Method for Extraction and Separation by Solid Phase Extraction of Neutral Lipid, Free Fatty Acids, and Polar Lipid from Mixed Microbial Cultures†

H. Gale Bateman, II, and Thomas C. Jenkins*

Department of Animal, Dairy, and Veterinary Sciences, 151 Poole Agricultural Center, P.O. Box 0361, Clemson University, Clemson, South Carolina 29634-0361

A method using solvent extraction followed by selective elution from aminopropyl bonded phase columns has been developed for the separation of lipid classes in mixed microbial cultures. Lipid extraction in heptane/2-propanol (1:4 v/v) of culture solids (pelleted by centrifugation) was in excess of 95% of total lipid in cultures with no lipid in the liquid portion of the culture. Lipid classes were separated from extracts using modifications of an existing procedure using aminopropyl bonded phase columns. Single lipid classes were verified by thin layer chromatography with comparison to known standards. Quantification of fatty acids in individual fractions was by gas−liquid chromatography and resulted in excess of 95% recovery of fatty acids from a standard lipid mixture.

Keywords: *Rumen; triglycerides; fatty acids; solid phase extraction*

INTRODUCTION

The antimicrobial effect of free fatty acids (FFA) has long been known (Galbraith et al., 1971; Henderson, 1973). However, addition of intact triglycerides (TG) to microbial culture will also inhibit microbial growth (Palmquist and Jenkins, 1980). To fully evaluate whether the TG or the FFA have antimicrobial action when lipases (either endogenous or exogenous to the culture) are present, separation of these lipid classes is imperative. Traditional techniques for separation of lipid classes included preparative thin layer chromatography (TLC), solvent partitioning, and preparative high-performance liquid chromatography (HPLC). Recently, solid phase extraction (SPE) on either silica gel or bonded phase columns (Kaluzny et al., 1985; Kim and Salem, 1990; Prieto et al., 1992) has been explored for separation of lipid classes.

Kaluzny et al. (1985) reported a SPE method using bonded phase aminopropyl disposable columns for separation of neutral lipid (NL), FFA, and polar lipid (PL) fractions of rat brain extract. Attempts to utilize this procedure in our laboratory for separation of lipid extracts from mixed microbial cultures indicated that not all PL was eluted from the columns with the indicated solvents.

This paper describes modifications we have made to the procedures of Kaluzny et al. (1985) to allow for quantification of these PL. This paper also describes procedures for extracting lipid from microbial cultures for subsequent partitioning, and quantification. Finally, this paper describes changes in ruminal lipid pools as determined by utilization of this method.

MATERIALS AND METHODS

Reagents. Lipids were extracted from microbial cultures in heptane/2-propanol (1:4 v/v). Solvents for separation of lipid classes were chloroform/2-propanol (2:1 v/v), 2% (v/v) acetic acid in ethyl ether, and methanol. All solvents were of HPLC grade. Methyl derivatives of fatty acids from each fraction were formed using 5% solution of acetyl chloride in methanol. [1-^{14}C]Linoleic acid was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Heptadecanoic acid (>99% pure) was purchased from Sigma Chemical Co. (St. Louis, MO).

Procedures. *Microbial Cultures.* Microbial cultures were conducted using modifications of procedures outlined by Goering and Van Soest (1970). Our system utilized capped 50 mL culture tubes in place of Erlenmeyer flasks as the sample container during fermentation and is based upon modifications proposed by Sayer and Van Soest (1972). All incubations were for 24 h and conducted in an anaerobic atmosphere at 39 °C. Individual cultures were mixed by gentle inversion every 4 h during incubation.

Substrate for the cultures was grass hay ground to pass a 1 mm screen. Lipid in cultures was varied by addition of either linoleic acid (technical grade, Eastman Kodak Co., Rochester, NH) or soybean oil (SBO) up to 20% of weight. Lipid was added to cultures by first mixing hay substrate with desired lipid and then adding the mixed substrate to cultures.

Total Lipid Recovery from Cultures. Four culture tubes were prepared with substrate containing 10% added lipid as SBO and [1-^{14}C]linoleic acid (100:1 v/v) and allowed to incubate for 24 h without being inoculated. Following incubation, 10 mL of whole culture contents was centrifuged at 36000*g* for 20 min at 4 °C in 50 mL polycarbonate centrifuge tubes with sealing caps. The supernate was decanted into glass scintillation vials and dried under vacuum. The pellet was extracted twice in 5 mL of heptane/2-propanol (1:4 v/v) with shaking on a wrist action shaker for 1 h. Following each extraction, samples were centrifuged at 36000*g* for 20 min at 4 °C. The supernates were combined by decanting into glass scintillation vials and dried under vacuum. Residual radioactivity in extracted pellet and centrifuge tubes was measured after the remaining contents were quantitatively transferred to scintillation vials. All samples were counted on a Packard 1900 CA liquid scintillation counter for 4 min.

