BIOACTIVITY OF *TRANS* OCTADECENOIC ACIDS: UTILIZATION FOR MILK FAT SYNTHESIS IN DAIRY COWS AND EFFECTS ON BIOMARKERS OF CORONARY HEART DISEASE RISK IN THE GOLDEN SYRIAN HAMSTER

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ABSTRACT

There are two main dietary sources of *trans* fatty acids (TFA): an industrial-derived source originating from the partial hydrogenation of polyunsaturated oils and a natural source associated with ruminant-derived food products. Industrial-derived TFA have been implicated in the progression of a number of chronic human diseases, especially coronary heart disease (CHD). These effects have not been observed for ruminant-derived TFA and differences could relate to the fatty acid composition of two TFA sources. Previous research has demonstrated that individual fatty acids, rather than dietary fats, are responsible for producing specific biological effects. With regard to TFA, however, the role of specific isomers and potential mechanisms through which individual isomers may elicit their bioactivity remain largely unclear. The present thesis research was carried out to investigate the biological activity of two *trans* 18:1 fatty acids, *trans*-9 18:1 (elaidic acid; EA) a major TFA in partially hydrogenated vegetable oil (PHVO) and *trans*-11 18:1 (vaccenic acid; VA), the predominant TFA of ruminant fat.

The objective of the first study was to investigate the biological activity of EA and VA in dairy cows with regard to plasma lipid transport and mammary utilization for milk fat synthesis. We utilized three ruminally cannulated, mid-lactation Holstein dairy cows in a 3 x 3 Latin square design, and treatments were a 4 d abomasal infusion (~45 g/d; free fatty acid, FFA form) of 1) *cis*-9 18:1 (oleic acid, OA), 2) EA and 3) VA. There were striking differences in the fatty acid composition of the individual plasma lipid fractions and in the plasma lipid distribution of specific 18-carbon fatty acids. Infusion

of the treatment isomers caused their specific increase in the various plasma lipid fractions, with OA and EA being predominantly associated with plasma phospholipids (PL), while VA was more evenly distributed between plasma PL and triglycerides. Treatments had no effect on milk production variables, including milk fat. Transfer efficiency of infused OA and EA to milk fat averaged 65.5 ± 3.0% and 59.7 ± 1.5%, respectively. Transfer of infused VA to milk fat averaged 54.3 ± 0.6%, of which approximately one-quarter was accounted for by its use for the endogenous synthesis of *cis*-9, *trans*-11 18:2 in milk fat. Notably, 18:2*n*6 and 18:3*n*3 accounted for 47.7% of total plasma fatty acids, but only 2.6% of fatty acids in milk fat. Overall, results demonstrated clear differences in the plasma transport and mammary uptake and utilization of 18-carbon fatty acids, and these relate to the location, orientation and number of double bonds.

The second study was designed to investigate the biological effects of EA and VA on biomarkers of CHD risk. Hamsters (n = 32) were randomly assigned to 1 of 4 treatment diets: 1) Control, “Western” diet, 2) PHVO, 3) FFA of EA and 4) FFA of VA. Fat supplements were fed at 2.5 g/100g of diet, and the EA and VA supplements averaged 84% purity for the respective *trans* 18:1 isomer. Hamsters fed the PHVO diet had higher total plasma cholesterol and higher concentrations of cholesterol in the atherogenic lipoprotein fractions, including very low-density lipoproteins, intermediate-density lipoproteins and low-density lipoproteins. Relative to the Control and PHVO treatments, hamsters fed the EA and VA diets had significantly lower total plasma cholesterol and lower cholesterol concentrations in the atherogenic lipoprotein fractions. Hamsters fed the EA treatment showed greater expression of the hepatic low density lipoprotein-receptor, indicating a

possible mechanism to explain the reduction in plasma cholesterol. Overall, results demonstrated that the hypercholesterolemic effects of the PHVO supplement were not dependent on the presence of EA or VA.

BIOGRAPHICAL SKETCH

Cynthia Tyburczy was born on November 10, 1978 in Red Bank, NJ. She grew up in Harrington Park, NJ, and graduated from Northern Valley Regional High School in Old Tappan in 1997. Her love of traveling, outdoors and adventure led her to Colorado where she worked as a bartender for a few years, but her passion for learning soon brought her back to school. She transferred to Cornell University in 2004, and received a Bachelor of Science degree in Animal Science in January 2006. She completed an undergraduate honors research project under the supervision of Dr. Dale E. Bauman in which she was able to blend her interests in animals, lipids and human health, and which led her to further pursue graduate studies. In January 2008, Cynthia received her Master of Science degree in Animal Science with a minor in Human Nutrition.

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LIST OF ABBREVIATIONS

CE	Cholesterol esters
CHD	Coronary heart disease
CLA	Conjugated linoleic acid
DMI	Dry matter intake
EA	Eladic acid; *trans*-9 18:1
FAME	Fatty acid methyl esters
FFA	Free fatty acids
HDL	High-density lipoproteins
IDL	Intermediate-density lipoproteins
LDL	Low-density lipoproteins
LDL-R	Low-density lipoprotein-receptor
LCPUFA	Long chain polyunsaturated fatty acids
MFD	Milk fat depression
NEFA	Non-esterified fatty acids
OA	Oleic acid; *cis*-9 18:1
PHVO	Partially hydrogenated vegetable oil
PL	Phospholipids
PUFA	Polyunsaturated fatty acids
RA	Rumenic acid; *cis*-9, *trans*-11 18:2
TC	Total cholesterol
TFA	*Trans* fatty acids
TG	Triglycerides
TLC	Thin layer chromatography
VA	Vaccenic acid; *trans*-11 18:1
VLDL	Very low-density lipoproteins

CHAPTER ONE

INTRODUCTION

The roles of dietary fatty acids in the body are numerous and diverse; for example, they serve as sources of energy, act as structural components of cell membranes, and provide precursors for eicosanoid signaling molecules. More recently, it has been recognized that some fatty acids have functional food properties, providing benefits to health beyond their traditional nutritive content (Lock and Bauman, 2004). These fatty acids, more collectively known as bioactive fatty acids, impact metabolism either through the regulation of biological processes or alteration of gene expression.

The most well researched bioactive fatty acids include conjugated linoleic acid (CLA) and the omega-3 fatty acids, eicosapentaenoic acid (20:5*n*3) and docosahexaenoic acid (22:6*n*3), known for their beneficial effects on cardiovascular disease risk factors (Connor, 2000). The majority of CLA in the human diet originates from ruminant-derived food products, arising as a consequence of the biohydrogenation of polyunsaturated fatty acids by rumen microbes, with *cis*-9, *trans*-11 CLA (rumenic acid; RA) being the major CLA isomer. This isomer is known for its anticarcinogenic and antiatherogenic properties in biomedical models of human disease (Parodi, 1997; Bauman et al., 2006). In addition to the formation of RA, the typical pathways for rumen biohydrogenation involve formation of *trans* 18:1 fatty acids before the final hydrogenation step to stearic acid. These isomers may be potentially bioactive with regard to milk fat synthesis and chronic diseases in humans.

Situations of diet-induced milk fat depression are characterized by a specific reduction in the yield and content of milk fat, and occur as a result of alterations in rumen fermentation associated with the production of unique, bioactive CLA isomers (Bauman and Griinari, 2003). In 1970, Davis and Brown were among the first to recognize that situations of diet-induced milk fat depression coincided with enhanced levels of *trans* 18:1 fatty acids in milk fat, and subsequent examinations revealed alterations in the patterns of *trans* 18:1 isomers in milk fat (Griinari et al., 1998). It was previously suggested that the *trans* 18:1 isomers produced under conditions of altered rumen fermentation were directly affecting milk fat synthesis (Erdman, 1996); limited availability of pure isomers, however, restricted direct examination. Few studies have directly investigated the effects of individual *trans* 18:1 fatty acids, and none have demonstrated adverse effects on milk fat production at the doses examined (Bauman and Griinari, 2003). On the other hand, preparations involving partially hydrogenated vegetable oil (PHVO) have demonstrated specific reductions in milk fat yield and content. PHVO is largely comprised of *trans* 18:1 fatty acids, but also contains a multitude of unique fatty acid isomers not typically observed in nature, and for which bioactivity is largely unknown.

With regard to human health, *trans* fatty acids (TFA) have been associated with the progression of a number of chronic diseases, including coronary heart disease (CHD), systemic inflammation and type-II diabetes (Willett, 2006), and may also impair essential fatty acid metabolism during growth and development (Hill et al., 1982; Innis, 2006). There are two major sources of TFA in the diets of humans: those originating from the industrial synthesis of PHVO, and those derived from the milk and meat of ruminant

animals. Where as the adverse effects of TFA have been documented for PHVO, these relationships have not been established for ruminant-derived TFA, and rather some studies utilizing biomedical models have demonstrated potentially beneficial effects of ruminant-derived RA and vaccenic acid (VA; *trans*-11 18:1; Parodi, 1997; Bauman et al., 2006). The health effects attributed to the two sources of TFA may relate to differences in *trans* 18:1 isomer content and profile; however, few studies have examined the extent to which the adverse effects are related to specific *trans* 18:1 fatty acids.

The overall objective of this thesis research was to examine the bioactivity of the two more prevalent *trans* 18:1 fatty acids, *trans*-9 18:1 (elaidic acid, EA) and *trans*-11 18:1, with regard to milk fat synthesis and biomarkers of CHD risk. In the first study, we utilized lactating dairy cows to examine EA and VA in terms of plasma lipid transport and utilization by the mammary gland for milk fat synthesis. For this study, we compared the EA and VA isomers to effects seen with oleic acid (*cis*-9 18:1), a *cis*-octadecenoic acid typically found in the milk fat of ruminant animals. In the second study, we examined the effects of EA and VA, in relation to PHVO on biomarkers of CHD risk, including hepatic lipid and plasma lipoprotein metabolism, using the cholesterol-fed Golden Syrian hamster as a biomedical model. For this study, it was of interest to determine if the adverse effects of PHVO with regard to CHD risk factors, are dependent on the presence of EA or VA.

CHAPTER TWO

LITERATURE REVIEW

As consumers are becoming aware of the links between dietary fat, health maintenance and disease prevention, there is an increasing need for clarification of the bioactivity and metabolic fate of dietary fatty acids. One group of fatty acids of particular interest and relevance are the *trans* fatty acids (TFA). In relation to human health, TFA have been implicated in the progression of a number of chronic human diseases, especially CHD (Willett, 2006). However, source of TFA can be important as ruminant-derived TFA may have differential effects on human health than those originating from the partial hydrogenation of polyunsaturated oils (Lock et al., 2004; Pfeuffer and Schrezenmeir, 2006). The following sections are designed to give an overview of TFA, with particular focus on the differences between dietary sources of TFA and related human health implications.

INTRODUCTION TO *TRANS* FATTY ACIDS

Fatty acids can be collectively described as a group of lipid macromolecules that consist of a hydrocarbon chain with a carboxyl group (COOH) at one end and a methyl group (CH_3) at the other. Fatty acids function alone or as components of larger lipid complexes, including phospholipids (PL), cholesterol esters (CE) and triglycerides (TG); these, together with nucleic acids, amino acids and carbohydrates, make up the core macromolecules of biological tissues.

Shorthand notation for a fatty acid involves description of the chain length and the number and location of the double bonds. Butyric acid, a fatty acid containing four carbons and no double bonds would be written as 4:0 or

C4:0. Fatty acids containing no double bonds are called saturated fatty acids, those containing one double bond are referred to as monounsaturated fatty acids, and fatty acids with two or more double bonds are considered polyunsaturated fatty acids (PUFA). Most fatty acids of nutritional significance have a straight hydrocarbon chain and contain an even number of carbon atoms. Other fatty acids not pertinent to this discussion include branch-chain fatty acids and fatty acids that contain additional functional groups, including hydroxyl, keto or epoxy groups.

The length of the fatty acid hydrocarbon chain varies among tissues, species and even kingdom. The majority of fatty acids in mammalian tissues range from 12 to 26 carbons in length, although shorter-chain fatty acids may also be found in the milk of many mammalian species (Enser, 1984). Nutritionally significant fatty acids from plant tissues and seeds typically range from 12 to 18-carbons in length, while longer chain dietary PUFA originate from marine species. Fatty acids are acquired either from the diet or are synthesized de novo from precursors such as acetate, propionate and butyrate in ruminants or glucose in nonruminants. The typical de novo synthesis pathway for fatty acids terminates in production of 16:0; the lactating mammary gland, however, has the capability of producing short and medium chain fatty acids (4:0-14:0) due to the presence of an acylthioester hydrolase enzyme (Enser, 1984). Likewise, the ability to synthesize long chain fatty acids (>16:0) relates to expression of elongase enzymes (Enser, 1984).

There are essentially two distinct categories of fatty acid nomenclature, and these involve naming the location of the double bond in relation to either the carboxyl end or methyl end of the hydrocarbon chain. Counting from the

methyl end, double bonds are numbered sequentially and given an *n* or omega label (e.g. *n*-3 or ω-3). This system of nomenclature is common for discussing fatty acids with nutritional implications (ω-3 vs. ω-6) for which the predominant common feature is the location of the initial double bond from the methyl end. For the present discussion and unless otherwise stated, the double bond orientation of PUFA indicates "all *cis*" (e.g. 18:2*n*6 indicates *cis*-9, *cis*-12 18:2 or 18:2*n*6, all *cis*). The other system of nomenclature relates to naming the initial double bond counting from the carboxyl end, also considered the more biologically reactive end of the fatty acid. The double bonds are numbered with regard to the adjacent carbon atom, and given a delta label (e.g. Δ^9). An enzyme that catalyzes a reaction at a specific carbon in the chain will also be numbered and given a delta label (e.g. Δ^9-desaturase, also known as stearoyl-CoA desaturase). Nutritionally speaking though, we tend to call fatty acids by the orientation and location of the double bond, from the carboxyl end, without adding the delta (e.g. *trans*-11 18:1; vaccenic acid, VA).

The orientation of the double bond describes the spatial distribution of the hydrogen atoms located adjacent to the double bond, and this can be in either a *cis* or *trans* configuration (Figure 2.1). The *cis* orientation indicates that the hydrogen atoms are located on the same side of the double bond. The *trans* orientation, stemming from the Latin prefix "on the opposite side [of]" indicates that the hydrogen atoms are located on opposite sides of the double bond. Double bond orientation impacts the structure of the fatty acid such that a *cis* double bond would introduce approximately a 45° kink in the fatty acid chain.

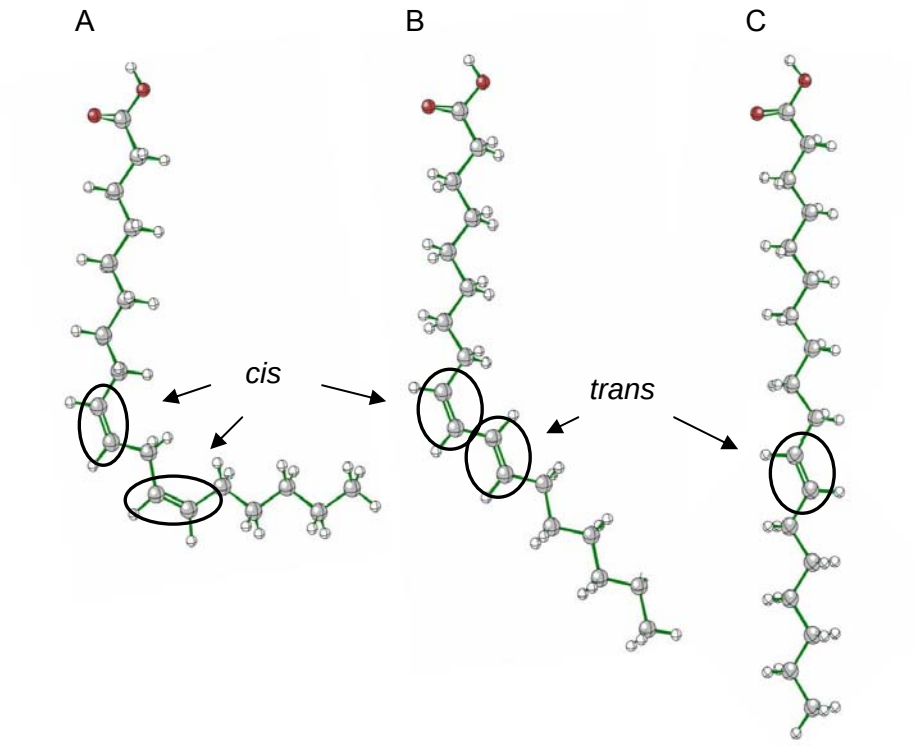


Figure 2.1. Effect of double bond orientation on the chemical structures of A) linoleic acid (*cis*-9, *cis*-12 18:2); B), rumenic acid (*cis*-9, *trans*-11 18:2); and C) vaccenic acid (*trans*-11 18:1). Adapted from Bauman et al. (2004).

In contrast, *trans* double bonds do not introduce a major kink; rather, TFA take on a straight-chain appearance, similar to saturated fatty acids. There is often debate regarding the metabolism of *trans* 18:1 fatty acids and whether they are metabolized similar to saturated fatty acids based on structure, or similar to *cis*-monounsaturated fatty acids based on the presence of a double bond.

With regard to the proximity of other double bonds, PUFA may contain two double bonds in either a conjugated or non-conjugated arrangement. Non-conjugated fatty acids contain a methylene group (CH_2) between or "interrupting" the two double bonds. In nature, this is the most commonly observed arrangement for PUFA in vegetable and plant oils, and examples

include linoleic acid (18:2*n*6) and linolenic acid (18:3*n*3). Conjugated fatty acids, on the other hand, have a double bond arrangement in which the interrupting methylene group is absent, and the double bonds are located adjacently. The conjugated double bond arrangement is naturally formed during the biohydrogenation of PUFA by rumen bacteria in ruminant animals, but may also arise during the industrial synthesis of PHVO (Banni et al., 1994). Many mammalian tissues express enzymes that facilitate the desaturation of fatty acids to produce conjugated double bonds, one such enzyme being Δ^9-desaturase. The most well-studied conjugated fatty acid is *cis*-9, *trans*-11 CLA, which is known for its anticarcinogenic and antiatherogenic properties (Parodi, 1997).

The melting point properties of a pure fatty acid are affected by its physical structure (Enser, 1984). In relation to chain length, short chain fatty acids melt at a lower temperature than longer chain fatty acids: 4:0 melts at -7.9°C, while 16:0 melts at 62.9°C. The presence of a double bond, however, also affects the melting point. Stearic acid (18:0), a straight-chain fatty acid with no double bonds, melts at 69.6°C, while presence of the *trans* double bond in *trans*-9 18:1 reduces the melting point to 43.7°C. *Cis* double bonds further decrease the melting point: *cis*-9 18:1 melts at 13-14°C and 18:2*n*6 melts at -5°C (Enser, 1984). The thermodynamic basis for the melting point of a fatty acid involves steric hindrance and van der Waals forces during crystallization. Because of the straight-chain nature, saturated fatty acids pack closely together, and van der Waals forces cause weak attractions between adjacent molecules that enable formation of a relatively stable crystal structure. The kink produced by a *cis* double bond, on the other hand, introduces a situation of steric hindrance where rotation of the hydrocarbon

chain at the double bond inhibits close packing of the fatty acid molecules and minimizes van der Waals forces. Intermediate of the two, hydrocarbon rotation at a *trans* double bond provides minimal steric hindrance, enabling formation of a more stable crystal structure than *cis*-monounsaturated fatty acids.

Based on the aforementioned discussion of the structural properties of fatty acids, we can now define TFA as a group of fatty acids that contains at least one double bond in the *trans* conformation. Although many TFA exist, the major TFA of nutritional relevance include *trans* 16:1, *trans* 18:1 and *trans* 18:2 isomers, with *trans* 18:1 fatty acids being of particular interest (Emken, 1984).

