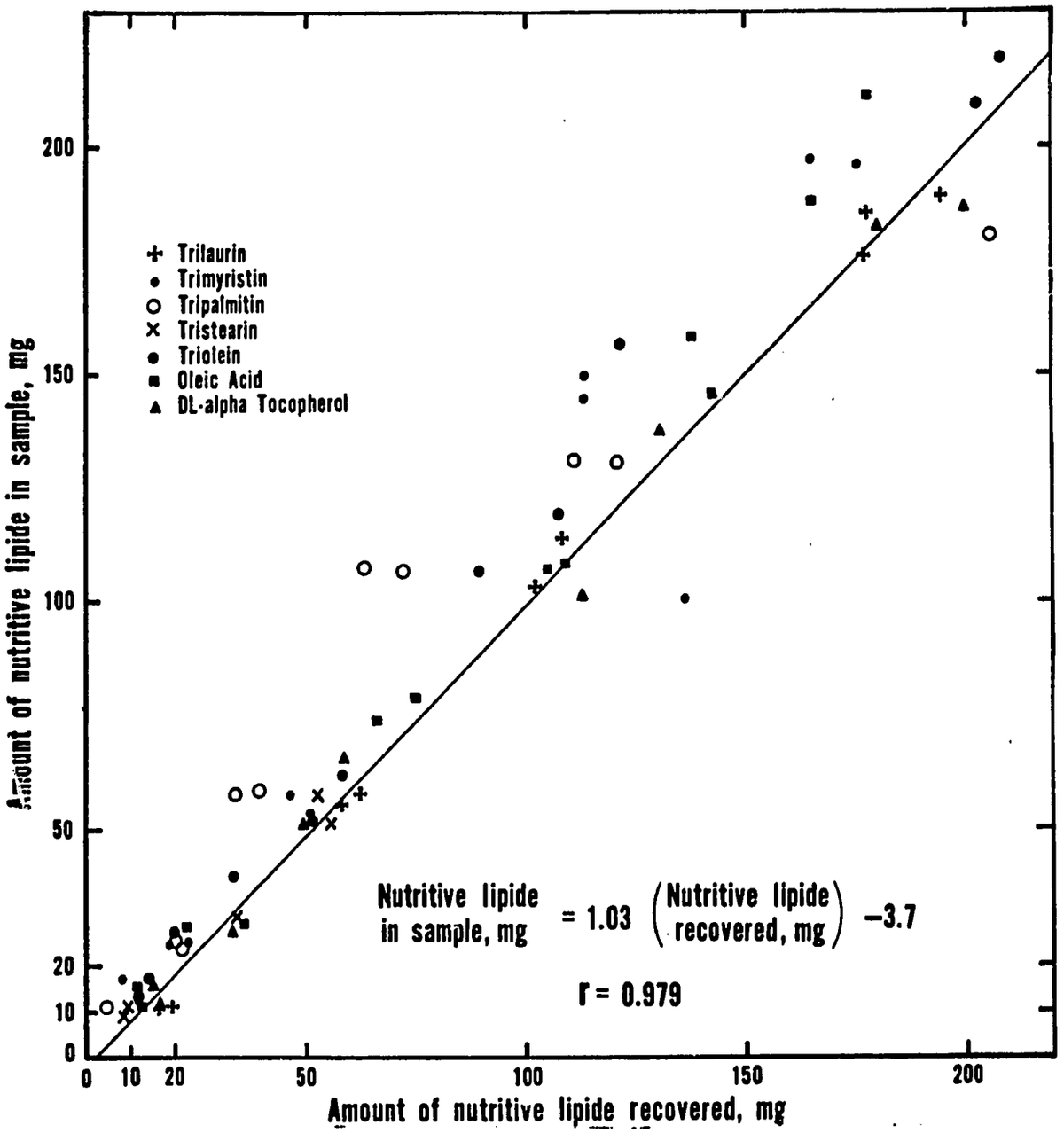

Figure 1. Recovery of various purified lipids from a silicic acid column with 85 ml ethyl ether-hexane (1:3, v/v)
Sample standard deviation from regression (Sy.x) was 13.6 mg

from the nutritive lipid fraction. Modifications in solvent concentrations and volumes could result in the recovery of the phospholipids, but would probably also provide unwanted nonnutritive lipid such as free sterols and bile acids (when analyzing feces samples), and additional plant pigments.