To check for loss of lipid during the extraction procedure, two separate cultures with no [^{14}C]linoleic acid were prepared to measure fatty acids retained in the pellet. Pellets from nonradiolabeled cultures were extracted, and then the pellet was quantitatively transferred to glass screw-top tubes and dried under vacuum. Fatty acid methyl esters from dried, nonradiolabeled pellets were formed using the procedures of Sukhija and Palmquist (1988). These were then quantified by gas−liquid chromatography (GLC) (Hewlett-Packard 5890 gas chromatograph) using the following conditions: 30 m ×

* Author to whom correspondence should be addressed (fax 864.656.3131; e-mail TJNKNS@Clemson.edu).

† Technical Contribution 4189 of the South Carolina Agricultural Experiment Station.

S0021-8561(96)00333-0 CCC: $14.00 © 1997 American Chemical Society

Table 1. Composition of Diet Fed to Fistulated Cow

ingredient	% of ration dry matter	ingredient	% of ration dry matter
Coastal Bermudagrass hay	50.0	dicalcium phosphate	3.8
cottonseed hulls	17.6	calcium carbonate	2.3
cracked corn	10.4	urea	1.0
soybean meal	10.3	trace mineralized salt	0.5
soybean oil	4.0		

25 μm polyamino glycol capillary column (Supelco Inc., Bellefonte, PA); injector, 260 °C; 1:100 split; flame ionization detector, 250 °C; oven temperature programmed at 150 °C for 2 min, then raised at 2 °C/min to 220 °C, and held for 8 min. Carrier gas (He) flow rate was 20 cm/s. Peak identification was by comparison of relative retention times to commercially prepared standards, and quantification was by internal standard (heptadecanoic acid).

Lipid Partitioning. Standard lipid mixtures of linoleic acid, SBO, and soy lecithin (American Lecithin Co., Woodside, NY) were dissolved in chloroform for verification of the separation procedures of Kaluzny et al. (1985). Briefly, 500 mg aminopropyl bonded phase SPE columns (Waters Corp., Milford, MA) were prewashed with 4 mL of hexane, and then 0.5 mL of the standard lipid mixture was loaded onto the column. Neutral lipid was eluted with 4 mL of chloroform/2-propanol, FFA were eluted with 8 mL of 2% acetic acid in ethyl ether, and polar lipid was eluted with 4 mL of methanol.

The presence of single lipid fractions within each elution step was verified by TLC on 20 × 20 cm silica gel plates (Alltech, Inc., Deerfield, IL). Plates were spotted with 10 μL of sample by hand. Additional lanes of each eluate were intentionally overloaded with 30 μL of sample to check for small contaminants. Plates were developed in a two-step procedure. Solvent for step 1 was 78% toluene, 10.4% ethyl ether, 10.4% ethyl acetate, and 1.2% acetic acid. Solvent for step 2 was 78.4% hexane, 19.6% ethyl ether, and 2% formic acid. Spots were visualized by spraying plates with 60% (v/v) H_2SO_4 followed by heating at 110 °C for 30 min.

Recovery of individual lipid fractions from separation procedures was evaluated by quantifying fatty acids in each fraction by GLC and comparison to calculated amounts present in standard lipid mixtures. To check for lipid remaining on separation columns, packing was removed by hand and any fatty acids remaining attached were quantified by GLC.

Verification of Procedures. A mature Holstein cow fitted with ruminal cannula was fed a hay-based diet with 4% of the diet dry matter (DM) as added SBO (Table 1). The cow was maintained for 16 days at 85% of her ad libitum feed intake prior to sampling of ruminal contents. On day 17, beginning immediately prior to feeding and every 2 h thereafter, approximately 500 g wet weight of total ruminal contents was removed via the ruminal cannula. This digestus was placed in resealable plastic bags and immediately frozen (0 °C) and stored. Prior to analysis, samples were thawed at room temperature and dried at 60 °C in a forced air oven. Dry samples were ground to pass a 1 mm screen. Dry ground samples were then extracted and NL and FFA separated by the procedure outlined above with PL discarded along with the SPE column packing material.

RESULTS AND DISCUSSION

Recovery of radioactive fatty acid from culture substrate was within 1 standard deviation (SD) of calculated total counts present in individual cultures (Table 2) with minimal residual contamination of extraction equipment. Recovery of radioactive fatty acid indicated that extraction of lipid from the solids portion of the microbial cultures was sufficient to remove all lipid present in cultures. No radioactivity was detected in the dried supernate from cultures. Minimal concentrations of fatty acids were detected in pellets following extraction (50 mg of lipid added, <0.5 mg of fatty acid recovered), indicating that two extractions were sufficient to remove the majority of lipid from the culture pellet.