ORIGIN OF *TRANS* FATTY ACIDS

There are two main dietary sources of TFA, an industrial source derived from the partial hydrogenation and purification of polyunsaturated vegetable and marine oils, and a natural source that originates from ruminant-derived food products. With regard to *trans* 18:1 isomers, the two sources differ in both total content and isomer profile (Figure 2.2). Shown in Figure 2.3 are chromatograms typical for the *trans* 18:1 region of anhydrous butter oil and PHVO (retention times 53.1-54.2 min). Both sources contain smaller amounts of *trans* 18:2 conjugated and non-conjugated isomers (Allison et al., 1995). In addition to *trans* 18:1 and *trans* 18:2 fatty acids, partially hydrogenated fish oils contain long-chain *trans* isomers 20-24 carbons in length, which can account for up to 40% of total fatty acids (Ackman, 1982). An often overlooked source of TFA is the deodorization process in which

vegetable oils are steam-treated to remove volatile odors (Kemény et al., 2001).

Industrial-derived Trans Fatty Acids

Hydrogenation is a process that involves the use of high heat, hydrogen atoms and a metal catalyst to convert unsaturated vegetable oil fatty acids to saturated fatty acids. The incomplete, or partial, hydrogenation process allows for production of a semi-solid fat comprised of saturated, unsaturated and PUFA. A side-reaction of the hydrogenation process is the isomerization of *cis* double bonds, and this results in the formation of unique *trans* isomers not typically found in vegetable oils (Steinhart et al., 2003). The presence of TFA in PHVO is desirable in terms of baking and manufacturing applications because they introduce "plasticity," a physical property that enables a cooking fat to melt over a wide range of temperatures. This has implications for the texture and mouth-feel of the final food product; for example, the flakiness of pastry crust occurs because some of the fatty acids are liquid at room temperature while others remain solid.

Starting in the 1950-1960's, the food industry saw major changes in the types of dietary fat being used for table spreads and in food manufacturing. During this period, researchers began associating dietary fat with heart disease risk, and saturated fat and dietary cholesterol became major targets of investigation (Keys et al., 1957; Hegsted et al., 1965). Because of their content of saturated fatty acids and cholesterol, the image of ruminant food products quickly turned from healthful to harmful, and alternative dietary fat sources were investigated (Maijala, 2000).

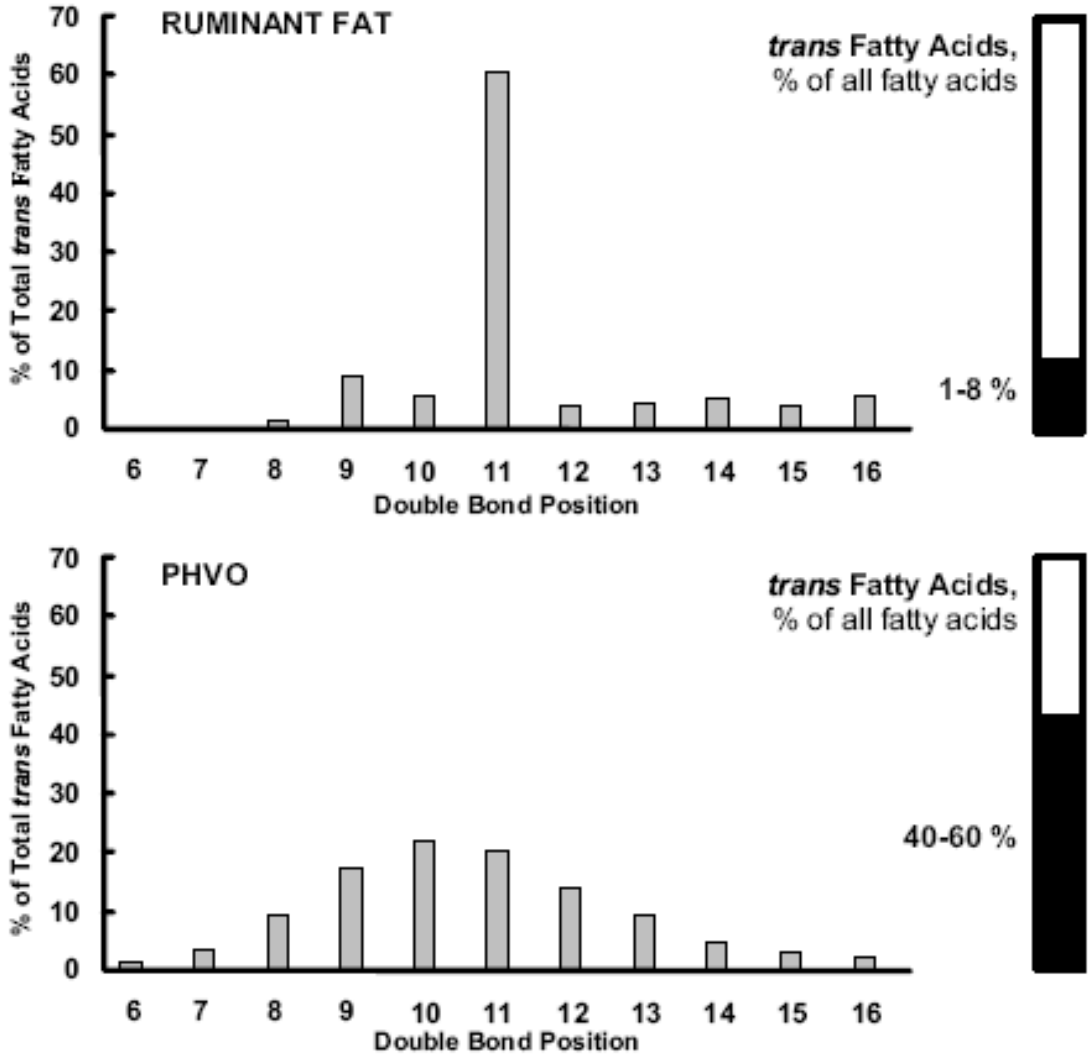
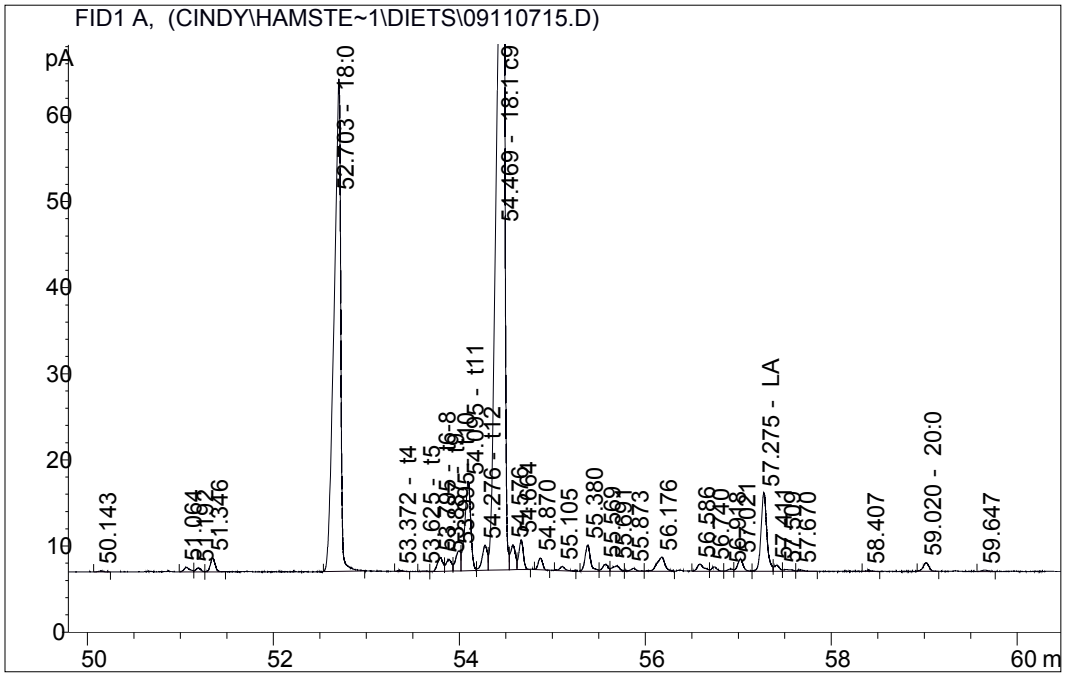
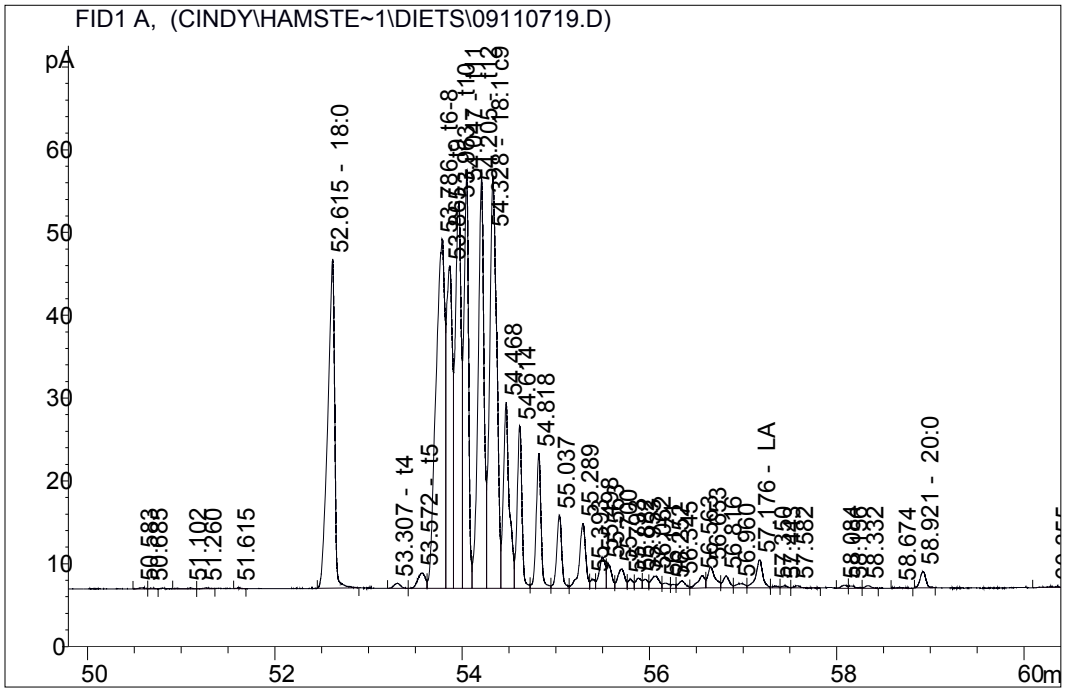


Figure 2.2. Distribution and content of *trans* 18:1 fatty acids in ruminant fat and partially hydrogenated vegetable oil (Adapted from Lock et al., 2004).



A. Anhydrous Butter Oil



B. PHVO

Figure 2.3. Chromatograms of the *trans* 18:1 region of A) anhydrous butter oil and B) partially hydrogenated vegetable oil (PHVO).

The introduction of PHVO as a replacement for ruminant fat was not new; Procter & Gamble first marketed Crisco in 1911, but with new dietary health recommendations to reduce total saturated fat and cholesterol (Committee on Nutrition, American Heart Association, 1968), PHVO regained public attention. This semi-solid, synthetic fat had the physical characteristics ideal for baking, but also showed improved shelf-life over animal based-fats and a decreased rate of oxidation compared with vegetable oils. Also a cheaper fat source than animal fat or semi-solid palm oil, PHVO quickly became assimilated into mainstream baking and food production. However, by the end of the 20th century, it was increasingly apparent that consumption of TFA from PHVO was associated with adverse effects on human health.

Data from 1994-1996 indicate that approximately 80% of the intake of dietary TFA in the United States originated from industrial-derived sources, with baked goods, margarine and fried potatoes being major contributors (Figure 2.4; U.S. Food and Drug Administration, 2003). It is expected that the total U. S. TFA intake from PHVO would have decreased since then, and this is most likely due to labeling regulations for TFA in the U.S. and Canada (U.S. Food and Drug Administration, 2006), public education on the adverse health effects of TFA and shifts in the types of fat used by food manufacturers. In response to consumer demand, the introduction of interesterified vegetable oils and oil blends has allowed for decreased use of PHVO in many food production applications (Hunter, 2006).

Trans Fatty Acids of Ruminant Origin

One uniqueness of the ruminant is the presence of bacterial fermentation in the rumen that aides in the digestion of fibrous plant materials.

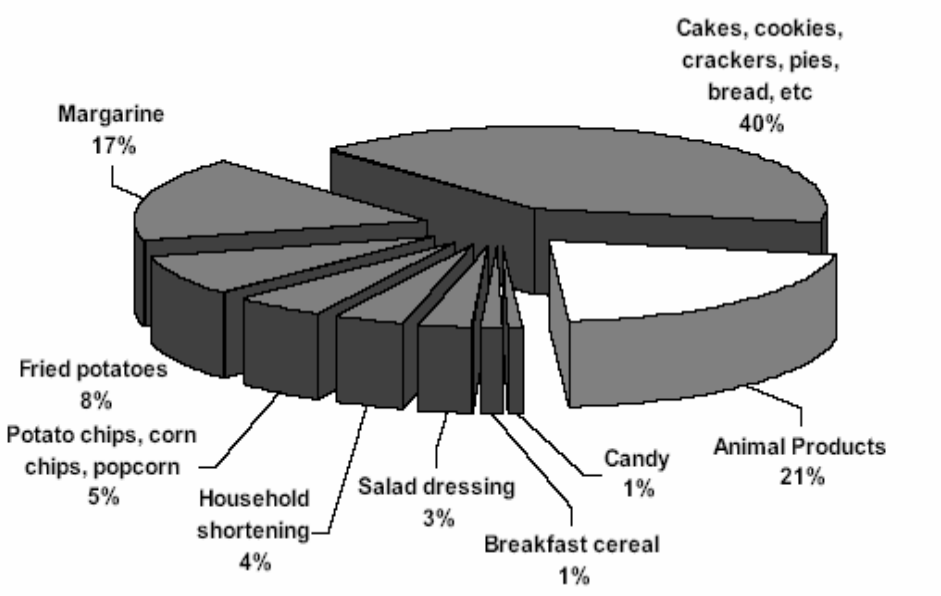


Figure 2.4. *Trans* fats in the U. S. diet (1994-1996): contribution of major food sources. Adapted from Lock et al. (2004) based on data from U.S. Food and Drug Administration (2003).

The rumen is essentially a large anaerobic fermentation vat containing, per gram of digesta, 10^6 ciliate protozoa, 10^{10} strictly anaerobic bacteria and 10^5 anaerobic fungi (personal communication, R. J. Wallace, Rowett Research Institute, Aberdeen, UK). Members of this microbial population work synergistically to break down plant materials, and volatile fatty acids, a side product of fiber digestion, are produced which the animal can absorb and use for energy. Dietary fat typically comprises 3-6% of the ruminant's diet; the unsaturated fatty acids in this fat are toxic to rumen bacteria. In response, the rumen bacteria secrete extracellular enzymes that convert PUFA to more saturated, less toxic forms. The majority of the dietary fatty acids consumed by ruminants are esterified, so the essential first step of digestion in the rumen is the hydrolysis of the ester linkages and release of free fatty acids.

The unsaturated fatty acids then undergo biohydrogenation via the extracellular enzymes secreted by rumen bacteria, involving a series of isomerization and hydrogenation reactions that ultimately result in a highly saturated fatty acid composition of the digesta leaving the rumen. Thus, the fatty acid composition of the ruminant's diet differs tremendously from the digesta that reaches the small intestine. In ruminants, fatty acids are absorbed in free fatty acid form, while monogastrics absorb free fatty acids and *sn*-2 monoglycerides (Doreau and Chilliard, 1997). This difference relates to formation and composition of the micelles prior to absorption.

The major fatty acids in the diets of ruminants include *cis*-9 18:1 and 18:2*n*6, which are found in many seed oils, and 18:3*n*3 which is the predominant fatty acid of glycolipids in forages. Of course, the content of these and other fatty acids in the diet depends on dietary formulation and extent of grazing. Under typical conditions of rumen fermentation, the major biohydrogenation pathway for 18:2*n*6 involves formation of intermediates such as RA and VA before the final hydrogenation step to 18:0 (Figure 2.5). The biohydrogenation pathway for 18:3*n*3 also involves the formation of VA, but not RA. Under conditions of altered rumen fermentation, unique biohydrogenation intermediates are formed as a result of changes in the microbial population of the rumen (Palmquist et al., 2005). Major factors leading to altered rumen fermentation include a high concentrate to forage ratio and dietary formulation with plant and fish oils. While industrial hydrogenation is typically a random process that results in the production of a multitude of *trans* and *cis* unsaturated fatty acids, the pathways for biohydrogenation in the rumen produce characteristic intermediates based on dominant microbial populations. We can therefore expect a relatively

consistent pattern of *trans* 18:1 fatty acids and CLA isomers in the plasma and milk fat of ruminants following consumption of specific dietary rations.

In 1970, Davis and Brown were among the first to recognize that situations of diet-induced milk fat depression (MFD) coincided with enhanced levels of *trans* 18:1 fatty acids in milk fat. Subsequent investigations by Griinari et al. (1998) demonstrated shifts in the milk fatty acid profile of *trans* 18:1 isomers, including increased content of *trans*-10 18:1. For years, many believed that the *trans* 18:1 isomers arising from the altered rumen fermentation were directly affecting milk fat synthesis, but the limited availability of pure isomers restricted direct examination. We recently demonstrated that *trans*-10 18:1, a *trans* 18:1 isomer strongly correlated with the extent of MFD (Hinrichsen et al., 2006), did not directly affect milk fat synthesis when abomasally infused at 45 g/d (Lock et al., 2007). Other *trans* 18:1 isomers previously tested include *trans*-9 18:1 at 25 g/d (Rindsig and Schultz, 1979), and *trans*-11 18:1 and *trans*-12 18:1 in a 50:50 mixture (dose range 7.14 g/d to 29.4 g/d for each isomer; Griinari et al., 2000; Shingfield et al., 2007), with none affecting milk fat synthesis at the doses examined. Thus, it appears that although *trans* 18:1 isomers do increase in milk fat during situations of diet-induced MFD, they function more as a surrogate marker for changes in rumen fermentation associated with MFD, rather than having a causative role.

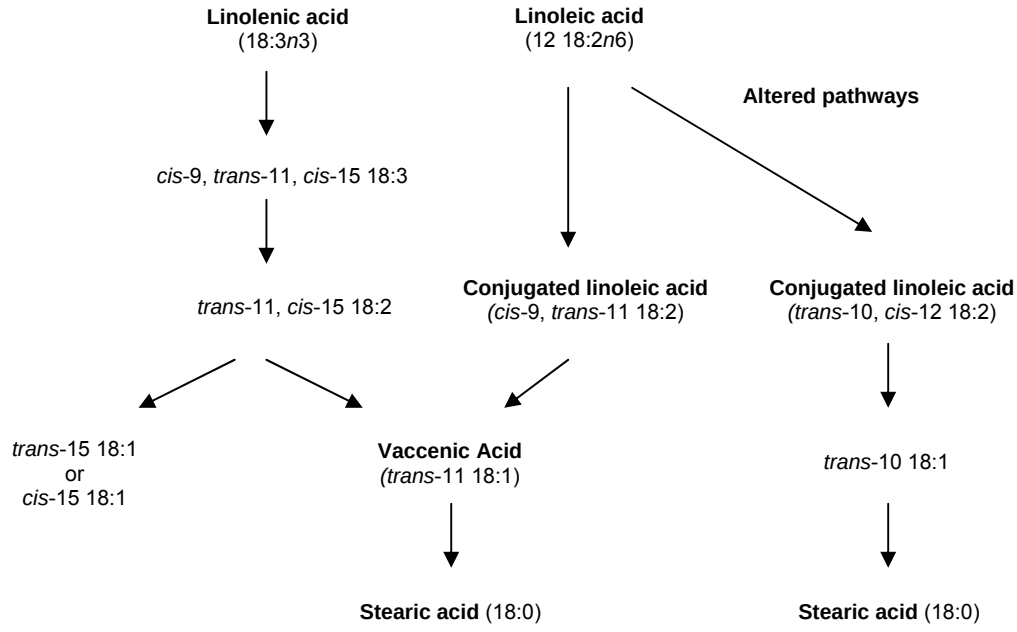


Figure 2.5. Pathways for the biohydrogenation of linoleic acid (18:2*n*6) and linolenic acid (18:3*n*3) during typical and altered conditions of rumen fermentation. Adapted from Bauman and Griinari (2003).