Waxes are also partially recovered in the nutritive lipid fraction. The wax content of the plant tissue consumed by animals and man may inject only a small error into the nutritive lipid fraction.

The digestibilities of the solvent extract and nutritive lipid in three diets for swine are compared in Table 3. In diets 1 and 2, much of the solvent extract was from alfalfa meal. In diet 3, corn oil provided part of the lipid. Apparent digestion coefficients do not provide estimates of the metabolizable energy value of the solvent extract, but digestion or absorption is the first step in metabolism. These data show a greater apparent digestibility of nutritive lipid over solvent extract. The apparent digestibility of the nonnutritive lipid fraction is decidedly lower than for the nutritive lipid. The apparent digestibility of the nutritive lipid was in the range of 85% for swine fed grain-alfalfa meal diets compared to apparent digestibilities of 55% for the nonnutritive fraction. These data support the assumption that this chemical method partitions the solvent extract approximately as it is utilized by animals.

TABLE 2. Comparison of solvent extract, ether extract and nutritive lipid of various feeds and foods (dry basis).

Item	Total Solvent	Ether Extract	Nutritive Lipid
	%	%	%
Feeds			
Alfalfa hay, s-c	4.1	1.8	0.8
Bluegrass, Kentucky, early vegetative	7.4	3.5	1.1
Oat, grain	3.9	2.6	1.3
Soybean, seeds, raw	20.8	18.1	17.7
Foods			
Broccoli, buds and stems, freeze dried	6.8	2.9	1.4
Cabbage, leaves, freeze dried	2.0	1.8	0.4
Lettuce, leaves, freeze dried	2.8	1.0	0.5
Peppers, green wo seeds, freeze dried	2.1	0.8	0.5
Squash, banana, wo seeds and skin, freeze dried	3.2	1.2	0.5

Summary

This report describes the development of a simplified method for determining the total solvent extract of foods and feed samples and a further partitioning of the solvent extract into fractions called nutritive lipid and nonnutritive lipid. The lipids are extracted with chloroform-methanol solution (2:1, v/v) at temperatures less than 30°C. A water rinsing of the solvent extract removes non-lipid contaminants. An ethyl ether-hexane (1:3, v/v) solution is used to carry the solvent extract through a silicic acid column, which separates the extract into nutritive and nonnutritive fractions. The main problems with this chromatographic separation is that waxes and sterol esters are carried into the nutritive lipid fraction but phospholipids are not. The method is intended as a replacement for the commonly used ether extract procedure.

The solvent extract of all foods and feeds is greater than ether extract while nutritive lipid is less than ether extract. The nonnutritive lipid of the leaves and stems of plants usually exceeds the nutritive portion.

TABLE 3. Apparent digestibility of solvent extract and nutritive lipid and nonnutritive lipid by swine.

		Solvent extract		Nutritive lipid		Nonnutritive lipid	
Diet No.	Animal No.	Content	Digestion	Content	Digestion	Content	Digestion
		%	%	%	%	%	%
1	1	3.7	65.4	1.2	88.8	2.5	54.6
	2	3.7	67.8	1.2	84.5	2.5	60.0
2	3	4.2	56.8	1.3	85.8	2.9	45.0
	4	4.2	50.8	1.3	80.8	2.9	38.5
	5	4.2	58.2	1.3	80.5	2.9	49.1
3	6	5.0	74.8	2.1	86.2	2.9	67.0
	7	5.0	77.6	2.1	89.2	2.9	69.8

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