Table 2. Recovery of [^{14}C]Linoleic Acid from Extraction of Microbial Cultures[a,b]

	DPM	% of DPM added
DPM added	50404	100
DPM recovered	59294	112.4
from pellet	55773 ± 5718	111.3
from supernate	38 ± 4	<0.1
from vial	483 ± 2	1.0

[a] Data are presented as mean ± SD. [b] For all analysis $n = 4$.

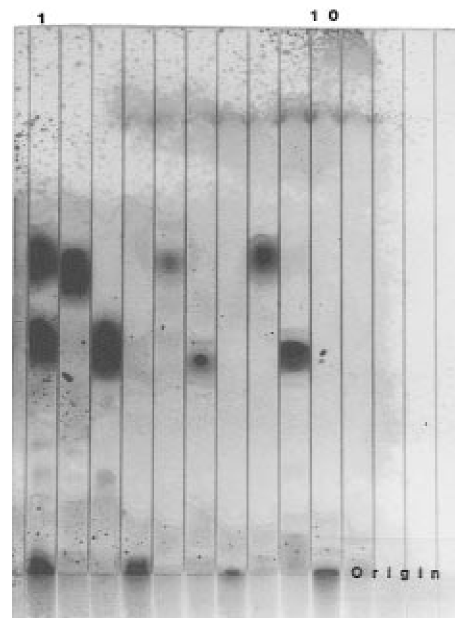

Figure 1. Thin layer chromatography of elutant from solid phase extraction on aminopropyl bonded phase columns: (lane 1) mixed standard containing soybean oil, linoleic acid, and soy lecithin; (lane 2) soybean oil standard; (lane 3) linoleic acid standard; (lane 4) soy lecithin standard; (lane 5) first elution fraction from SPE columns (10 μL); (lane 6) second elution fraction from SPE columns (10 μL); (lane 7) third elution fraction from SPE column (10 μL); (lane 8) first elution fraction from SPE columns (30 μL); (lane 9) second elution fraction from SPE columns (30 μL); (lane 10) third elution fraction from SPE column (30 μL).

Individual lipid classes (NL, FFA, and PL) were recovered using the solid phase extraction procedure (Figure 1). Also, identification of individual classes of lipid was verified by comparison of relative position of spots to those of known standards. Separation of individual lipid classes by SPE was complete, with no cross-contamination detected even when lanes were intentionally overloaded with sample.

Recoveries of NL and FFA from the SPE column were within 5% of theoretical (Table 3). However, recovery of PL in the eluate was only 39% of theoretical. A portion (16.2%) of the PL remained attached to the column packing, with the remainder (44.4%) unaccounted for. Overall recovery of total lipid from cultures was 89.2% of added fatty acid. Kaluzny et al. (1985) reported that all PL was removed from the columns by the methanol wash. However, Kim and Salem (1990) have reported that acidic PL may remain on these columns following separation procedures and have recommended a fourth wash with acidified methanol to remove them. For our purposes, methylating the packing material and column to determine PL is both quicker and easier than eluting two fractions of PL. Although removal of packing material followed by methylation of

Table 3. Recovery of Individual Lipid Fractions following Separation on Aminopropyl Solid Phase Extraction Columns[a,b]

culture fraction	theor mg of fatty acid	recovered fatty acid (mg)	% of theor[c]
neutral lipid	20.3	19.25 ± 0.218	94.8
free fatty acid	20.9	22.02 ± 0.046	105.4
total polar lipid	13.5	7.51	55.6
eluted from column		5.32 ± 0.133	39.4
left on packing		2.19 ± 0.027	16.2
total lipid	54.7	48.79	89.2

[a] Data are presented as mean ± SD. [b] For all analyses $n = 4$. [c] Amount of fatty acids present in each fraction as determined by analysis.

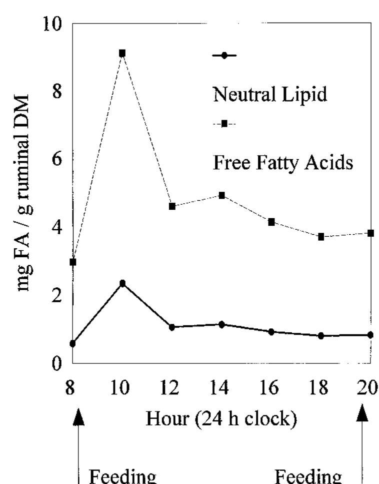

Figure 2. Neutral lipid and free fatty acid concentrations in ruminal samples taken from a cow fed a diet containing 4% of the total dry matter as soybean oil.

attached PL has potential for loss of fatty acids, this is not a large concern when lipolysis and biohydrogenation are the only aspects of microbial lipid metabolism being studied. Our method offers virtually complete recovery of both NL and FFA, which are the two lipid fractions involved in lipolysis and biohydrogenation. If microbial cellular metabolism was being studied, methylating packing material should allow for rapid and easy quantification of fatty acids present in PL.