Uptake and Utilization of Fatty Acids in Ruminants

Rate of digesta passage from the rumen is continuous and as a result, some fatty acid intermediates escape complete biohydrogenation and become absorbed at the small intestine. *Trans* 18:1 isomers, more so than other intermediates, tend to escape the rumen because the final biohydrogenation step to 18:0 is a rate-limiting reaction (Palmquist et al., 2005). This is especially apparent as dietary formulation with fish oil, a known inhibitor of the final hydrogenation step, results in marked increases of *trans* 18:1 fatty acids, and subsequently CLA isomers, in plasma and milk fat (Lock and Bauman, 2004). Evidence suggests that the rate and extent of absorption of *trans* 18:1 fatty acids in ruminants is constant, averaging 82-

98% for individual isomers, and is not significantly affected by the location of the *trans* double bond (Loor et al., 2004). Similar results have also been observed in studies with rats and humans (Emken, 1984).

The unique distribution of fatty acids among plasma lipid fractions (PL, CE, TG and non-esterified fatty acids; NEFA) clearly demonstrates the body's ability to recognize and allocate fatty acids for specific metabolic purposes. This distribution is based predominantly on the physical properties of the fatty acids, including chain length, and degree of unsaturation and orientation of the double bonds (Christie, 1981). Following absorption, free fatty acids are packaged as components of the plasma lipid fractions, and enter the bloodstream in the form of chylomicrons and very low density-lipoproteins (VLDL). Utilization of fatty acids is then impacted by their incorporation in specific plasma lipid fractions. With regard to milk fat synthesis, the mammary gland preferentially extracts fatty acids from the plasma TG and NEFA fractions, and uptake is proportional to their concentration in plasma (reviewed by Moore and Christie, 1979). In contrast, 18:2*n*6 and 18:3*n*3 are major fatty acids comprising the plasma PL and CE fractions, but the low milk fat content of these isomers suggests that plasma PL and CE may be less well utilized as sources of fatty acids for mammary uptake (Moore and Christie, 1979). Additional physiological factors pertinent to the uptake of fatty acids by the lactating mammary gland, including energy balance and genetic regulation of milk fat synthesis and milk fatty acid composition, are beyond the scope of this discussion.

The transfer efficiency of individual fatty acids to milk fat is one example of the body's ability to differentially allocate and utilize specific fatty acids. Two major factors affecting transfer efficiency include the plasma lipid

distribution of fatty acids and the selective mammary uptake of fatty acids from the various plasma lipid fractions. Transfer efficiency is calculated as the incremental increase in the secretion of a fatty acid in milk fat when supplied exogenously, and is taken as a proportion of the isomer available for mammary uptake. Two experimental approaches involve comparison of transfer efficiency to milk fat when fatty acids of known concentration are infused into the abomasum or when fatty acid supply is based on estimates of rumen or abomasal outflow for individual fatty acids. A review of the available literature indicates that the transfer efficiency of *trans* 18:1 isomers ranges from 15-49% for isomers infused into the abomasum, and ranges from 24-86% when using measurements based on rumen or abomasal outflow (Table 2.1). It is also evident from these observations, that few studies have examined the transfer efficiency of individual *trans* 18:1 isomers, and for those that have, it appears the location of the double bond may impact the utilization of the *trans* 18:1 isomer, either through plasma lipid distribution or selective mammary uptake.

Conjugated Linoleic Acid and Trans Fatty Acids

When considering ruminant-derived TFA, a discussion of CLA is pertinent, partly due to the potent bioactivity associated with CLA isomers but more so because by definition, many CLA isomers are also *trans* isomers. RA is the major CLA isomer produced in the rumen, and accounts for approximately 73-91% of the total CLA in milk fat (Lock and Bauman, 2004). It was previously thought that the content of RA in milk fat was directly proportional to the amount taken up by the mammary gland, but Griinari et al.

Table 2.1. Summary of research examining the transfer efficiency of *trans* 18:1 isomers to milk fat

Reference	Experimental design[1]	*Trans* isomer(s)	Amount, g/d	Transfer efficiency, %
			Infused	
Gaynor et al. (1994)	AI	*trans* 18:1	308	22[3]
Romo et al. (1996)	AI	*trans* 18:1	261.7	49[3]
Griinari et al. (2000)	AI	*trans*-11 18:1	12.5	40[4]
		trans-12 18:1	12.5	64[4]
Lock et al. (2007)	AI	*trans*-10 18:1	42.6	15
Shingfield et al. (2007)	AI	*trans*-11 18:1	7.4, 14.7 or 29.4	29[4]
		trans-12 18:1	7.1, 14.3 or 28.5	34[4]
			Flow	
Wonsil et al. (1994)	RO	*trans* 18:1	126	86[3]
		trans 18:1	115	73[3]
Kalscheur et al. (1997)	DF	*trans* 18:1	287	24[3]
		trans 18:1	295	24[3]
		trans 18:1	266	28[3]
Piperova et al. (2002)	DF[2]	*trans*-9 18:1	5	49[3]
		trans-10 18:1	29	55[3]
		trans-11 18:1	34	29[3,4]
		trans-12 18:1	10	32[3]
		trans 18:1	120	39[3]

[1]AI, abomasal infusion; RO, rumen outflow, DF, duodenal flow.

[2]LFNB - low forage, no buffer treatment, compared with high forage, no buffer treatment.

[3]Calculated using data presented in publications. Calculations for transfer efficiency are based on the incremental secretion of specific fatty acid(s) in milk fat when supplied exogenously, either infused or based on flow estimates, and taken as a proportion of the isomer available for mammary uptake.

[4]Accounts for increased yield of Δ^9-desaturase products in milk fat.

(2000) demonstrated that the majority of RA in milk fat occurs as a result of the conversion of VA to RA via the mammary enzyme, Δ^9-desaturase. Furthermore, across a range of dietary treatments, ruminant-derived fats typically contain VA and RA in a 3:1 concentration ratio, indicative of a precursor-product relationship (Bauman et al., 2006). Altered fermentation patterns, such as those observed with situations of diet-induced MFD, coincide with significant increases in the milk fat content of unique biohydrogenation intermediates, including *trans*-10, *cis*-12 CLA. *Trans*-10, *cis*-12 CLA was the first CLA isomer identified as a potent inhibitor of milk fat synthesis (Baumgard et al., 2000), functioning through the SREBP1 transcription factor system to decrease mammary expression of lipogenic enzymes (Harvatine and Bauman, 2006). To date, a range of CLA isomers have been tested in the dairy cow, but only three isomers (*trans*-10, *cis*-12 CLA; *trans*-9, *cis*-11 CLA and *cis*-10, *trans*-12 CLA) have been shown to decrease milk fat synthesis (Bauman et al., 2008).

With regard to human health, the most well-studied CLA isomer is RA, known for its anticarcinogenic and antiatherogenic properties (Lock and Bauman, 2004). These effects are observed when RA is supplied as a chemical supplement, but they also occur when RA is provided as a natural food component (i.e. VA/RA-enhanced dairy foods). Investigations using dietary supplements of CLA isomer mixtures, most often RA plus *trans*-10, *cis*-12 CLA, show a range of biological effects in biomedical studies with animal models (Pariza, 2004; Poirier et al., 2005; Battacharya et al., 2006). A portion of these effects relate to reduced fat deposition and body fat content, but undesirable effects such as insulin resistance and tissue-specific fat accumulation have also been observed (Wang and Jones, 2004; Kelley and

Erickson, 2003). A recent meta-analysis of 18 clinical trials with humans found that *trans*-10, *cis*-12 CLA, taken as a mixed dietary supplement at 3.2 g/d, is effective for aiding in moderate weight loss (Whigham et al., 2007), however, safety concerns relating to the adverse biological effects of *trans*-10, *cis*-12 CLA still remain in question (Wang and Jones, 2004).

TRANS FATTY ACIDS AND IMPLICATIONS FOR HUMAN HEALTH

Industrial-derived *trans* fatty acids are associated with adverse effects on human health, including cardiovascular disease, chronic inflammation and type-II diabetes (Willett, 2006). These relationships have not been established for TFA from ruminant fat, and rather, some *trans* isomers (i.e. VA and RA) in ruminant fat have been shown to offer promising beneficial health effects in animal models of chronic disease (Parodi, 1997; Bauman et al., 2006). Furthermore, as reviewed by Lock et al. (2005a), a summary of the epidemiological data indicate a neutral or potentially beneficial effect of ruminant-derived *trans* fats on heart disease risk factors. The differential health effects attributed to these two sources have been traditionally thought to be related to differences in the *trans* 18:1 isomer content and profile; however, few studies have examined the extent to which the adverse effects relate to specific *trans* 18:1 isomers or other unique fatty acid isomers found in PHVO.

Trans Fats and Coronary Heart Disease

The etiology of many cardiovascular diseases begins with hyperlipidemia and chronic inflammation. Atherogenesis, the formation of the atherosclerotic plaque, evolves as a consequence of the accumulation and

oxidation of low-density lipoprotein (LDL) cholesterol in the arterial endothelium, and is further promoted by an inflammatory response that progressively leads to a hardening and thickening of arterial walls (Lusis, 2000). Further complications such as thrombosis and stroke may occur following rupture of the atherosclerotic plaque (Lusis, 2000). Major risk factors for CHD include hyperlipidemia, smoking, diet and stress; however, the pathology has both a genetic and lifestyle component, which renders some individuals inherently more susceptible than others (Breslow, 2000). For years, plasma concentrations of total cholesterol (TC) and LDL-cholesterol were used as biomarkers of CHD and increases were considered major risk factors. With recognition of high-density lipoproteins (HDL) and their role in reverse cholesterol transport, the ratios TC:HDL-C and nonHDL-C:HDL-C (where nonHDL-C is equal to the sum of the concentrations of cholesterol in the atherogenic lipoproteins: VLDL plus intermediate-density lipoprotein [IDL] plus LDL) have become the major biomarkers used. These biomarkers are considered very effective predictors of CHD risk (Mensink et al., 2003). Whereas the initial research on CHD demonstrated that intake of dietary cholesterol and three saturated fatty acids (12:0, 14:0, 16:0) explained 88% of the changes in total serum cholesterol (Hegsted et al., 1965), subsequent research showed the biology of CHD was much more complicated, and in fact, as of 2000, 270 risk factors have been identified for CHD (Maijala, 2000).

Adverse health effects associated with TFA have been recognized for over a decade. Data from the Nurse's Health Study were among the first to demonstrate an adverse association between intake of TFA from industrial sources and risk of CHD (Willett et al., 1993); subsequent epidemiological

and case-control studies confirmed this relationship (Aro et al., 1995; Pietinen et al., 1997; Oomen et al., 2001). Direct examination of TFA intake from PHVO revealed that consumption is associated with changes in plasma biomarkers indicative of an increased risk of CHD, including increased plasma TC, LDL-cholesterol, TG and inflammatory markers, as well as decreased plasma HDL-cholesterol (Mensink et al., 1992; Ascherio et al., 1994; Baer et al., 2004). Other studies have demonstrated a correlation between the content of TFA in a variety of tissues, including adipose tissue, platelets, erythrocyte membranes and atherosclerotic plaques, and the risk and occurrence of CHD (Hodgson et al., 1996; Pedersen et al., 2000; Lemaitre et al., 2002; Stachowska et al., 2004). On the whole, a substantial amount of evidence, accumulated over the past two decades, points to TFA as being a major risk factor for CHD and associated pathologies. In response to these data, public health organizations have proposed that consumption of TFA be avoided altogether (Food and Nutrition Board, 2005).

Examination of the epidemiological data relating to TFA intake and CHD revealed differences following consumption of ruminant-derived and industrial-derived *trans* fats. Whereas the expected increase in CHD risk was observed with increasing relative intake of total TFA, ruminant-derived TFA appeared neutral or even inversely related to CHD risk (Figure 2.6). One explanation for this relates to differences in the quantity of intake of *trans* isomers between the two sources (Weggemans et al., 2004). The most recent data for the U.S. consumption of TFA indicate an average intake of 5.3 g/d or 2.6% en for total TFA (U.S. Food and Drug Administration, 2003), with approximately 1.1 g/d of TFA intake originating from ruminant-derived sources and 4.2 g/d from industrial-derived sources, as calculated based on

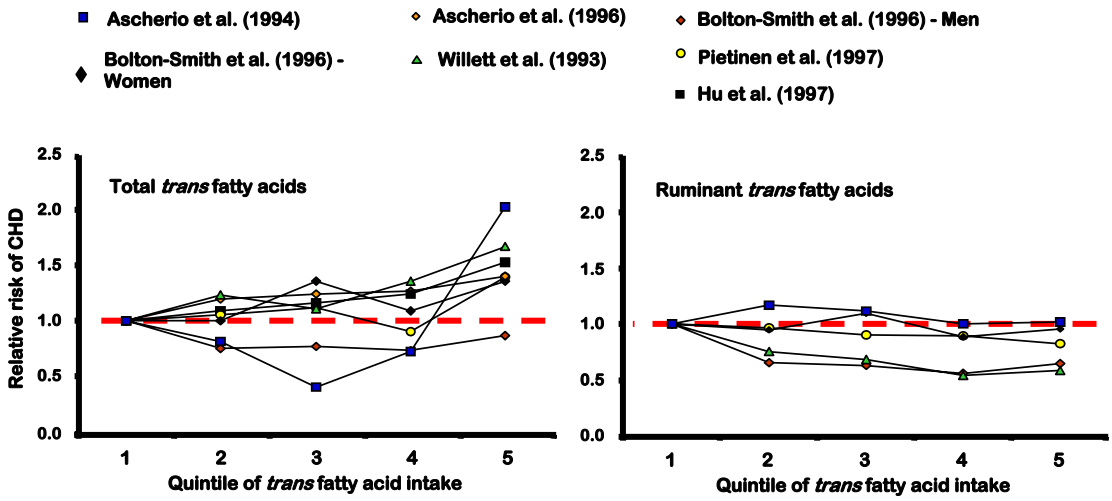


Figure 2.6. Comparison of the relative risks of CHD based on quintiles of TFA intake (% en) by source: an analysis of the epidemiological data. Adapted from Lock et al. (2005a).

patterns of TFA intake (Figure 2.4). While effects on CHD risk appear neutral at current U.S. intakes of ruminant-derived TFA, a divergence in the relative risk is apparent at greater, absolute (g/d) intakes (Figure 2.7). A second explanation for the divergent effects of TFA source on CHD risk relates to differences in the *trans* 18:1 isomer profile between the two sources. Approximately 60% of ruminant-derived *trans* fats are found as VA. The conversion of VA to RA in tissues may provide potential health benefits associated with the RA isomer, but would also reduce the total content of *trans* 18:1 isomers in the tissue (Bauman et al., 2006). PHVO, on the other hand, typically contains a Gaussian distribution of *trans* 18:1 isomers; the adverse effects of these individual isomers (excluding VA) are largely unknown. Thus, it is apparent from these two lines of evidence (total intake of TFA and TFA profile) that both factors may contribute to the divergent effects of TFA source on CHD risk. However, with its content of saturated fatty acids

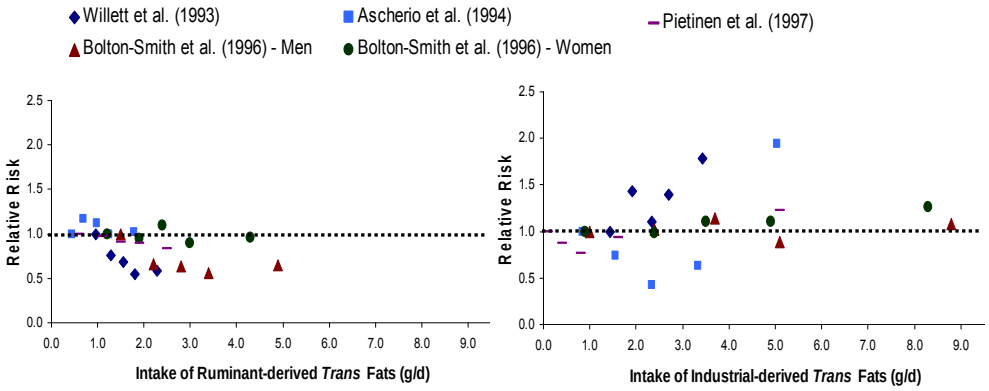


Figure 2.7. Comparison of the relative risks of CHD based on TFA intake (g/d) by source: an analysis of the epidemiological data. Calculated by author using data from individual studies as indicated. Studies selected for use in Figures 2.6 and 2.7 differ based on form that data were reported by authors (% en versus g/d intakes).

and cholesterol, the dogmas regarding the healthfulness of ruminant fat still remain a topic of debate (German and Dillard, 2006).

Of the *trans* 18:1 fatty acids in PHVO, intake of EA has traditionally been considered most synonymous with consumption of PHVO. This is most likely due to content, as EA is often a major TFA in PHVO. Furthermore, the majority of dietary EA arises from intake of PHVO as the milk fat content of EA is very low, accounting for approximately 0.3% of total milk fatty acids. Therefore, intake of EA may serve as a proxy for PHVO intake; however, interchangeable use of EA and PHVO assumes that the effects of EA would mask the effects of other isomers in PHVO, or that all *trans* fatty acids in PHVO are behaving similarly to EA. This trend for the interchangeable use of EA and PHVO started appearing in studies published in the 1990's (Kromhout et al. 1995; Sundram et al., 1997), at a time when separation of individual

trans 18:1 isomers by gas chromatography was difficult, and it is unclear whether EA, or total *trans* 18:1 isomers, were actually measured in these studies. While we are not aware of any studies examining pure EA in humans, previous investigations using biomedical models of CHD have indicated that intake of EA may not be as detrimental as once considered (Woollet et al., 1994; Meijer et al., 2001).

Other Adverse Health Effects Associated with Trans Fatty Acids

The adverse effects of industrial-derived TFA on CHD risk factors are well established, and numerous reviews have summarized the biological effects of TFA on lipoprotein metabolism (Zock et al., 1995; Willett, 2006). However, as reviewed by Willett (2006), the relative risks associated with the intake of industrial-derived TFA are greater than expected based on changes in plasma TC:HDL-C ratios, alone. This suggests that other biological effects (e.g. systemic inflammation) of TFA must be occurring. The adverse effects demonstrated for TFA on systemic inflammation also provide a basis for relating TFA to the development of type-II diabetes and insulin resistance (Willett, 2006). Furthermore, consumption of industrial-derived TFA by lactating women has been a particular concern for the pediatric community as dietary TFA are efficiently transferred in breast milk. TFA may impair the metabolism of essential fatty acids in the nursing infant, either by being converted to biologically inactive precursors for eicosanoid molecules or by directly replacing essential fatty acids (18:*n*6, 18:3*n*3 and 22:6*n*3) in breast milk (Innis, 2006).

CHAPTER THREE

Uptake and Utilization of *Trans* Octadecenoic Acids in Lactating Dairy Cows

INTRODUCTION

As consumers are becoming aware of the links between dietary fat, health maintenance and disease prevention, there is an increasing need for clarification of the metabolic fate and bioactivity of dietary fatty acids. When considering milk and dairy products, we are particularly concerned with the content of saturated and unsaturated fatty acids, bioactive fatty acids (e.g. conjugated linoleic acid and omega-3 fatty acids) and *trans* fatty acids (Lock and Bauman, 2004). The current interest in *trans* fatty acids with regard to milk fat is twofold. In relation to human health, *trans* fatty acids have been associated with increased risk of chronic diseases, including cardiovascular disease, systemic inflammation and type-II diabetes (Willett, 2006). With respect to milk fat depression (MFD), an inverse association is typically observed between the milk fat content of *trans* 18:1 acid isomers and the extent of the reduction in milk fat yield or content (Bauman and Griinari, 2003).

Factors affecting the fatty acid composition of milk fat include the supply of fatty acids, arising from dietary lipid intake and ruminal biohydrogenation of polyunsaturated fatty acids (PUFA), and also the plasma transport of fatty acids and their selective uptake and utilization by the mammary gland. Transport of fatty acids in the bloodstream is complex, and involves various plasma lipid fractions, including phospholipids (PL), cholesterol esters (CE), triglycerides (TG) and non-esterified fatty acids (NEFA). With regard to milk fat synthesis, the mammary gland preferentially

utilizes fatty acids from the plasma TG and NEFA fractions, and uptake is proportional to their concentration (Moore and Christie, 1979). In contrast, comparisons of the fatty acid composition of bovine plasma and milk lipids indicate that some fatty acids are distributed to plasma lipid fractions (e.g. PL and CE) that are less well utilized as a source of fatty acids for mammary uptake (Moore and Christie, 1979).

The metabolism of octadecenoic acids is largely impacted by the location and orientation of the double bond (Allison et al., 1995). In terms of the plasma transport of *trans* 18:1 fatty acids, Loor et al. (2002) demonstrated that as a proportion of fatty acids within a lipid fraction, vaccenic acid (VA) was greatest in plasma TG, while Mosley et al. (2006) found that across all fractions, the greatest concentration of VA was in plasma PL. These analyses have not been reported for other *trans* 18:1 isomers in the plasma of lactating dairy cows. We hypothesize that differences in the physical structure between *cis* and *trans* double bonds may affect the distribution of octadecenoic acids among plasma lipid fractions, and this would subsequently affect the uptake and use of individual isomers for mammary synthesis of milk fat. Thus, our objective was to compare two *trans* octadecenoic acids, *trans*-9 18:1 (elaidic acid; EA) and *trans*-11 18:1 (VA) with *cis*-9 18:1 (oleic acid, OA), in terms of plasma lipid transport and utilization by the mammary gland for milk fat synthesis.

MATERIALS AND METHODS

Animals and Experimental Methods

All procedures involving animals were approved by the Cornell University Institutional Animal Care and Use Committee. Three ruminally

cannulated, Holstein dairy cows, 259 ± 6 DIM (mean ± SEM) were randomly assigned in a 3 x 3 Latin square design. Prior to the start of the first treatment period, cows were fitted with abomasal infusion lines, 0.5 cm i.d. polyvinyl chloride tubing, passing through the rumen fistula and sulcus omasi (Lock et al., 2007). Lines were checked daily during treatment periods to ensure that infused isomers were properly delivered into the abomasum. Treatments were free fatty acids of 1) OA (45.5 g/d), 2) EA (41.7 g/d) and 3) VA (41.4 g/d) solubilized in 95% ethanol and heated to 37°C before infusion. Infusions were designed to provide similar amounts of the treatment isomer 6 x/d and treatment periods were 4 d, with a 7 d washout interval between treatments.

Cows consumed a corn silage based diet (Table 3.1) formulated using the Cornell Net Carbohydrate and Protein System (Fox et al., 2004) to meet or exceed nutrient requirements for energy, protein, minerals and vitamins (NRC, 2001), and had free access to water at all times. Cows were fed ad libitum with fresh feed offered twice daily at 1100 and 1800 h, and orts determined daily. Feed samples were collected twice during each treatment period, and composites were formed and analyzed by wet chemistry procedures for CP, ADF, NDF and ether extract (Dairy One Cooperative Inc. Ithaca, NY).

Synthesis and Characterization of Fatty Acid Supplements

Three octadecenoic acid supplements were used in this investigation. The OA supplement (95% purity) was purchased from Sigma-Aldrich (St. Louis, MO).

The EA and VA supplements were synthesized and purified as previously described in Lock et al. (2007). The EA product (83% purity) was

a yellow solid, melting at 42-43°C; the VA product (83% purity) was similar in appearance and melted at 45-46°C. During the synthesis and purification processes, the EA and VA products were characterized by several different procedures. Gas chromatography analyses involved a Varian 3800 (Varian, Inc., Les Ulis, France) using a CP-Select CB-FAME column (50 m x 0.25 mm i.d. with 0.25 μm film thickness; Varian, Inc., France). Samples were derivitized with trimethylsulfonium hydroxide (Macherey-Nagel, France) before injection (1 μl). The oven was kept at 185°C for 40 min then ramped to 250°C at 15°C/min and held at 250°C for 10 min. The injector and flame ionization detector temperatures were 250°C, and helium was used as the carrier gas with a flow of 1.2 ml/min. Melting point was evaluated on an electrothermal digital melting point apparatus (Bioblock, Illkirch, France). Infrared spectroscopy was performed on a Fourier transform infrared 460 Plus spectrometer (Jasco, Nantes, France) in KBr pellets (Sigma-Aldrich, France). ^{1}H and ^{13}C NMR spectra were recorded from a Bruker Advance apparatus at 500 MHz (Bruker, France), and samples were analyzed in 99% deuterated chloroform (Sigma-Aldrich, France).

Experimental Procedures and Analysis

Cows were housed in individual tie-stalls at the Large Animal Research and Teaching Unit in Ithaca, NY. Cows were milked twice daily at 0700 and 1900 h, and two aliquots of milk were collected at each milking. One aliquot was stored with preservative (bronopol tablet; D&F Control System, San Ramon, CA) at 4°C until infrared analysis was performed for fat, true protein, lactose, and somatic cells (Dairy One Cooperative Inc., Ithaca, NY) as

described by Bernal-Santos et al. (2003). A second aliquot was stored without preservative at -20°C until fatty acid analysis was performed.

Milk lipids were extracted according to Hara and Radin (1978) using hexane:isopropanol (3:2) and sodium sulfate, and transmethylated using sodium methoxide according to Christie (1982) with modifications by Chouinard et al. (1999). Fatty acid methyl esters were quantified as described by Perfield et al. (2002) involving gas chromatography (GCD system HP 6890+, Hewlett Packard, Avondale, PA) equipped with a CP-Sil 88 fused silica capillary column (100 m x 0.25 mm i.d. with 0.2 μm film thickness; Varian, Inc., Walnut Creek, CA). Fatty acid peaks were identified using pure methyl ester standards (Nu-Chek Prep, Inc. Elysian, MN) and a butter oil reference standard (CRM 164; Commission of the European Community Bureau of References, Brussels, Belgium) was used for routine quality control and internal correction factors. This same gas chromatography analysis was used for the final characterization of the octadecenoic acid supplements and these results are presented in Table 3.2.

Blood samples were collected by coccygeal venipuncture in vacutainers containing sodium heparin (100 U/ml of blood; Becton Dickinson, Franklin Lakes, NJ) at the start and end of each treatment period. Plasma was harvested by centrifuging blood at 2300 x*g* for 15 min at 4°C, and stored at -20°C until analyzed for lipids. Total plasma lipids were extracted according to Hara and Radin (1978) and methylated using 2% methanolic sulfuric acid and toluene overnight at 40°C according to Christie (1989) with modifications. Plasma lipid fractions were separated by thin layer chromatography using a hexane:diethyl ether:acetic acid developing solvent (80:20:2) according to Christie (1989) with modifications. Briefly, extracted

plasma lipids were resolubilized in chloroform, spotted on channeled, silica G plates (Analtech, Inc., Newark, DE) and allowed to develop for approximately 45 min in the solvent system. Plates were sprayed with 2',7'-dicholorfluorescein in 95% methanol and bands were identified under ultra-violet light against known standards for PL, cholesterol, NEFA, TG and CE (Nu-Chek Prep, Inc., Elysian, MN). Lipid fractions were scraped from the plates, extracted using chloroform:methanol (2:1), and methylated as previously described for total plasma lipids. Gas chromatography system for analysis of plasma fatty acids was as described for milk fatty acids and conditions were as follows: the oven temperature was initially set at 80°C, then ramped at 2°C/min to 190°C and held for 20 min, then ramped at 10°C/min to 225°C and held for 12 min. The inlet and flame ionization detector temperatures were 250°C, the split ratio was 50:1 and a 4 µl injection volume was used. The hydrogen carrier gas flow to the detector was 25 ml/min, airflow was 400 ml/min and the flow of nitrogen make-up gas was 40 ml/min. Fatty acid peaks were identified using pure methyl ester standards (Nu-Chek Prep, Inc.).

Quantification of plasma lipid fractions (total cholesterol, free cholesterol, PL, TG and NEFA) was carried out enzymatically using commercially available kits according to manufacturer's directions (Wako Chemicals USA, Inc.), and read on a Beckman DU640 spectrophotometer (Beckman Coulter, Inc. Fullerton, CA). Maximum acceptable coefficient of variation was 5.0%. Concentration of plasma CE was determined as the difference between total cholesterol and free cholesterol.

Calculations

Calculations for the proportion of fatty acids comprising individual plasma lipid fractions were based on the molecular weight and molar percent of fatty acids within the lipid fractions. These values were adjusted for the loss of hydrogen and oxygen during the esterification of fatty acids to glycerol or cholesterol, and for the formation of the amide bond in sphingomyelin. The proportion of individual PL in bovine plasma has been characterized by Christie (1981), and these values were used in calculating the average fatty acid component of plasma PL.

The method of Glasser et al. (2007) was used to calculate yields of fatty acids in the milk fat TG using milk fat yield, milk fatty acid composition, and fatty acid molecular weights. This method attributes the oxygen in the ester linkage to the esterified fatty acid. Transfer efficiency of infused isomers to milk fat was calculated on an individual cow basis and accounted for the incremental increase in the secretion of the infused isomer in milk fat. The difference between baseline secretion (pre-treatment) and the average of d 3 and d 4 secretions of infused isomers in milk fat was determined and this was expressed as a proportion of the amount of isomer infused daily. In the case of VA, the transfer efficiency calculation also accounted for the incremental increase in the secretion of *cis*-9, *trans*-11 18:2 (rumenic acid; RA) in milk fat.

Statistical Analysis

Statistical analysis was carried out using the fit model procedure of JMP (2006 SAS Institute Inc., 6.0.2) and involved the fixed effects of treatment and period, and random effect of cow. Period was removed from the model when considered insignificant at $P > 0.35$. When applicable,

baseline values were used as a covariate in the model. Matched pairs analysis was used to determine the overall effect of treatment as compared to baseline values for concentrations of individual plasma lipid fractions. Effects were declared significant at $P < 0.05$.

RESULTS AND DISCUSSION

Animal Performance

Abomasal infusion is a commonly used experimental method for providing unsaturated fatty acids to ruminant animals while bypassing alterations from rumen fermentation. In the current study, abomasal infusion provided 40-45 g/d of OA, EA and VA, in the respective treatments. The amount of infusate was selected based on isomer availability and on previously reported data from rumen outflow estimates or abomasal infusion of these fatty acids (Gaynor et al., 1994; Loor et al., 2004). The infused isomers were provided as free fatty acids, the form that rumen biohydrogenation intermediates and the majority of dietary-derived fatty acids would typically pass to the small intestine in ruminants; thus, we expected a high efficiency of absorption at the small intestine (Moore and Christie, 1979).

Dry matter intake and milk yield were similar across treatments, averaging 19.6 ± 0.3 kg/d and 24.1 ± 0.7 kg/d (mean ± SEM), respectively (Table 3.3). Yields of milk fat, milk protein and milk lactose were unaffected by treatment; there were small differences, however, in the content of protein and lactose in milk which may be related to the small number of animals, short duration and limited sampling associated with this intense experiment. Somatic cell count was unaffected by treatment, averaging 1.0 x 10^5/ml across treatments.

Situations of diet-induced MFD are often correlated with enhanced levels of *trans* 18:1 fatty acids in milk fat (Davis and Brown, 1970; Bauman and Griinari, 2003); the role of specific *trans* 18:1 isomers in the regulation of milk fat synthesis however, has not been examined extensively. In the current study, the infusion of EA and VA had no effect on milk fat yield or content. In the study by Lock et al. (2007), we similarly demonstrated that abomasal infusion of *trans*-10 18:1 at 45 g/d, did not affect milk fat synthesis. Other investigations utilizing *trans* 18:1 isomers have also observed no effects on milk fat production for EA (25 g/d; Rindsig and Schultz, 1974) or VA and *trans*-12 18:1 in a 50:50 mixture (dose range 7.1 g/d to 29.4 g/d, Griinari et al., 2000; Shingfield et al., 2007). Furthermore, an indirect examination of *trans* 18:1 isomers, by feeding cows approximately 500 g/d of OA resulted in a 1.7-fold increase in the milk fat content of total *trans* isomers (profile not specified), but had no effect on milk fat yield or content (Selner and Schultz, 1980). Overall, these studies provide no evidence for a causative role for specific *trans* 18:1 fatty acids in diet-induced MFD. Nevertheless, the content of *trans* 18:1 isomers in milk fat may be a useful surrogate marker for changes in rumen fermentation associated with MFD.

Plasma Lipid Fractions and Treatment Effects

Baseline (pretreatment) concentrations of the individual plasma lipid fractions and the fatty acid content of these plasma fractions are presented in Table 3.4. These values are in agreement with those typically seen for mature cattle in late lactation (Christie, 1981). The proportion of total plasma fatty acids associated with each lipid fraction at baseline were 62.6 ± 0.6%,

26.1 ± 0.6%, 9.8 ± 0.4% and 1.5 ± 0.1% for PL, CE, TG and NEFA, respectively.

The fatty acid composition of the individual plasma lipid fractions at baseline was strikingly different. For plasma PL, the fatty acids of greatest content were linoleic acid (*cis*-9, *cis*-12 18:2; 23.3%) and stearic acid (18:0, 22.8%), followed by palmitic acid (16:0, 12.3%) and OA (9.9%). For the plasma CE fraction, linoleic acid was the predominant fatty acid, accounting for 61.8% of total fatty acids, followed by linolenic acid (18:3*n*3, all *cis*; 10.2%). Stearic acid was the major fatty acid in plasma TG, accounting for 39.3% of the fatty acids, followed by palmitic acid (20.5%) and OA (11.6%). The fatty acid composition of the NEFA fraction was similar to plasma TG, with the major fatty acid components being stearic acid (44.4%), palmitic acid (16.5%) and OA (11.6%).

Quantitatively, across all fractions, the distribution of individual fatty acid isomers at baseline varied with chain length and degree of unsaturation. Palmitic acid accounted for 8.8 ± 0.2% of total plasma fatty acids, and its plasma distribution was 67.4% PL, 9.9% CE, 19.3% TG and 3.4% NEFA (data not shown). The distribution of 18-carbon fatty acids is presented in Figure 3.1. Stearic acid accounted for 13.4 ± 0.4% of total plasma fatty acids at baseline, and its distribution was 77.9% PL, 1.2% CE, 18.8% TG and 2.1% NEFA. OA accounted for 6.4 ± 0.3% of total plasma fatty acids and was distributed 77.3% to PL, 11.5% to CE, 9.1% to TG and 2.1% to NEFA. Linoleic acid accounted for 41.7 ± 0.8% of total plasma fatty acids, and its distribution among plasma lipid fractions was 46.8%, 52.0%, 0.9% and 0.2% for PL, CE, TG and NEFA, respectively. Linolenic acid accounted for 6.0 ± 0.1% of total plasma fatty acids, and its plasma distribution was 31.7% PL,

66.3% CE, 1.7% TG and 0.3% NEFA. In contrast, the long chain PUFA, eicosapentaenoic acid (20:5*n*3, all *cis*) and docosahexaenoic acid (22:6*n*3, all *cis*) together with their intermediate metabolite, docosapentaenoic acid (22:5*n*3, all *cis*) accounted for approximately 1.0% of total plasma fatty acids; over 95% of these isomers were distributed in the plasma PL and CE fractions (data not shown). Thus, it is apparent that with increasing number of *cis* double bonds there are major shifts in the pattern of distribution such that more of the fatty acid is distributed to the plasma CE fraction, with concomitant decreases in plasma PL and TG (Figure 3.1).

On d 4 of the treatment periods, the proportion of circulating fatty acids in the different plasma fractions was similar to baseline values, averaging 59.7 ± 0.9%, 28.0 ± 0.7%, 11.2 ± 0.4%, and 1.2 ± 0.1% for PL, CE, TG and NEFA, respectively. Infusion of the *trans*-18:1 isomers resulted in increases in the total lipid concentration for plasma CE, TG, and free cholesterol, as compared to values observed during the OA infusion (Table 3.5).

The major effect of treatment on fatty acid composition was to cause an increase in the content of the infused fatty acid in the various plasma lipid fractions. Plasma PL and TG fractions were most affected by treatment (Tables 3.5 and 3.7), the response in NEFA was intermediate (Table 3.8) and the fatty acid composition of plasma CE was least affected (Table 3.6). For the VA infusion, the content of RA in total plasma fatty acids averaged 0.12%, and this did not differ from baseline (0.08% of total fatty acids; $P = 0.42$; data not shown).

The total increase of the infused isomers in plasma was 6.0 ± 0.3 mg/dl, 1.9 ± 0.2 mg/dl and 2.3 ± 0.5 mg/dl for the infusion of OA, EA and VA, respectively. Infused isomers were predominantly incorporated in the PL

fraction, although differences among isomers were apparent (Figure 3.2). When OA was infused, 75% of the increased plasma content of OA occurred in the PL fraction, 10% in CE and 15% in TG. Infusion of EA resulted in a plasma increase of this fatty acid that was distributed 80% to PL and about 15% to the TG fraction. OA was the only infused isomer that resulted in its content increasing in the CE fraction, and only minimal changes were observed for the NEFA fraction for any of the infused fatty acids. The preferential incorporation of infused EA in plasma PL has been demonstrated previously, and may come as a consequence of the modest incorporation of EA in plasma CE (Coots, 1964). Furthermore, we observed that infusion of EA and VA had little effect on the content of the infused fatty acid in the plasma CE fraction; this contrasts with Offer et al. (2001) who reported increases of *trans* 18:1 isomers in plasma CE, but not plasma TG, in dairy cows after consuming 300 g/d of fish oil.

The increase in the plasma content of VA when it was infused was about equally distributed between PL and TG. This was unexpected because increases during infusion of OA and EA were predominantly associated with plasma PL. A preferential distribution of VA to plasma TG has been previously reported for lactating dairy cows (Loor et al., 2002) and with humans (Innis and King, 1999; Shahin et al., 2006). On the other hand, Mosley et al. (2006) found that across all fractions, VA was in greatest amount in the plasma PL of lactating dairy cows. Differences among studies most likely relate to differences in the absolute concentration of PL and TG in bovine plasma.

The unique distribution of fatty acids among plasma lipid fractions is one example of the body's ability to differentially recognize and allocate fatty

acids for specific metabolic purposes. For example, in the current study 59% of total linoleic acid and 74% of total linolenic acid were distributed to the plasma CE fraction. These fatty acids have been shown to be good substrates for lecithin-cholesterol acyltransferase (LCAT), the enzyme involved in plasma CE formation (Jonas, 1986). On the other hand, stearic acid and *trans* 18:1 fatty acids are less preferred substrates for LCAT and this may explain the modest incorporation of the infused EA and VA, as well as stearic acid, in the plasma CE fraction (Table 6).

Milk Fatty Acid Composition and Treatment Effects

Treatments resulted in increases in the milk fatty acid content of the specific isomers being infused (Table 3.9). The infused fatty acid progressively increased during the treatment period, and incorporation into milk fat reached an apparent plateau by d 3 (data not shown). Milk fatty acid content of EA increased from 0.33% of milk fatty acids prior to infusion to 3.21% on d 3-4 of the EA infusion. Milk fat concentration of OA averaged 22.74% of milk fatty acids prior to treatment and increased to 25.27% by d 3-4 of the OA infusion. For the VA infusion, there was a 1.5 fold increase in the concentrations of both VA and RA in milk fat, and the ratio of VA to RA remained unaffected and averaged 2.8 ± 0.1 over the 4 d infusion period. This increase of RA in milk fat is consistent with the endogenous conversion of VA to RA via the mammary enzyme Δ^9-desaturase (Bauman and Lock, 2006).

After correcting for endogenous levels in milk fat, the transfer efficiency of infused OA and EA to milk fat averaged 65.5 ± 3.0% and 59.7 ± 1.5%, respectively. Transfer of infused VA to milk fat averaged 54.3 ± 0.6%, of

which, 24.6 ± 1.1% was accounted for by the increased yield of RA in milk fat. A similar high rate of transfer is often seen for fatty acids that are well taken up by the mammary gland, including stearic, oleic and palmitic acids (LaCount et al., 1994; Wu et al., 1993). However, as the chain length and number of double bonds increases, transfer efficiency to milk fat decreases. As reviewed by Lock and Bauman (2004), the transfer of abomasally infused eicosapentaenoic acid and docosahexaenoic acid to milk fat is much lower, averaging approximately 20-30%.