Application of this technique to samples of stomach contents from a fistulated cow are shown in Figure 2. Following feeding there was an increase in ruminal NL and FFA concentrations. Concentrations of these lipid fractions then declined. The decline was rapid during the first 2 h, and then the rate decreased over the following 8 h. This transient increase was expected. At feeding, the cow would consume her diet, in its entirety, within 2 h. This would imply a rapid increase in ruminal NL from the diet following feeding. Because ruminal microbes possess lipase, which rapidly and completely hydrolyzes FFA from NL, it was also expected that FFA concentrations would increase. We expected to see these increases as transient because as digestion continued both NL and FFA would be washed out of the rumen. It was expected, however, that the increase in NL would precede the increase in FFA. We believe that the long time period between our sampling times prohibited our observation of this phenomenon.

This method was considered as an alternative to solvent partitioning or preparative TLC for separation of lipid classes. Our SPE system for separation of lipid classes offers advantages over both solvent partitioning and preparative TLC. Solvent partitioning involves manipulation of large volumes of organic solvents (Jenkins and Palmquist, 1982) that must be evaporated prior to analysis of fatty acids. The separation procedure presented here uses small volumes (4 and 8 mL for each separation step), which will decrease solvent cost and waste disposal. Automated equipment is available for manipulation of commercial SPE columns which would allow for reduction in manual labor required for separation of lipid classes.

Preparative TLC involves loading samples onto specialized TLC plates and then scraping individual spots into vessels for further analysis. There is a large possibility for loss of NL, FFA, and PL when TLC plates are scraped, similar to our observed loss of PL with manual removal of packing material from SPE columns. Additionally, often multiple lanes on TLC plates must be used for each sample to provide enough individual lipid classes for analysis. Our method allows for much larger samples to be separated at one time.

CONCLUSION

We believe that this procedure will allow rapid and efficient separation of lipid classes recovered from microbial cultures. Extraction of culture solids that have been pelleted by centrifugation is sufficient for removal of lipid from microbial cultures. Recovery of individual lipid classes from SPE columns is in acceptable limits. Separation of lipid classes is excellent. This procedure should allow for the influence of lipids on microbial fermentation to be explored in detail.

LITERATURE CITED

Galbraith, H.; Miller, T. B.; Patton, A. M.; Thompson, J. K. Antibacterial activity of long chain fatty acids and the reversal with calcium, magnesium, ergocalciferol, and cholesterol. *J. Appl. Bacteriol.* **1971**, *34*, 803−814.

Goering, H. K.; Van Soest, P. J. *Forage Fiber Analysis (Apparatus, Reagents, Procedures, and Some Applications)*; Agricultural Handbook 379; ARS-USDA, U.S. Government Printing Office: Washington, DC, 1970.

Henderson, C. The effects of fatty acids on pure cultures of rumen bacteria. *J. Agric. Sci. (Cambridge)* **1973**, *81*, 107−111.

Jenkins, T. C.; Palmquist, D. L. Effect of added fat and calcium on in vitro formation of insoluble fatty acid soaps and cell wall digestibility. *J. Anim. Sci.* **1982**, *55*, 957−963.

Kaluzny, M. A.; Duncan, L. A.; Merritt, M. V.; Epps, D. E. Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.* **1985**, *26*, 135−140.

Kim, H.; Salem, N. Separation of lipid classes by solid phase extraction. *J. Lipid. Res.* **1990**, *31*, 2285−2289.

Palmquist, D. L.; Jenkins, T. C. Fat in lactation rations: review. *J. Dairy Sci.* **1980**, *63*, 1−14.

Prieto, J. A.; Ebri, A.; Collar, C. Optimized separation of nonpolar and polar lipid classes from wheat flour by solid-phase extraction. *J. Am. Oil Chem. Soc.* **1992**, *69*, 387−391.

Sayer, K. D.; Van Soest, P. J. Comparison of types of fermentation vessels for an in vitro artificial rumen procedure. *J. Dairy Sci.* **1972**, *55*, 1496−1498.

Sukhija, P. S.; Palmquist, D. L. Rapid method for determination of total fatty acid content and composition of feedstuffs and feces. *J. Agric. Food Chem.* **1988**, *36*, 1202−1206.

Received for review May 15, 1996. Accepted October 8, 1996.[⊗]

JF9603339

[⊗] Abstract published in *Advance ACS Abstracts,* November 15, 1996.