Reports on the transfer efficiency of individual *trans* 18:1 isomers to milk fat is limited to only a few studies. We recently demonstrated a 15% transfer of abomasally infused *trans*-10 18:1 to milk fat, and postulated that this low transfer efficiency may be due to inefficient absorption at the small intestine or the fact that this *trans* isomer may be metabolized differently in the body (Lock et al., 2007). Griinari et al. (2000) demonstrated that 40% of abomasally infused VA and 64% of infused *trans*-12 18:1 were transferred to milk fat, with 31% and 10%, respectively, of the transfer being accounted for by increases in yield of the Δ^9-desaturation products of the infused isomers (RA and *cis*-9, *trans*-12 18:2, respectively). Shingfield et al. (2007) reported comparatively lower transfer efficiencies for VA and *trans*-12 18:1 (29% and 34%, respectively), with 8% and 2.0%, respectively, of the transfer being accounted for by the increased yield of the Δ^9-desaturation products.

Measurements on the arteriovenous difference of fatty acids across the mammary gland have been useful for investigating the extraction of fatty acids from the various plasma lipid fractions for milk fat synthesis. Glascock et al. (1966) demonstrated that plasma TG provide the main source of fatty acids taken up by the bovine mammary gland, and these results were

confirmed by Annison et al. (1967) in lactating goats. Annison et al (1967) also observed there was little net uptake of fatty acids from the NEFA pool; however, the use of labeled fatty acids enabled the authors to demonstrate significant mammary extraction of labeled NEFA with concomitant release of unlabeled NEFA. Plasma PL and CE are generally not considered major suppliers of fatty acids for mammary uptake, although some studies suggest a modest contribution of fatty acids from the plasma PL fraction for milk fat synthesis (Glascock et al., 1966; Christie et al., 1986; Nielsen and Jakobsen, 1994).

Consistent with the preferential mammary uptake of fatty acids from plasma TG and NEFA, the long chain fatty acid composition of milk fat from the cows in the current study most closely mimicked the composition of fatty acids in the plasma TG and NEFA fractions. On the other hand, plasma PL and CE contained a number of long chain PUFA, branch chain fatty acids, and alcohols not observed in milk fat (data not shown), indicating that these fatty acids may not be well taken up by the mammary gland, or that plasma PL or CE may be less well utilized as a source of fatty acids for mammary uptake. Most notably, we observed that linoleic and linolenic acids comprised approximately 47.7% of the total fatty acids in plasma, but only about 2.6% of the fatty acids in milk fat. Thus, the low transfer of these fatty acids to milk fat appears to relate to their distribution predominantly to plasma lipid fractions less well utilized for mammary uptake, rather than a consequence of low concentrations in plasma. Furthermore, we hypothesize that fatty acids predominantly distributed to plasma CE and PL may have specific biological functions (e.g. synthesis of essential fatty acids, cholesterol and lipid signaling molecules, provides cell membrane structure) that demand their preservation

within plasma, while fatty acids distributed to plasma TG and NEFA may provide a more general role in lipid metabolism (e.g. energy source, fat storage, milk fat synthesis).

In conclusion, OA, EA and VA were taken up by the mammary gland and secreted in milk fat at a high rate of transfer; treatments, however, had no effect on milk fat yield or content. Infusion of OA and EA resulted in their preferential incorporation in plasma PL, while VA was more evenly distributed between plasma PL and TG. For the VA infusion, there was an increase of RA in milk lipids, consistent with the endogenous conversion of VA to RA via the mammary enzyme Δ^9-desaturase. Distribution of 18-carbon fatty acids among the various plasma lipid fractions was particularly striking, and this along with the selective uptake of fatty acids by the mammary gland, provides evidence for the low transfer efficiency of long chain PUFA to milk fat. Thus, results demonstrate there are clear differences in the plasma transport and mammary uptake and utilization of 18-carbon fatty acids, and this relates to the location, orientation and number of double bonds.

Table 3.1. Ingredient and chemical composition of diet

Composition	Content
Dietary ingredient, % of DM	
Corn silage[1]	31.4
Alfalfa silage[2]	24.0
Straw	1.0
High moisture corn	9.6
Ground corn	10.6
Grain mix[3]	23.4
Chemical analysis, % of DM	
Neutral detergent fiber	34.2
Acid detergent fiber	22.6
Crude protein	16.8
Ether extract	4.8
NE_L,[4] MJ/kg DM	6.9

[1]Corn silage contained 30.0% DM (as fed).

[2]Alfalfa silage contained 29.6% DM (as fed).

[3]Grain mix contained 33.1% corn gluten feed, 29.4% corn distiller grains, 17.1% wheat midds, 5.3% limestone, 3.2% blood meal, 2.6% sodium bicarbonate, 1.9% tallow, 1.9% urea, 1.7% salt, 1.7% corn gluten meal, 0.8% calcium sulfate, 0.6% citrus pulp, 0.4% magnesium oxide, 0.19% selenium blend (contained 0.06% sodium selenite), 0.16% trace mineral mixture, 0.1% vitamin ADE mixture, 0.05% Mepron and 0.02% vitamin E (contained 69.1 g/kg dl-α-tocopherol). The trace mineral mixture contained (g/100g): 12.3 calcium, 12.5 sulfur, 0.32 magnesium, 1.9 copper, 9.0 magnesium, 0.25 cobalt, 0.19 iodine and 11.0 zinc. The vitamin ADE mixture contained (g/kg): 10.8 retinyl acetate, 0.18 cholecalciferol and 0.047 dl-α-tocopherol.

[4]Net energy for lactation.

Table 3.2. Fatty acid composition of infusates[1]

	Infusate[2]		
Fatty acid, g/100 g	OA	EA	VA
16:0	0.3	2.6	2.6
18:0	2.4	3.2	3.2
18:1, *trans*-9	2.9	83.4	0.0
18:1, *trans*-11	0.0	0.0	82.8
18:1, *cis*-9	91.0	8.9	8.9
18:2, *cis*-9, *cis*-12	2.6	0.0	0.0
Other	0.7	2.0	2.4

[1]Fatty acids were solubilized in 95% ethanol and equal quantities infused into the abomasum 6 x/d.

[2]OA = oleic acid, EA = elaidic acid and VA = vaccenic acid.

Table 3.3. Performance of lactating cows during infusion periods[1]

	Fatty acid infusion[2]				
Variable	OA	EA	VA	SEM	*P*[3]
DMI, kg/d	19.6	20.0	19.3	0.60	0.69
Milk yield, kg/d	24.3	24.4	23.5	1.39	0.77
Milk fat					
%	3.80	3.74	3.71	0.12	0.60
kg/d	0.90	0.90	0.90	0.04	0.80
Milk protein					
%	3.17[a]	3.06[b]	3.03[b]	0.15	0.05
kg/d	0.75	0.74	0.74	0.04	0.88
Milk lactose					
%	4.72[b]	4.72[b]	4.76[a]	0.06	0.03
kg/d	1.12	1.14	1.15	0.04	0.67

[1]Values represent least squares means ± SEM (n = 3 cows) during d 3-4 of infusion periods.

[2]Abomasal infusions were oleic acid (OA; 45.5 g/d), elaidic acid (EA; 41.7 g/d) and vaccenic acid (VA; 41.4 g/d).

[3]*P*-value represents probability of a treatment effect. Means in a row without a common letter differ, $P < 0.05$.

Table 3.4. Concentration of individual plasma lipid fractions and plasma fatty acids[1]

		Fatty acid infusion[2]					
Variable	Baseline	OA	EA	VA	SEM	*P*[3]	*P'*[4]
Plasma lipids, mg/dl							
PL	255.1	241.9	280.0	232.1	23.1	0.47	0.72
CE	147.2	157.8	171.2	155.1	7.7	0.57	0.01
TG	24.7	29.3	26.4	31.2	0.9	0.11	0.002
NEFA	3.7	3.1	3.0	2.8	0.3	0.40	0.08
FC	38.9	49.3	50.7	50.8	1.2	0.99	<0.001
Plasma fatty acids, mg/dl							
PL	151.6	166.5	143.9	137.9	13.6	0.46	0.72
CE	63.0	67.5	73.4	66.5	3.2	0.55	0.01
TG	23.6	28.0	25.3	29.9	0.8	0.11	0.002
NEFA	3.7	3.1	3.0	2.8	0.3	0.40	0.08

[1]Values represent least squares means ± SEM (n = 3 cows) at baseline (pre-treatment) and on d 4 of infusion.

[2]Abomasal infusions were oleic acid (OA; 45.5 g/d), elaidic acid (EA; 41.7 g/d) and vaccenic acid (VA; 41.4 g/d).

[3]*P*-value represents probability of an effect among treatments.
[4]*P*'-value represents probability of a non-specific treatment effect in relation to baseline values. Analyses carried out using matched pairs comparisons.

Table 3.5. Fatty acid composition of plasma phospholipid fraction on d 4 of infusion[1]

	Fatty acid infusion[2]				
Fatty acid, g/100 g	OA	EA	VA	SEM	P^3
14:0	1.04	1.00	1.03	0.10	0.69
14:1, *cis*-9	0.32	0.29	0.33	0.02	0.35
15:0	0.37	0.35	0.36	0.02	0.75
16:0	12.44	11.87	12.07	0.41	0.31
16:1, *cis*-9	0.70	0.71	0.69	0.04	0.39
17:0	1.25	1.24	1.25	0.04	0.90
18:0	23.28[a]	23.28[a]	22.87[b]	0.41	0.02
18:1, total *trans*	1.65[c]	2.63[a]	2.35[b]	0.03	<0.001
18:1, *trans*-4	0.05	0.05	0.06	0.03	0.50
18:1, *trans*-5	0.03	0.03	0.03	0.01	0.98
18:1, *trans*-6-8	0.11	0.07	0.12	0.02	0.44
18:1, *trans*-9	0.17[b]	1.22[a]	0.14[b]	0.03	<0.001
18:1, *trans*-10	0.13	0.13	0.10	0.01	0.25
18:1, *trans*-11	0.68[b]	0.64[b]	1.35[a]	0.04	<0.001
18:1, *trans*-12	0.53	0.49	0.54	0.02	0.21
18:1, *cis*-9	11.03[ab]	12.20[a]	10.37[b]	0.63	0.02
18:2, *cis*-9, *cis*-12	22.44	23.52	24.09	1.05	0.19
20:0	0.07	0.06	0.06	0.00	0.09
18:3*n*6, all *cis*	0.19	0.24	0.23	0.03	0.18
18:3*n*3, all *cis*	1.62	2.08	2.05	0.11	0.17
18:2, *cis*-9, *trans*-11	0.08	0.08	0.12	0.01	0.07
20:3*n*6, all *cis*	3.97	4.01	4.11	0.22	0.59
20:4*n*6, all *cis*	2.66	2.96	2.96	0.20	0.38
23:0	0.33	0.24	0.29	0.07	0.39
20:5*n*3, all *cis*	0.48	0.53	0.58	0.05	0.06
24:0	0.25	0.23	0.22	0.04	0.72
22:4*n*6, all *cis*	0.58	0.61	0.60	0.04	0.76
22:5*n*3, all *cis*	0.89	0.96	0.97	0.07	0.57
22:6*n*3, all *cis*	0.06	0.06	0.06	0.01	0.12
Other	12.28	12.21	12.69	0.55	0.30

[1]Values represent least squares means ± SEM (n = 3 cows) on d 4 of infusion.

[2]Abomasal infusions were oleic acid (OA; 45.5 g/d), elaidic acid (EA; 41.7 g/d) and vaccenic acid (VA; 41.4 g/d).

[3]*P*-value represents probability of a treatment effect. Means in a row without a common letter differ, $P < 0.05$.

Table 3.6. Fatty acid composition of plasma cholesterol ester fraction on d 4 of infusion[1]

	Fatty acid infusion[2]				
Fatty acid, g/100 g	OA	EA	VA	SEM	*P*[3]
14:0	0.71	0.71	0.64	0.09	0.65
14:1, *cis*-9	1.04	1.12	1.12	0.04	0.15
15:0	0.67	0.68	0.69	0.03	0.18
16:0	4.00	4.07	4.04	0.08	0.27
16:1, *cis*-9	1.06	1.15	1.12	0.07	0.19
17:0	0.22[b]	0.30[a]	0.30[a]	0.02	0.04
18:0	0.68	0.67	0.66	0.10	0.21
Trans 18:1	0.26	0.27	0.29	0.02	0.49
18:1, *trans*-4	0.00	0.00	0.00	0.00	1.00
18:1, *trans*-5	0.00	0.00	0.00	0.00	1.00
18:1, *trans*-6-8	0.01	0.00	0.02	0.00	0.08
18:1, *trans*-9	0.02	0.04	0.03	0.01	0.56
18:1, *trans*-10	0.03	0.03	0.03	0.01	0.97
18:1, *trans*-11	0.06	0.05	0.07	0.01	0.11
18:1, *trans*-12	0.14	0.14	0.15	0.02	0.65
18:1, *cis*-9	3.82	3.81	3.59	0.33	0.30
18:2, *cis*-9, *cis*-12	64.30	63.44	62.78	0.85	0.06
20:0	0.06	0.03	0.04	0.01	0.09
18:3*n*6, all *cis*	2.08	2.10	2.14	0.11	0.81
18:3*n*3, all *cis*	10.38	10.63	10.69	0.39	0.64
18:2, *cis*-9, *trans*-11	0.05	0.05	0.06	0.01	0.21
20:3*n*6, all *cis*	0.66	0.65	0.76	0.06	0.39
20:4*n*6, all *cis*	1.70	1.66	1.65	0.06	0.23
23:0	0.01	0.01	0.02	0.01	0.24
20:5*n*3, all *cis*	0.77	0.76	0.84	0.11	0.77
24:0	0.01	0.00	0.10	0.03	0.10
22:4*n*6, all *cis*	0.00	0.00	0.00	0.00	0.98
22:5*n*3, all *cis*	0.06	0.02	0.02	0.02	0.28
22:6*n*3, all *cis*	0.00	0.00	0.01	0.01	0.44
Other	7.45[b]	7.66[b]	8.43[a]	0.35	0.02

[1]Values represent least squares means ± SEM (n = 3 cows) on d 4 of infusion.

[2]Abomasal infusions were oleic acid (OA; 45.5 g/d), elaidic acid (EA; 41.7 g/d) and vaccenic acid (VA; 41.4 g/d).

[3]*P*-value represents probability of a treatment effect. Means in a row without a common letter differ, $P < 0.05$.

Table 3.7. Fatty acid distribution of plasma triglyceride fraction on d 4 of infusion[1]

	Fatty acid infusion[2]				
Fatty acid, g/100 g	OA	EA	VA	SEM	*P*[3]
14:0	1.35	1.74	1.51	0.28	0.44
14:1, *cis*-9	0.85	0.97	0.87	0.08	0.15
15:0	1.08	1.17	1.15	0.07	0.26
16:0	20.55	20.92	20.54	0.89	0.88
16:1, *cis*-9	0.79	0.88	0.88	0.09	0.40
17:0	1.10	1.11	1.12	0.07	0.95
18:0	43.23[a]	39.88[b]	39.30[b]	2.18	0.04
Trans 18:1	4.64	5.75	7.47	0.53	0.09
18:1, *trans*-4	0.07	0.05	0.07	0.02	0.43
18:1, *trans*-5	0.07	0.04	0.05	0.02	0.43
18:1, *trans*-6-8	0.55[a]	0.44[b]	0.51[a]	0.02	0.02
18:1, *trans*-9	0.39[b]	1.50[a]	0.33[b]	0.21	0.01
18:1, *trans*-10	0.54	0.64	0.53	0.03	0.24
18:1, *trans*-11	2.34[b]	2.38[b]	5.13[a]	0.48	0.03
18:1, *trans*-12	0.93	0.71	0.84	0.08	0.20
18:1, *cis*-9	8.88[a]	8.56[a]	8.01[b]	0.30	0.03
18:2, *cis*-9, *cis*-12	2.42	2.24	2.66	0.46	0.57
20:0	0.55	0.64	0.50	0.10	0.43
18:3*n*6, all *cis*	0.00	0.00	0.00	0.00	1.00
18:3*n*3, all *cis*	0.66	0.57	0.64	0.12	0.82
18:2, *cis*-9, *trans*-11	0.03	0.10	0.12	0.05	0.42
20:3*n*6, all *cis*	0.11	0.23	0.09	0.11	0.45
20:4*n*6, all *cis*	0.00	0.00	0.00	0.00	1.00
23:0	0.18	0.29	0.22	0.04	0.09
20:5*n*3, all *cis*	0.00	0.00	0.00	0.00	1.00
24:0	0.30	0.28	0.27	0.02	0.15
22:4*n*6, all *cis*	0.08	0.02	0.02	0.01	0.30
22:5*n*3, all *cis*	0.17	0.13	0.08	0.04	0.32
22:6*n*3, all *cis*	0.00	0.00	0.00	0.00	1.00
Other	13.24	14.29	14.32	1.17	0.63

[1]Values represent least squares means ± SEM (n = 3 cows) on d 4 of infusion.

[2]Abomasal infusions were oleic acid (OA; 45.5 g/d), elaidic acid (EA; 41.7 g/d) and vaccenic acid (VA; 41.4 g/d).

[3]*P*-value represents probability of a treatment effect. Means in a row without a common letter differ, $P < 0.05$.

Table 3.8. Fatty acid distribution of plasma non-esterified fatty acid fraction on d 4 of infusion[1]

	Fatty acid infusion[2]				
Fatty acid, g/100 g	OA	EA	VA	SEM	P^3
14:0	0.68	0.91	1.20	0.19	0.13
14:1, *cis*-9	0.33	0.40	0.41	0.05	0.22
15:0	0.42	0.51	0.57	0.08	0.24
16:0	15.58	16.37	17.46	1.00	0.37
16:1, *cis*-9	0.79	0.92	0.94	0.05	0.13
17:0	1.05	1.06	1.11	0.09	0.88
18:0	46.92	44.18	43.84	2.56	0.54
18:1, total *trans*	4.14	4.59	5.47	0.60	0.31
18:1, *trans*-4	0.00	0.00	0.00	0.00	1.00
18:1, *trans*-5	0.00	0.00	0.00	0.00	1.00
18:1, *trans*-6-8	0.36	0.18	0.18	0.10	0.44
18:1, *trans*-9	0.24[b]	1.39[a]	0.34[b]	0.09	0.04
18:1, *trans*-10	0.75	0.77	0.75	0.12	0.98
18:1, *trans*-11	1.98[a]	1.67[a]	3.49[b]	0.33	0.02
18:1, *trans*-12	0.72	0.58	0.72	0.22	0.79
18:1, *cis*-9	12.03	10.86	12.15	0.83	0.34
18:2, *cis*-9, *cis*-12	5.59	5.63	6.28	0.72	0.23
20:0	0.59	0.59	0.49	0.05	0.21
18:3*n*6, all *cis*	0.00	0.00	0.00	0.00	1.00
18:3*n*3, all *cis*	0.65	0.83	0.67	0.10	0.51
18:2, *cis*-9, *trans*-11	0.00	0.05	0.07	0.07	0.70
20:3*n*6, all *cis*	0.00	0.00	0.39	0.16	0.28
20:4*n*6, all *cis*	0.36	0.27	0.14	0.30	0.81
23:0	0.64	0.67	0.13	0.11	0.07
20:5*n*3, all *cis*	0.00	0.00	0.00	0.00	1.00
24:0	0.80	1.08	0.75	0.13	0.12
22:4*n*6, all *cis*	0.17	0.00	0.00	0.07	0.28
22:5*n*3, all *cis*	0.29	0.18	0.07	0.07	0.27
22:6*n*3, all *cis*	0.00	0.00	0.00	0.00	1.00
Other	10.25	8.07	8.15	1.25	0.46

[1]Values represent least squares means and SEM (n = 3 cows) on d 4 of infusion periods.

[2]Abomasal infusions were oleic acid (OA; 45.5 g/d), elaidic acid (EA; 41.7 g/d) and vaccenic acid (VA; 41.4 g/d).

[3]*P*-value represents probability of a treatment effect. Means in a row without a common letter differ, $P < 0.05$.

Table 3.9. Fatty acid composition of milk fat in response to infusion of treatment fatty acids[1]

	Fatty acid infusion[2]				
Fatty acid, g/100 g	OA	EA	VA	SEM	*P*[3]
4:0	3.96	3.96	3.91	0.19	0.31
6:0	2.11	2.07	2.13	0.04	0.47
8:0	1.12	1.09	1.16	0.04	0.42
10:0	2.31	2.20	2.45	0.12	0.25
12:0	2.60	2.51	2.79	0.15	0.25
14:0	9.85[b]	10.02[a]	10.22[a]	0.29	0.04
14:1, *cis*-9	0.90	1.07	0.95	0.21	0.15
15:0	0.85	0.84	0.90	0.04	0.17
16:0	28.39	28.81	28.78	1.77	0.93
16:1, *cis*-9	1.46	1.55	1.40	0.15	0.10
17:0	0.54[a]	0.51[b]	0.55[a]	0.01	0.05
18:0	11.63	10.83	11.60	0.39	0.38
18:1, *trans*	2.93[c]	5.77[a]	4.71[b]	0.21	0.02
18:1, *trans*-4	0.02	0.02	0.02	0.00	0.45
18:1, *trans*-5	0.02	0.01	0.02	0.00	0.06
18:1, *trans*-6-8	0.31[a]	0.22[b]	0.30[a]	0.01	0.02
18:1, *trans*-9	0.39[b]	3.21[a]	0.27[b]	0.04	<0.001
18:1, *trans*-10	0.40	0.47	0.35	0.05	0.26
18:1, *trans*-11	1.26[b]	1.32[b]	3.20[a]	0.21	0.005
18:1, *trans*-12	0.53	0.52	0.57	0.02	0.23
18:1, *cis*-9	25.27[a]	22.85[b]	21.70[b]	1.09	0.03
18:2, *cis*-9, *cis*-12	2.20	1.98	2.13	0.07	0.12
20:0	0.10	0.10	0.10	0.01	0.65
18:3*n*3, all *cis*	0.40	0.37	0.39	0.01	0.30
18:2, *cis*-9, *trans*-11	0.48[b]	0.52[b]	1.11[a]	0.06	<0.001
Other	2.90	2.96	3.02	0.05	0.25

[1]Values represent least squares means ± SEM (n = 3 cows) during d 3-4 of infusion.

[2]Abomasal infusions were oleic acid (OA; 45.5 g/d), elaidic acid (EA; 41.7 g/d) and vaccenic acid (VA; 41.4 g/d).

[3]*P*-value represents probability of a treatment effect. Means in a row without a common letter differ, $P < 0.05$.

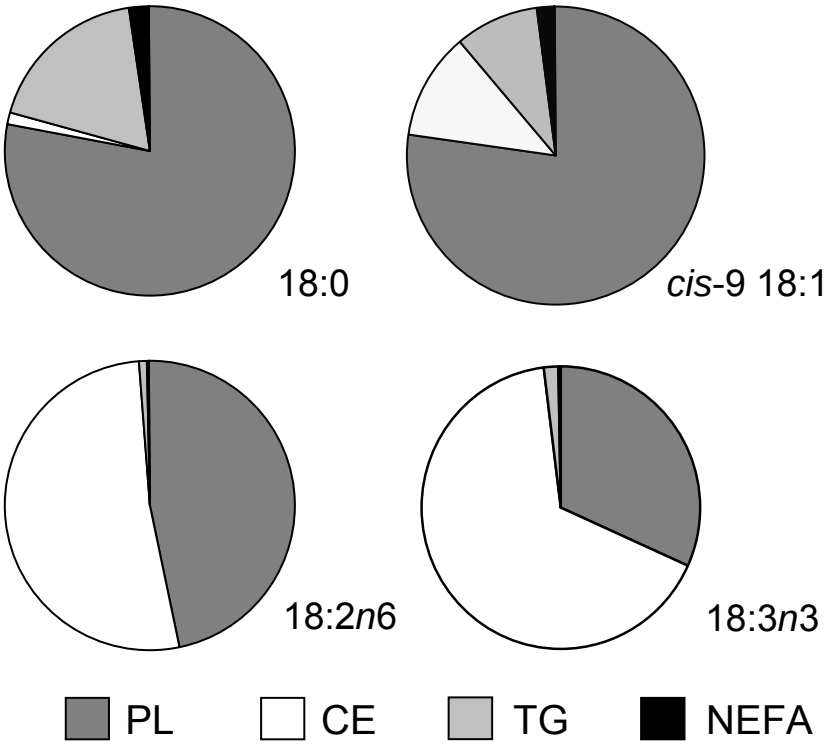


Figure 3.1. Plasma distribution of 18:0 and *cis* double bond-containing fatty acids at baseline (pre-treatment values; based on total concentration of individual isomers in plasma). Abbreviations for plasma lipid fractions are: PL = phospholipids, CE = cholesterol esters, TG = triglycerides and NEFA = non-esterified fatty acids.

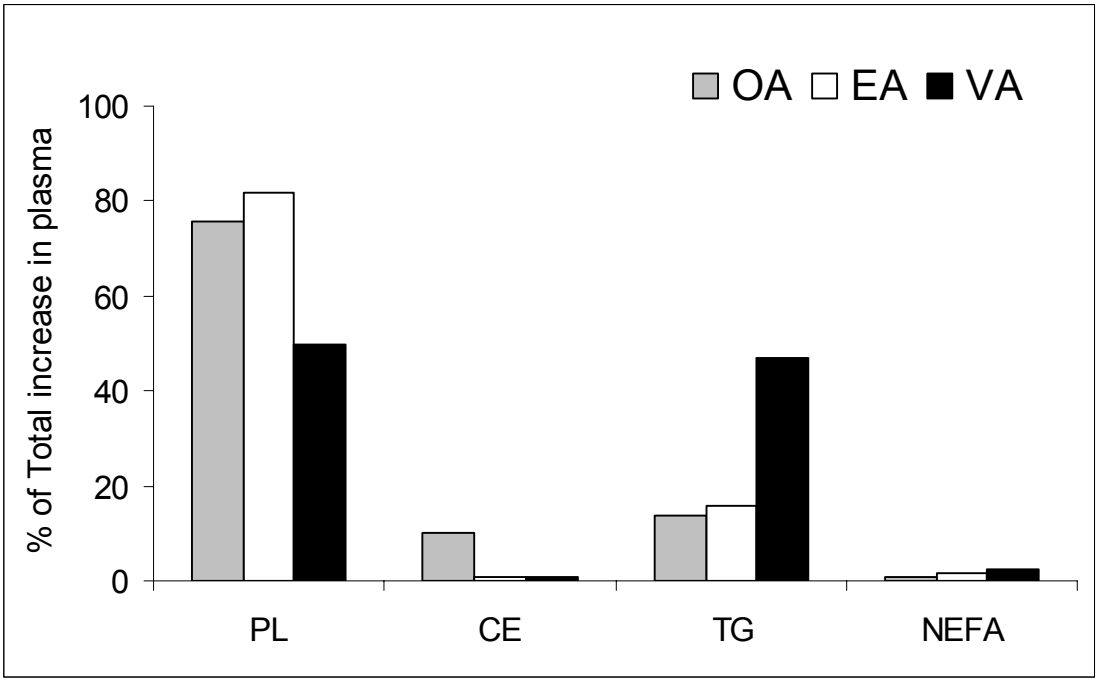


Figure 3.2. Distribution of infused isomers among plasma lipid fractions: phospholipids (PL), cholesterol esters (CE), triglycerides (TG) and non-esterified fatty acids (NEFA). The total increase of the abomasally infused fatty acids in plasma was 6.0 ± 0.3 mg/dl, 1.9 ± 0.2 mg/dl and 2.3 ± 0.5 mg/dl (mean ± SEM) for oleic acid (OA), elaidic acid (EA) and vaccenic acid (VA). Samples for the baseline analysis of the concentrations of lipid in the plasma lipid fractions and the related fatty acid profiles were obtained prior to treatments.

CHAPTER FOUR

Differential Effects of Individual *Trans* Octadecenoic Acids and Partially Hydrogenated Vegetable Oil on Hepatic Lipid and Lipoprotein Metabolism in the Golden Syrian Hamster

INTRODUCTION

There are two main dietary sources of *trans* fatty acids (TFA), an industrial source derived from the partial hydrogenation and purification of polyunsaturated oils and a natural source that originates from ruminant-derived food products. Intake of industrial-derived TFA has been associated with the progression of a number of chronic human diseases, in particular, coronary heart disease (CHD; Willett, 2006), and may also impair essential fatty acid metabolism (Hill et al., 1982; Innis, 2006). These relationships have not been established for TFA from ruminant products; rather, as reviewed by Lock et al. (2005a), the available epidemiological data suggests a neutral or potentially beneficial effect of ruminant-derived TFA on the relative risk of CHD.

The adverse human health effects typically observed with industrial-synthesized TFA have traditionally been associated with the presence of *trans* 18:1 fatty acids in partially hydrogenated vegetable oil (PHVO). *Trans* 18:1 fatty acids comprise nearly half the fatty acids in PHVO, with a Gaussian distribution of isomers predominating, including *trans*-9, -10, -11 and -12 18:1 (Stender and Dyerburg, 2003). In contrast, the TFA content of ruminant-derived sources is mainly *trans*-11 18:1 (vaccenic acid; VA); VA is the precursor for the endogenous synthesis of *cis*-9, *trans*-11 18:2 (rumenic acid; RA), a fatty acid shown to have anticarcinogenic and antiatherogenic activity

in biomedical studies with animal models (Bauman and Lock, 2006). The possible contribution of specific *trans* 18:1 isomers, or other unique fatty acids in PHVO, to overall CHD risk has not been well investigated. Evidence for differences, however, stems from epidemiological studies which suggest differential health effects between the two TFA sources (Lock et al., 2005a; Pfeuffer and Schrezenmeir, 2006). We are not aware of any study that has examined the effects of pure *trans* 18:1 fatty acid isomers on CHD risk factors in humans. Studies with biomedical models, however, have indicated a relatively neutral effect of *trans*-9 18:1 (elaidic acid; EA) compared with VA and *cis*-9 18:1 (oleic acid) on biomarkers of CHD risk using the Golden Syrian hamster (Woollett et al., 1994; Meijer et al., 2001). The effects of pure, individual *trans* 18:1 fatty acids relative to PHVO remain unknown.

The objective of the current study was to examine the effects of the two more prevalent dietary *trans* 18:1 isomers, EA, a major fatty acid component of PHVO, and VA, the predominant TFA in ruminant fat, on biomarkers of CHD risk using the cholesterol-fed Golden Syrian hamster as our biomedical model. PHVO was included as a positive-control treatment for determining if the adverse effects associated with industrial-derived TFA are dependent on the presence of EA or VA. We are not aware of any previous investigations that have directly compared these *trans* 18:1 isomers with the effects seen for PHVO.

MATERIALS AND METHODS

Animals and Diets

All procedures involving animals were subject to UK Home Office regulations. Thirty-two male Golden Syrian Hamsters (16-24 wk old; weight

134-175 g; Harlan UK, Bicester, Oxon, UK) were individually housed and maintained in a controlled environment (21°C, 55% humidity) with a 12 hr light-dark cycle. Following acclimatization, hamsters were randomly assigned to 1 of 4 treatment diets: 1) Control "Western" diet, 2) PHVO supplement, 3) EA supplement and 4) VA supplement. Per 100 g of diet, treatment diets contained 84.8 g of chow (detailed diet composition is available as Appendix I; Rat and Mouse Breeding Diet No. 3, Special Diet Services, Chelmsford, Essex, UK), 15.0 g of fat and 0.2 g of crystalline cholesterol (Sigma, Poole, Dorset, UK; Table 4.1). The fat blend for the Control diet consisted of a mixture of dietary fats formulated to approximate the quantity and quality of fat consumed in a typical "Western" diet, averaging 50% tallow, 10% sunflower oil, 20% tripalmatin and 20% rapeseed oil. The experimental fat supplements (PHVO, EA and VA) were incorporated into the respective treatment diets at 2.5 g/100g of diet, in exchange for an equivalent amount of rapeseed oil from the Western fat blend. Experimental diets were fed for 4 wk, during which, hamsters were allowed free access to food and water, with fresh feed being offered and orts determined every 3 d. Hamsters were checked daily for signs of ill-health, and body weights were measured every 3 d. One hamster was removed from the study due to excessive loss of body weight. At the end of the trial, hamsters were anesthetized using an intraperitoneal injection of sodium pentabarbitone (Sagatal, 1 ml/kg body weight), and 3-4 ml blood was obtained by cardiac puncture and collected into EDTA-tubes. Livers and two adipose tissue depots, perirenal and epididymal, were removed, weighed and snap frozen in liquid nitrogen. Plasma was isolated from blood by centrifugation at 2,500 x*g* and 4°C for 20 min.

Source and Purification of Fatty Acid Supplements

The PHVO supplement was purchased from Cargill, Inc. (Minneapolis, MN).

The EA and VA supplements (free fatty acid form) were synthesized and purified according to procedures previously described (Lock et al., 2007). The EA product (83% purity) was a yellow solid melting at 42-43°C; the VA product (84% purity) was similar in appearance and melted at 45-46°C. During the synthesis and purification processes, the EA and VA products were characterized by several different procedures. Gas chromatography analyses involved a Varian 3800 (Varian, Inc., Les Ulis, France) using a CP-Select CB-FAME column (50 m x 0.25 mm i.d. with 0.25 µm film thickness; Varian, Inc., France). Samples were derivitized with trimethylsulfonium hydroxide (Macherey-Nagel, France) before injection (1 µl). The oven was kept at 185°C for 40 min then ramped to 250°C at 15°C/min and finally held at 250°C for 10 min. The temperatures of injector and flame ionization detector were 250°C, and helium was used as carrier gas with a flow of 1.2 ml/min. Melting point was evaluated on an electrothermal digital melting point apparatus (Bioblock, Illkirch, France). Infrared spectroscopy was performed on a Fourier transform infrared 460 Plus spectrometer (Jasco, Nantes, France) in KBr pellets (potassium bromide; Sigma-Aldrich, France). ^{1}H and ^{13}C NMR spectra were recorded from a Bruker Advance apparatus at 500 MHz (Bruker, France), and the samples were analyzed in 99% deuterated chloroform (Sigma-Aldrich, France).

Separation and Analysis of Lipoproteins

Lipoprotein fractions were separated by sequential ultracentrifugation

using a Beckman Optima Ultracentrifuge XL-70 and a 50.4 rotor. Chylomicrons were separated by spinning ~1.5 ml plasma at 12,000 x*g* for 20 min under vacuum at 12°C in 1.006 g/ml potassium bromide solution. Very-low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were separated with the density ranges <1.006, 1.006-1.020, 1.020-1.060 and >1.060 g/ml, respectively by spinning at 39,000 x*g* for 20 hr at 12°C according to McAteer et al. (2003).

Hepatic cholesterol and triglyceride concentrations were quantified for total plasma and for the individual lipoprotein fractions using enzymatic kits according to manufacturer's directions with modifications for microplate analysis (Infinity kits for cholesterol and triglyceride; Thermo Scientific, Waltham, MA).

Hepatic Cholesterol and Triglyceride Analyses

Frozen livers were homogenized using a Polytron PT 3000 (Kinematica Cincinnati, OH) in sodium sulfate, and total lipids were extracted according to the procedure of Hara and Radin (1978) using hexane:isopropanol (3:2) and sodium sulfate. Lipids were resuspended in isopropanol to prevent denaturation of color reagent enzymes, and analyzed for hepatic concentrations of cholesterol and triglyceride as described for plasma.

Fatty Acid Analyses

Fatty acids from the basal diet were extracted according to Hara and Radin (1978) using hexane:isopropanol (3:2) and sodium sulfate, and

transmethylated using sodium methoxide according to Christie (1982) with modifications by Chouinard et al. (1999). Fat supplements were methylated using 2% methanolic sulfuric acid and toluene for 4 hr at 50°C according to Christie (1989). Liver lipids were extracted as described for hepatic cholesterol and triglyceride analyses, and methylated using 2% methanolic sulfuric acid and toluene overnight at 40°C. Following methylation, solid phase extraction with aminopropyl columns (Varian, Inc., Walnut Creek, CA) was performed to separate fatty acid methyl esters from free cholesterol. This procedure was based on Agren et al. (1992) with modifications (Appendix II), and validated by thin layer chromatography.

Fatty acid methyl esters were quantified by gas chromatography (GCD system HP 6890+; Hewlett Packard, Avondale, PA) equipped with a CP-Sil 88 fused silica capillary column (100 m x 0.25 mm i.d. with 0.2 μm film thickness; Varian, Inc., Walnut Creek, CA). Conditions for analysis of the diet and fat supplements were as described by Perfield et al. (2002). Conditions for the analysis of hamster liver lipids involved the oven being initially set at 80°C, then ramped at 2°C/min to 190°C and held for 20 min, then ramped at 10°C/min to 225°C and held for 12 min. The inlet and flame ionization detector temperatures were 250°C, the split ratio was 100:1, and a 2 μl injection volume was used. The hydrogen carrier gas flow to the detector was 25 ml/min, airflow was 400 ml/min and the flow of nitrogen make-up gas was 40 ml/min. A second isotherm run was used to further separate the *trans* 18:1 region for samples in which hamsters consumed EA or VA diets. The oven was set at 160°C and held for 40 min, then ramped at 15°C/min to 225°C and held for 33 min. Fatty acid peaks were identified against pure fatty acid methyl ester standards (Nu-Chek Prep, Inc. Elysian, MN) and a butter oil

reference standard (CRM 164; Commission of the European Community Bureau of References, Brussels, Belgium) was used for routine quality control and verification of correction factors.

In the data presentation and discussion sections, the double bond orientation of long-chain polyunsaturated fatty acids (LCPUFA) will be "all *cis*," unless otherwise stated.

Isolation of Hepatic Total RNA and Determination of mRNA Abundance

Frozen livers were homogenized on ice in Trizol (Invitrogen Ltd, Paisley, UK) according to manufacturer's directions and RNA was subsequently treated with DNase I (Promega, Southampton, UK) to remove any residual DNA. RNA yield was determined by measurement at 260 nm on a UV spectrophotometer (Gene Quant Pro, Amersham, Buckinghamshire, UK), where 1 absorbance unit was equal to 40 µg RNA/ml, and protein contamination was measured at 280 nm. Integrity was assessed by running RNA on an agarose gel and staining with ethidium bromide. The gel was viewed using a UV transilluminator 2000 (Bio-Rad, Hertfordshire, UK), and integrity was indicated by the absence of a low molecular weight smear. cDNA synthesis was carried out using reagents from Promega, and standard curves were generated from dilutions of cDNA pooled from all animals included in the trial. Quantitative, real-time PCR for the measurement of relative steady-state mRNA concentrations was carried out on an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Primers and dual-labeled fluorescent probes (Taqman, Applied Biosystems) were designed based on hamsters-specific sequences as previously described (Groot et al., 1992). Standards and unknown samples were run in triplicate.

Statistical Analyses

Statistical analysis was carried out using the fit model procedure of JMP (2006 SAS Institute Inc., 6.0.2) and involved the fixed effect of treatment. For gene expression data, mRNA abundance was corrected using the geometric mean of three housekeeping genes (beta-actin, glyceraldehyde-3-phosphate dehydrogenase, and TATA-box binding protein). Effects were declared significant at $P < 0.05$, and non-significant (NS) at $P \geq 0.05$.

RESULTS

The fatty acid composition of the Control diet and fat supplements (PHVO, EA and VA) is detailed in Table 4.2. The fat blend of the Control diet was designed to approximate the fatty acid composition of a typical "Western" diet, and contained 44% saturated fatty acids, 28% *cis*-9 18:1, 16% 18:2*n*6, and 2.0% *trans* 18:1 fatty acids. The content of *trans* 18:1 fatty acids differed among fat supplements, averaging 46.2%, 83.1% and 84.5% of total fatty acids for PHVO, EA and VA, respectively, and provided an additional 1.1 g, 2.1 g and 2.1 g of *trans* 18:1 fatty acids per 100 g of diet, for the respective treatments. The EA and VA supplements (~84% purity) also contained small amounts of *cis*-9 18:1, 18:0 and 16:0. Fat supplements differed in the total content of saturated fatty acids, averaging 23.0%, 6.1% and 6.4% of fatty acids for PHVO, EA and VA, respectively. Differences in the dietary contribution of saturated fatty acids were an effect of supplying the fat supplements on an isoenergetic basis.

Dietary treatment had no effect on final body weight or feed intake, which averaged 109.7 ± 1.4 g (mean ± SEM) and 5.9 ± 0.1 g/d, respectively across all treatments (Table 4.3). Hamsters fed the Control diet had greater

epidydimal adipose and perirenal adipose tissue weights (% body weight), although for the perirenal adipose tissue, data did not reach significance ($P < 0.10$). For hamsters fed the PHVO, EA and VA diets, liver weights (% body weight) were similar, and significantly greater than liver weights of hamsters fed the Control diet. Hepatic concentrations of cholesterol and triglycerides were within the normal range typically observed for all treatments, averaging 301.2 ± 8.9 µmol/liver and 74.9 ± 2.4 µmol/liver, respectively. Treatments, however, had no effect on these variables.

Effect of Diet on Plasma Lipoproteins

Dietary treatments greatly affected the concentrations of total plasma cholesterol and cholesterol within the individual lipoprotein fractions; the effects on plasma triglycerides, however, were not significant (Table 4.4). Animals consuming the PHVO diet had higher total plasma cholesterol and higher concentrations of cholesterol in the atherogenic lipoprotein fractions, including VLDL, IDL and LDL. Compared with the PHVO treatment, consumption of the EA and VA diets resulted in significantly lower levels of cholesterol in the atherogenic lipoprotein fractions, although for VA, these differences were less pronounced.

Compared with the Control diet, the PHVO treatment increased the plasma TC:HDL-C and nonHDL-C:HDL-C ratios by 17% and 23%, respectively (Figure 4.1). In contrast, values were decreased by an average 27% and 46% for EA, and 8% and 14% for VA, respectively. For all treatments, changes in the plasma ratios were most affected by changes in the content of cholesterol in the atherogenic lipoprotein fractions, as the concentration of cholesterol in the HDL fraction was not significantly affected.

There was a trend, however, for HDL-cholesterol to be higher for hamsters fed the EA diet ($P < 0.10$).

Expression of the hepatic low-density lipoprotein receptor (LDL-R) was greatest for hamsters consuming the EA diet (Figure 4.2). Expression of hepatic LDL-R did not differ among the Control, PHVO or VA treatments. The effect of treatment on plasma LDL-cholesterol correlated with the expression of hepatic LDL-R mRNA abundance. Hamsters fed the PHVO treatment tended to have the highest LDL-cholesterol concentrations and the lowest expression of LDL-R, while hamsters consuming the EA diet had the lowest LDL-cholesterol concentrations and the greatest expression of hepatic LDL-R.

Effect of Treatment on Liver Fatty Acids

Composition of liver fatty acids reflected dietary intake, indicating that the supplemental test fats were absorbed and incorporated into tissue lipids (Table 4.5). Four fatty acids, 16:0, 18:0, *cis*-9 18:1 and 18:2*n*6, accounted for approximately 75% of total liver fatty acids. Differences existed among treatments for the total liver content of *trans* 18:1 isomers, ranging from 0.5% of fatty acids for Control animals to approximately 3.0% of fatty acids for hamsters fed the VA diet. Liver content of VA was greater than EA in the respective diets, and may in part, relate to the greater content of VA than EA in the Control diet (Table 4.2). VA treatment also increased the content of RA in liver fatty acids, averaging 0.2% of fatty acids for animals fed the Control diet and >1.0% of fatty acids for hamsters consuming the VA diet. Livers from hamsters consuming the PHVO diet contained the greatest distribution of *trans* 18:1 isomers, reflecting dietary intake. There was also a significant effect of the PHVO diet to increase the content of RA in liver fatty acids.

When compared with the other treatments, livers from hamsters fed the EA diet had significantly higher levels of 18:2*n*6 and 20:3*n*6, and lower concentrations of several LCPUFA, including 20:4*n*6, 22:4*n*6, 20:5*n*3, 22:5*n*3 and 22:6*n*3 (Table 4.5). The liver content of LCPUFA did not differ among hamsters fed the Control, PHVO or VA diets. The liver content of *cis*-9 18:1 was similar for animals fed the Control and EA diets (~22-24% fatty acids), and greater than for animals consuming the PHVO and VA treatments (~19% fatty acids). Total liver content of saturated fatty acids was slightly greater for hamsters fed PHVO, and lowest for hamsters fed the EA diet. Differences in the liver content of saturated fatty acids were small, but reflected the fact that the PHVO supplement provided more dietary saturated fatty acids than the EA or VA supplements.

DISCUSSION

Industrial-derived TFA have been associated with the progression of a number of chronic human diseases, especially CHD (Willett, 2006). The specific biological mechanisms through which individual TFA elicit their adverse health effects remain largely unknown. The current study was designed to investigate the effects of specific *trans* 18:1 fatty acid isomers, including EA, a predominant TFA of PHVO, and VA, the major TFA in ruminant fat, on biomarkers of CHD risk using the Golden Syrian hamster as a biomedical model. We utilized a PHVO treatment to serve as a positive control for determining if the adverse effects associated with industrial-derived TFA are dependent on the presence of EA or VA.

The Golden Syrian hamster has often been used as a biomedical model for investigating the effects of dietary fat on CHD risk. This is because

its lipoprotein metabolism, low rates of endogenous cholesterol synthesis and high level of cholesterol ester transfer protein activity are most similar to humans, compared with other rodent species (Spady et al., 1993; Bennett et al., 1995). Furthermore, hamsters, similar to humans, produce VLDL containing the apolipoprotein, apoB100 rather than apoB48 (Salter et al., 1998). As validation for the use of our model in the present study, the observed effects of PHVO on the plasma lipoprotein profile were as expected, and consistent with changes in plasma cholesterol typically observed when humans consume diets rich in PHVO (Mensink and Katan, 1990).

The plasma ratios, TC:HDL-C and nonHDL-C:HDL-C are considered effective predictors of CHD risk because they take into account HDL and its role in reverse-cholesterol transport (Mensink et al., 2003). Increases in the plasma ratios are associated with increased CHD risk, relating to elevated levels of cholesterol in the atherogenic lipoprotein fractions or impaired reverse-cholesterol transport activity. In the present study, PHVO increased plasma TC:HDL-C and nonHDL-C:HDL-C ratios by 15% and 20%, respectively, compared with the Control diet (Table 4.4). In contrast, consumption of the EA and VA diets produced major decreases in the plasma cholesterol ratios, with the EA treatment reducing plasma nonHDL-C:HDL-C by 50% of that for hamsters fed the Control diet. The magnitude of these observations was particularly striking, especially because the EA isomer has typically been considered an atherogenic component of PHVO (Kromhout et al., 1995; Sundram et al., 1997). On the other hand, we are not aware of any investigations with biomedical models that have reported adverse effects of pure EA on plasma lipid biomarkers of CHD risk. In studies with the Golden Syrian hamster, Meijer et al. (2001) observed little differences in the effects of

EA, VA or *cis*-9 18:1 on plasma cholesterol and liver lipids, while Woollett et al. (1994) demonstrated a relatively neutral effect of EA, compared with *cis*-9 18:1, to regulate activity of the hepatic LDL-R. In contrast to Woollett et al. (1994), we observed that the EA treatment increased hepatic LDL-R expression, relative to the Control, PHVO and VA diets (Figure 4.2). Measurements for the expression of hepatic LDL-R mRNA serve as a proxy for determining hepatic LDL uptake and clearance from plasma, with increased expression indicating a reduction in CHD risk (Spady et al., 1993). Furthermore, several dietary fatty acids have been shown to regulate hepatic LDL-R mRNA in a manner that is parallel with changes in LDL-R protein abundance (Mustad et al., 1996), suggesting that regulation of hepatic LDL-R expression may be a useful tool for predicting changes in plasma LDL-C concentrations. For hamsters fed EA in the present study, the increased expression of hepatic LDL-R correlated with a decrease in plasma LDL-cholesterol, indicating improvement of the plasma lipid biomarkers and reduced CHD risk.

Effects of the EA and VA treatments on the plasma lipoprotein profile were similar, and reflected a reduced CHD risk compared with the PHVO and Control diets (Table 4.4). Effects were especially dramatic for the EA treatment. Consistent with these observations, previous investigations in hamsters have indicated that a dietary supplement of EA resulted in a small, but significant improvement (reduction) in the plasma LDL:HDL-cholesterol ratio as compared with VA (Meijer et al., 2001). These results are somewhat surprising because dietary VA has the potential to be converted to RA via Δ^9-desaturase in a number of mammalian tissues, and RA is a fatty acid known for its antiatherogenic properties (Bauman and Lock, 2006). While not

studied extensively, the use of dairy products naturally-enriched in specific *trans* 18:1 fatty acids have indicated beneficial effects of VA/RA on biomarkers of CHD risk. We previously observed that a VA/RA-enriched butter reduced plasma LDL:HDL-cholesterol by 58% of that for the Control in cholesterol-fed hamsters (Lock et al., 2005b). More recently, Bauchart et al. (2007) demonstrated beneficial effects of VA/RA-enriched butter on plasma lipid biomarkers of CHD risk in rabbits, but treatment with *trans*-10 18:1-enriched butter tended to increase plasma cholesterol and adversely affect the plasma lipoprotein profile. It cannot be excluded that the effects observed with PHVO, in the current study, may be related to the content of *trans*-10 18:1 in the PHVO supplement; we are not aware, however, of any study that has directly examined the effects of pure *trans*-10 18:1 on biomarkers of CHD risk.

Fatty acid composition of the liver lipids reflected dietary intake, and provided evidence that the fat supplements were absorbed and incorporated into tissue lipids (Table 4.5). The total liver content of *trans* 18:1 isomers differed among treatments, and differences in the *trans* 18:1 isomer profile reflected treatment differences in the fatty acid composition of the fat supplements. Although there was no RA in the VA supplement, the liver content of RA increased to >1.0% of fatty acids for animals fed the VA diet, indicating endogenous conversion of VA to RA via Δ^9-desaturase (Bauman and Lock, 2006). Liver content of RA also increased for animals fed the PHVO diet, consistent with the fact that VA comprised approximately 8% of fatty acids in the PHVO supplement. Small increases in the content of RA in tissue lipids have been previously observed for liver, as well as epididymal and perirenal fat pads following treatment with PHVO (Lock et al., 2005b).

PHVO, when incorporated at high levels in the diet, has been shown to adversely affect essential fatty acid metabolism (Hill et al., 1982) and exacerbate essential fatty acid deficiency (Hill et al., 1979). These effects are most likely related to inhibition of enzymes involved in the elongation and desaturation pathways of linoleic and linolenic acids. In a study with weanling rats, partially hydrogenated soybean oil fed at 20 g/100g of diet, inhibited hepatic Δ^5- and Δ^6-desaturase activities and resulted in a decreased liver content of LCPUFA (Hill et al., 1982). For the current study, PHVO appeared to have no adverse effect on the content of LCPUFA in liver lipids, but the dietary contribution of the PHVO supplement (2.5 g/100g of diet) may have been too low to elicit these effects.

In contrast to our observations with PHVO, the EA treatment significantly reduced the content of several LCPUFA in liver lipids, but increased the content of *cis*-9 18:1, 18:2*n*6 and 20:3*n*6 (Table 4.5). Reductions in the content of LCPUFA occurred in both the *n*-6 and *n*-3 pathways, suggesting an inhibitory effect on enzymes common to the two pathways. In support of this, EA, but not VA, has been previously shown to directly inhibit Δ^5- and Δ^6-desaturase activities in human fibroblast cells (Rosenthal and Whitehurst, 1983; Rosenthal and Doloresco, 1984). These effects have also been observed in vivo: dietary trieladin, when fed to rats at 6.75 g/100g of diet, decreased the content of *n*-6 and *n*-3 LCPUFA and increased the content of 18:2*n*6 in heart phospholipids (Astorg and Chevalier, 1987). The inhibition of Δ^5- and Δ^6-desaturase enzymes is highly undesirable as LCPUFA have numerous, beneficial effects related to health maintenance and growth and development. Furthermore, the reduction of 20:4*n*6 in liver fatty acids, and possibly its associated pro-inflammatory prostaglandins

(Calder, 2001), may also be one mechanism through which the EA treatment elicited its beneficial effects, in the current study.

It has been recognized for some time now that PHVO adversely affects biomarkers of CHD risk. The lack of evidence for dietary EA or VA to adversely affect the plasma lipoprotein profile in the present study suggested that other components present in the PHVO supplement were responsible for eliciting the hypercholesterolemic effects. Utilizing gas chromatography-mass spectrometry analyses (J. Thomas Brenna's laboratory) according to procedures of Michaud et al. (2003) and Lawrence and Brenna (2006), we further investigated the composition of the PHVO supplement to identify fatty acids from the "other" category, which comprised nearly 18% of total fatty acids. Results revealed the presence of a multitude of fatty acid isomers, including 6 different *cis* 18:1 isomers (isomer range *cis*-8 18:1 through *cis*-13 18:1; 17% of total fatty acids), 10 different *trans* 18:1 isomers (isomer range *trans*-4 18:1 through *trans*-13 18:1; 53% of total fatty acids), 8 unique *cis* and *trans* 20:1 isomers (<1% of total fatty acids), and at least a dozen different conjugated linoleic acid isomers (<1% of total fatty acids). The content and distribution of isomeric 18:1 fatty acids typically occurs as a side reaction of the partial hydrogenation process (Steinhart et al., 2003); the multitude of 20:1 isomers and range of CLA isomers in the PHVO supplement, albeit at small concentrations, was striking.

There is increasing support for the role of bioactive fatty acids to elicit dramatic biological effects when fed at very small proportions of the diet. This raises the possibility that the content of unique fatty acids found as minor components of PHVO may play a previously unrecognized role with regard to biological mechanisms and effects on CHD risk factors. Evidence for the role

of bioactive fatty acids in PHVO is based on lactation studies which demonstrate an inhibitory effect of PHVO to specifically reduce milk fat yield and content in several mammalian species (Teter et al., 1990; Bauman and Griinari, 2003). In those studies, PHVO may have been supplied in great enough quantity (2.7% - 15% DM) to allow for even the minor fatty acid components to elicit potential biological activities. With regard to CHD, however, it remains unclear whether the adverse health effects associated with PHVO relate to the presence of highly bioactive compounds found in minute quantities, or the ultimate accumulation of unique fatty acid isomers and their metabolites over time, for which biological activity is relatively unknown. Furthermore, we cannot rule out the potential effects of other *trans* 18:1 isomers which were not tested in the current study.

With regard to the content of saturated fatty acids in the PHVO supplement, data from the meta-analysis of 60 controlled trials by Mensink et al. (2003) indicate that saturated fatty acids, when replacing carbohydrates at 1.0% of the diet, increase total plasma cholesterol, LDL-cholesterol, and also HDL-cholesterol. In contrast, PHVO increases total plasma and LDL-cholesterol, but decreases HDL-cholesterol, thus, adversely affecting the plasma lipoprotein profile. It seems possible that some of the observed hypercholesterolemic effects of PHVO were due to the content of saturated fatty acids; the undesirable effects on HDL-cholesterol, however, most likely originated from other components present in PHVO.

In conclusion, dietary supplementation with EA or VA supplements (~84% purity and fed at 2.5 g/100g of diet), produced differential effects on the plasma lipoprotein profile than the PHVO treatment. When compared with the PHVO treatment, dietary EA and VA reduced total plasma cholesterol

and the content of cholesterol in the atherogenic lipoprotein fractions. These effects translated into decreased plasma TC:HDL-C and nonHDL-C:HDL-C ratios, indicative of a reduced CHD risk. Industrial-derived TFA have been implicated the progression of CHD, however few studies have examined the specific effects of individual *trans* 18:1 fatty acid components. Data from the present study are the first to demonstrate that the hypercholesterolemic effects of PHVO are not dependent on the presence of EA or VA.

Table 4.1. Composition of experimental diets[1, 2]

Diet component, g/100g of diet	Control	PHVO	EA	VA
Rodent diet[3]	84.8	84.8	84.8	84.8
Western fat blend	15.0	12.5	12.5	12.5
Tallow	7.5	7.5	7.5	7.5
Sunflower oil	1.5	1.5	1.5	1.5
Tripalmatin	3.0	3.0	3.0	3.0
Rapeseed oil	3.0	0.5	0.5	0.5
PHVO	-	2.5	-	-
EA	-	-	2.5	-
VA	-	-	-	2.5
Cholesterol	0.2	0.2	0.2	0.2

[1]Experimental diets were Control ("Western" diet), PHVO (partially hydrogenated vegetable oil supplement), EA (elaidic acid supplement) and VA (vaccenic acid supplement). Fat supplements (PHVO, EA and VA; detailed fatty acid composition provided in Table 4.2) were added to the Control diet in exchange for high-oleic rapeseed oil.

[2]Per 100 g of diet, the Control diet supplied 0.3 g *trans* 18:1 fatty acids. The additional amount of *trans* 18:1 fatty acids supplied by the fat supplements was 1.1 g/100g of diet for PHVO, 2.1 g/100g of diet for EA and 2.1 g/100g of diet for VA.

[3]Detailed composition of Rat and Mouse No. 3 Breeding Diet (Special Diet Supplies, Chelmsford, Essex, UK) is provided in Appendix I.

Table 4.2. Fatty acid composition of Control diet and fat supplements[1]

		Fat Supplement		
Fatty acid, g/100g	Control Diet	PHVO	EA	VA
12:0	0.05	0.08	-	-
14:0	1.46	0.25	0.02	0.14
14:1, *cis*-9	0.19	-	-	-
15:0	0.29	0.02	-	-
16:0	29.77	13.48	2.58	2.67
16:1, *cis*-9	1.04	0.02	-	-
17:0	0.69	0.09	0.06	0.06
18:0	11.60	8.76	3.17	3.23
18:1, total *trans*	2.03	46.15	83.07	84.46
18:1, *trans*-4	-	0.13	-	-
18:1, *trans*-5	-	0.46	-	-
18:1, *trans*-6-8	0.14	12.02	-	-
18:1, *trans*-9	0.12	7.43	82.99	-
18:1, *trans*-10	0.49	8.98	-	-
18:1, *trans*-11	1.14	7.71	-	84.39
18:1, *trans*-12	0.14	9.42	-	-
18:1, *cis*-9	28.55	11.36	8.68	9.12
18:2, *cis*-9, *cis*-12	15.49	0.78	-	-
20:0	0.38	0.38	0.26	0.26
18:3*n*3, all *cis*	2.11	0.04	0.16	0.03
18:2, *cis*-9, *trans*-11	0.11	-	-	-
Other	6.20	18.69	2.00	0.03

[1]Per 100 g of diet, experimental diets contained 84.8 g chow, 15.0 g of fat and 0.2 g of cholesterol. Fat supplements were PHVO (partially hydrogenated vegetable oil), EA (elaidic acid supplement) and VA (vaccenic acid supplement). Fat supplements were added to the Control diet, at 2.5 g/100g of diet, in exchange for high-oleic rapeseed oil.

Table 4.3. Effect of treatment on feed intake, body weight, perirenal and epididymal adipose tissue depots and liver variables[1]

	Treatment[2]					
Variable	Control	PHVO	EA	VA	SEM	*P*[3]
Feed intake, g/d	5.8	6.1	5.7	5.8	0.17	NS
Body weight, g	111.0	110.6	108.2	109.2	3.14	NS
Adipose tissue						
Perirenal, % BW[4]	1.7	1.4	1.5	1.4	0.10	NS
Epididymal, % BW	1.8[a]	1.5[b]	1.4[b]	1.3[b]	0.10	0.003
Liver						
Weight, g	5.1	5.5	5.3	5.4	0.16	NS
Weight, % BW	4.6[b]	5.0[a]	4.9[a]	4.9[a]	0.10	0.03
TC, µmol/liver	314.9	294.8	302.8	289.5	20.71	NS
TG, µmol/liver	74.8	74.5	75.6	74.5	5.23	NS

[1]Values represent least squares means and pooled SEM; n = 8 for Control, PHVO and EA diets; n = 7 for VA diet.

[2]Diets are detailed in Tables 4.1 and 4.2

[3]*P*-value represents probability of a treatment effect. Means in a row without common letter differ, $P < 0.05$; NS = $P \geq 0.05$.

[4]BW, body weight; TC, total cholesterol; TG, triglycerides.

Table 4.4. Effect of treatment on plasma cholesterol and triglyceride concentrations[1]

	Treatment[2]					
Plasma variable[3]	Control	PHVO	EA	VA	SEM	*P*[5]
Cholesterol, mmol/L						
Total plasma	7.40^{ab}	8.02^{a}	6.16^{c}	6.38^{bc}	0.42	0.01
Chylomicron	1.43^{b}	1.98^{a}	1.42^{b}	1.38^{b}	0.12	0.004
VLDL	1.73^{ab}	2.00^{a}	1.21^{c}	1.36^{bc}	0.09	<0.001
IDL	0.26^{ab}	0.39^{a}	0.10^{c}	0.16^{bc}	0.05	0.003
LDL	0.94^{ab}	1.10^{a}	0.46^{c}	0.76^{bc}	0.09	<0.001
HDL	2.17	2.06	2.39	1.98	0.11	NS
TC:HDL-C	3.46^{b}	3.89^{a}	2.60^{c}	3.27^{bc}	0.19	<0.001
nonHDL-C:HDL-C	1.36^{b}	1.69^{a}	0.74^{c}	1.18^{b}	0.12	<0.001
Recovery, %[4]	88.58	87.18	90.59	88.70	2.50	NS
Triglyceride, mmol/L						
Total Plasma	4.88	5.31	5.76	5.15	0.57	NS
Chylomicron	2.00	2.19	2.35	2.07	0.23	NS
VLDL	2.51	2.37	2.55	2.42	0.23	NS

[1]Values represent least squares means and pooled SEM; n = 8 for PHVO and EA treatments; n = 7 for Control and VA treatments.

[2]Diets are detailed in Tables 4.1 and 4.2.

[3]VLDL, very low-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TC:HDL-C, total cholesterol:high-density lipoprotein-cholesterol; nonHDL-C:HDL-C, non-high-density lipoprotein-cholesterol:high-density lipoprotein-cholesterol, where nonHDL-C is equal to the sum of cholesterol in the VLDL + IDL + LDL lipoprotein fractions.

[4]Recovery was calculated as the sum of cholesterol in the individual lipoprotein fractions taken as a proportion of total plasma cholesterol.

[5]*P*-value represents probability of a treatment effect. Means in a row without common letter differ, $P < 0.05$; NS = $P \geq 0.05$.

Table 4.5. Composition of total liver fatty acids[1, 2]

	Treatment[3]					
Fatty acid, g/100g	Control	PHVO	EA	VA	SEM	*P*[4]
14:0	0.11	0.11	0.12	0.11	0.01	NS
14:1, *cis*-9	0.01	0.02	0.02	0.02	0.00	NS
15:0	0.12	0.11	0.09	0.11	0.01	NS
16:0	19.25^{b}	20.59^{a}	17.91^{c}	18.91^{bc}	0.38	<0.001
16:1, *cis*-9	0.94^{b}	0.83^{b}	1.25^{a}	0.86^{b}	0.05	<0.001
17:0	0.72^{a}	0.69^{a}	0.63^{b}	0.69^{a}	0.03	0.04
18:0	16.68^{ab}	17.07^{a}	16.07^{b}	16.33^{ab}	0.28	0.04
18:1, *trans*	0.50^{d}	1.95^{c}	2.61^{b}	2.88^{a}	0.05	<0.001
18:1, *trans*-4	0.00	0.00	0.00	0.00	0.00	NS
18:1, *trans*-5	0.00	0.00	0.00	0.00	0.00	NS
18:1, *trans*-6-8	0.04^{b}	0.22^{a}	0.06^{b}	0.06^{b}	0.01	<0.001
18:1, *trans*-9	0.08^{c}	0.35^{b}	2.14^{a}	0.07^{c}	0.02	<0.001
18:1, *trans*-10	0.13^{b}	0.47^{a}	0.17^{b}	0.06^{c}	0.02	<0.001
18:1, *trans*-11	0.25^{c}	0.50^{b}	0.27^{c}	2.74^{a}	0.03	<0.001
18:1, *trans*-12	0.00^{b}	0.40^{a}	0.00^{b}	0.00^{b}	0.01	<0.001
18:1, *cis*-9	21.86^{a}	18.88^{b}	23.60^{a}	18.60^{b}	0.66	<0.001
18:2, *cis*-9, cis-12	16.63^{b}	16.62^{b}	18.09^{a}	17.04^{b}	0.30	0.003
20:0	0.11	0.10	0.11	0.10	0.00	NS
18:3*n*6, all *cis*	0.04^{b}	0.03^{b}	0.06^{a}	0.04^{b}	0.00	<0.001
18:3*n*3, all *cis*	0.54^{a}	0.47^{b}	0.44^{bc}	0.42^{c}	0.01	<0.001
18:2, *cis*-9, *trans*-11	0.15^{c}	0.28^{b}	0.18^{c}	1.04^{a}	0.03	<0.001
22:0	0.07^{b}	0.07^{b}	0.10^{a}	0.05^{b}	0.01	0.001
20:3*n*6, all *cis*	0.82^{b}	0.82^{b}	1.10^{a}	0.86^{b}	0.04	<0.001
20:4*n*6, all *cis*	5.68^{a}	5.82^{a}	4.59^{b}	6.09^{a}	0.23	<0.001
23:0	0.06	0.05	0.04	0.04	0.01	NS
20:5*n*3, all *cis*	0.26^{a}	0.26^{a}	0.18^{b}	0.23^{a}	0.01	<0.001
24:0	0.07	0.07	0.07	0.05	0.01	NS
22:4*n*6, all *cis*	0.14^{b}	0.15^{a}	0.12^{c}	0.15^{ab}	0.00	<0.001
22:5*n*3, all *cis*	0.32^{b}	0.40^{a}	0.24^{c}	0.33^{b}	0.01	<0.001
22:6*n*3, all *cis*	8.28^{a}	8.75^{a}	6.62^{b}	9.07^{a}	0.30	<0.001
Other	5.82	5.90	5.80	5.57	0.17	NS
∑ SFA	37.15^{b}	38.86^{a}	35.12^{c}	36.81^{b}	0.58	<0.001
∑ EPA + DPA + DHA	8.86^{a}	9.39^{a}	7.04^{b}	9.63^{a}	0.30	<0.001
∑ ARA + ADA	5.82^{a}	5.96^{a}	4.70^{b}	6.24^{a}	0.23	<0.001

[1]Values represent least squares means and pooled SEM; n = 8 for Control, PHVO and EA diets; n = 7 for VA diet.

[2]Fatty acid abbreviations include SFA = saturated fatty acids; EPA = 20:5*n*3, DPA = 22:5*n*3, DHA = 22:6*n*3, ARA = 20:4*n*6, ADA = 22:4*n*6.

[3]Diets are detailed in Tables 4.1 and 4.2.

[4]*P*-value represents probability of a treatment effect. Means in a row without common letter differ, $P < 0.05$; NS = $P \geq 0.05$.

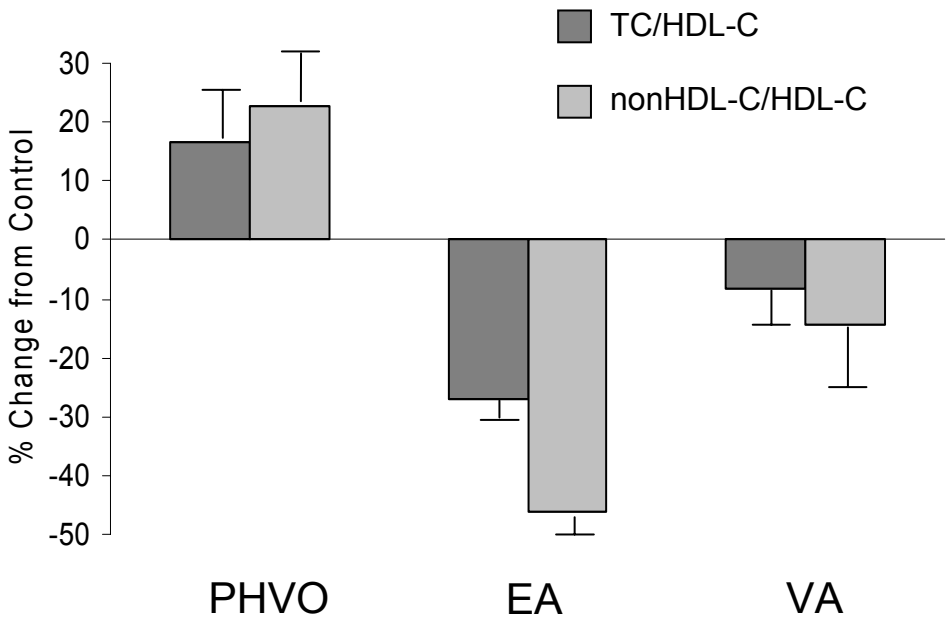


Figure 4.1. Effect of partially hydrogenated vegetable (PHVO), elaidic acid (EA; *trans*-9 18:1) and vaccenic acid (VA; *trans*-11 18:1) treatments, compared with the Control ("Western") diet, on ratios for total plasma cholesterol to high density lipoprotein-cholesterol (TC:HDL-C) and non-high density lipoprotein cholesterol to high density lipoprotein-cholesterol (nonHDL-C:HDL-C). Bars represent least squares means and SEM.

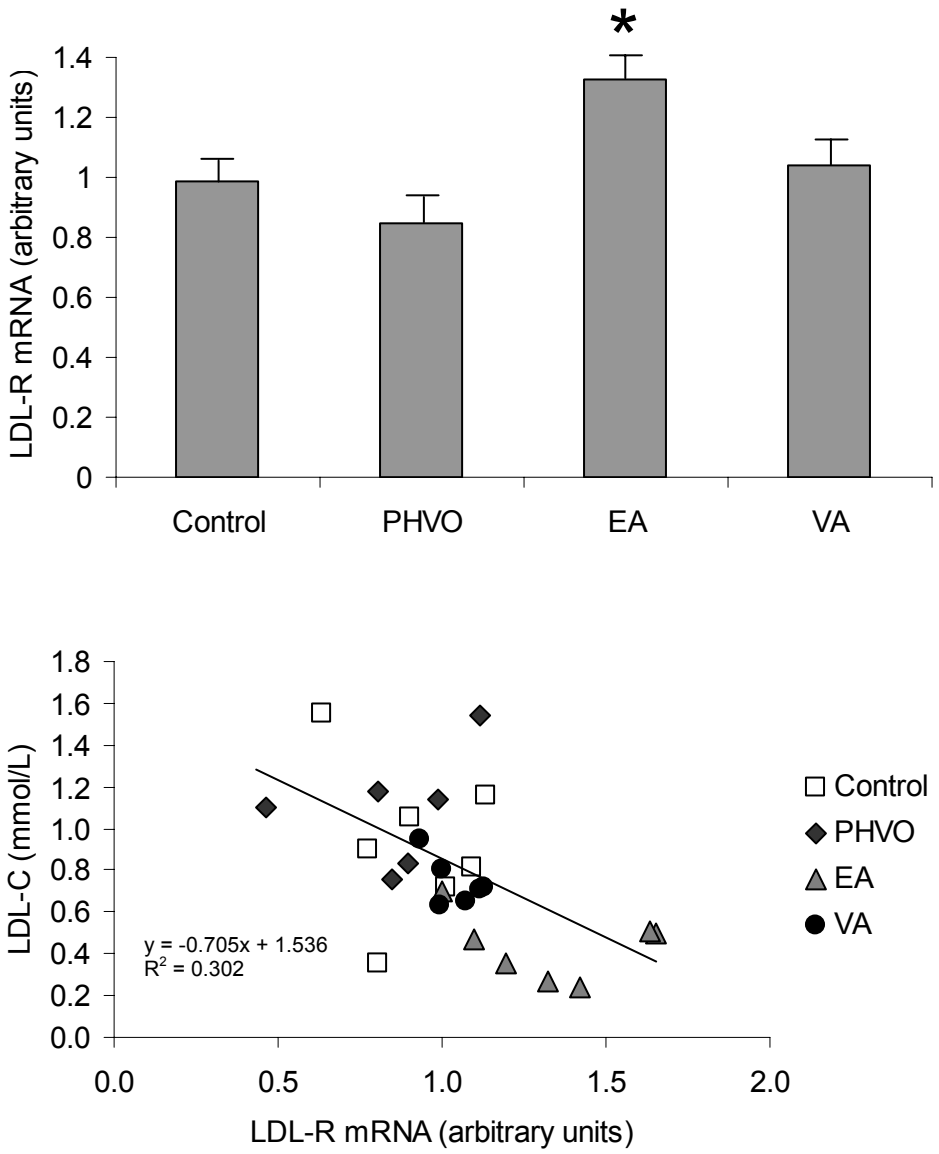


Figure 4.2. Panel A) Effect of diet on relative hepatic low-density lipoprotein receptor (LDL-R) mRNA abundance; *EA (elaidic acid supplement) diet was significantly different from Control ("Western"), PHVO (partially hydrogenated vegetable oil supplement) and VA (vaccenic acid supplement) treatments (P = 0.008). Bars represent least squares means and SEM. Panel B) Correlation between relative hepatic LDL-R mRNA abundance and concentration of plasma low-density lipoprotein cholesterol (LDL-C). Each data point represents an individual animal. Treatments are detailed in Tables 4.1 and 4.2.

CHAPTER FIVE

INTEGRATED DISCUSSION & SUMMARY

The roles of dietary fatty acids in the body are numerous and diverse: e.g. fatty acids provide energy, function as components of cell membranes and serve as precursors for the synthesis of essential fatty acids and signaling molecules. It has also been recognized that some fatty acids, collectively known as bioactive fatty acids, possess a unique ability to regulate metabolic processes and/or impact gene expression. Previous research in the field of bioactive lipids has focused largely on the ability of specific fatty acid isomers to impact chronic disease susceptibility, often relating to alterations in the expression of enzymes involved in lipogenesis and lipoprotein metabolism. Examples include dietary long-chain polyunsaturated fatty acids which are known suppress hepatic de novo lipid synthesis (Jump and Clarke, 1999) and several conjugated linoleic acid (CLA) isomers which have been recognized as potent inhibitors of milk fat synthesis (Bauman et al., 2008). While it is well established that industrial-derived *trans* fatty acids (TFA) pose numerous adverse effects on human health, the primary mechanisms through which individual isomers function remain largely unknown. Thus, the major objective of this thesis research was to investigate the bioactivity of two *trans* octadecenoic acids, *trans*-9 18:1 (elaidic acid; EA) and *trans*-11 18:1 (vaccenic acid; VA), with regard to milk fat synthesis in lactating dairy cows and effects on biomarkers of coronary heart disease (CHD) risk using the cholesterol-fed Golden Syrian hamster as biomedical model.

Trans Fatty Acids and Milk Fat Synthesis

The first study was designed to investigate the biology of EA and VA with regard to plasma lipid distribution and subsequent utilization for milk fat synthesis (Chapter 3). This investigation involved a 3 x 3 Latin square design utilizing 3 mid-lactation Holstein dairy cows, and treatments were abomasal infusion (45 g/d) of *cis*-9 18:1 (oleic acid), EA and VA. Plasma lipid analysis revealed striking differences in the fatty acid profiles among the various lipid fractions; there were also marked differences in the plasma lipid distribution of individual isomers based on chain length and degree of unsaturation. For 18-carbon fatty acids, it was apparent that with an increasing number of *cis* double bonds, more of the fatty acid would be associated with the plasma cholesterol ester (CE) fraction, while there were concomitant decreases in distribution to the phospholipid (PL) and triglyceride (TG) fractions. Likewise, several of the long chain polyunsaturated fatty acids (LCPUFA), including 20:5*n*3, 22:5*n*3 and 22:6*n*3, were predominantly associated with the plasma CE and PL fractions. This raises the interesting possibility that the body has evolved specialized mechanisms for identifying and preserving fatty acids which have specific biological functions (e.g. essential fatty acid synthesis, cell membrane structure), partitioning them largely to the plasma CE and PL fractions, while fatty acids involved in more general metabolic processes (e.g. oxidation for energy, fat storage, milk fat synthesis) would be distributed more to the plasma TG and non-esterified fatty acid (NEFA) fractions.

Plasma lipid distribution is a major factor affecting the transfer efficiency of absorbed fatty acids to milk fat. The mammary gland preferentially extracts fatty acids from the plasma TG and NEFA fractions, while plasma CE and PL appear to be less well utilized sources of fatty acids for mammary uptake

(Moore and Christie, 1979). With regard to the infused isomers, EA and OA were predominantly distributed to the plasma PL fraction, and to a lesser extent plasma TG, while VA was nearly equally distributed between the PL and TG fractions. There was a high rate of transfer for the infused isomers to milk fat, consistent with other isomers known to be well taken up by the mammary gland, and this may have been explained by their incorporation into plasma lipid fractions that were preferentially utilized by the mammary gland for milk fat synthesis (i.e. plasma TG). In contrast, 18:2*n*6 and 18:3*n*3 comprised approximately 48% of the total fatty acids in plasma, but accounted for less than 3% of the fatty acids in milk fat. These isomers were predominantly associated with the plasma CE and PL fractions, providing evidence that the low transfer of 18:2*n*6 and 18:3*n*3 to milk fat may be based on selective mammary uptake and not low concentrations in plasma. Overall, it is clear that the utilization of 18-carbon fatty acids for milk fat synthesis is markedly affected by the location, orientation and number of double bonds.

A secondary objective of the first study was to investigate the bioactivity of the infused *trans* 18:1 isomers with regard to effects on milk fat synthesis. Situations of diet-induced milk fat depression (MFD) coincide with alterations in rumen fermentation leading to the production of unique biohydrogenation intermediates. For years it was believed that the increase of *trans* 18:1 fatty acids in milk fat, derived from the altered pathways of rumen fermentation, were playing a causative role in MFD (Erdman, 1996). This theory was based on a review of the published literature by Davis and Brown (1970) who recognized an association between diet-induced MFD and an increased content of *trans* 18:1 fatty acids in milk fat, and by Griinari et al. (1998) who demonstrated that the milk fat content of specific *trans* 18:1 isomers was

increased during diet-induced MFD. With limited availability of pure *trans* octadecenoic acid isomers, however, direct examination of the effects on milk fat synthesis have been restricted.

Few studies have examined the effects of pure *trans* octadecenoic acids on milk fat synthesis. These data are summarized in Table 5.1, including those reported in this thesis, and none have demonstrated an inhibitory effect. On the other hand, studies in which partially hydrogenated vegetable oil (PHVO) was administered to dairy cows via abomasal infusion or fed to monogastrics (e.g. mice, women) demonstrated significant reductions in the yield and content of milk fat. It is apparent that PHVO does affect milk fat synthesis, however, based on the available data, the effect is most likely not dependent on the presence of the *trans* 18:1 fatty acids which have been previously examined. Further evidence that *trans* 18:1 isomers do not affect milk fat synthesis comes from a study by Selner and Schultz (1980) who fed large quantities of either PHVO or oleic acid to dairy cows. While both treatments increased the content of *trans* 18:1 fatty acids in milk fat, only the PHVO treatment caused MFD.

With regard to bioactivity, PHVO contains a range of unique fatty acids, including *cis* and *trans* 18:1 isomers, as well as smaller amounts of saturated fatty acids, 20:1 fatty acids and CLA isomers (Chapter 4; Banni et al., 1994; Phillips et al.,1997). Several biohydrogenation intermediates formed in the rumen during diet-induced MFD (e.g. *trans*-10, *cis*-12 CLA, *trans*-9, *cis*-11 CLA, *cis*-10, *trans*-12 CLA) have been identified as having an inhibitory effect on milk fat synthesis, and more recently it was recognized that they function through the SREBP1 transcription family to down regulate expression of mammary enzymes involved in lipogenesis (Harvatine and Bauman, 2006).

Trans-10, *cis*-12 CLA was the first CLA isomer identified to have potent inhibitory effects on milk fat synthesis (Baumgard et al., 2000), and based on the dose-response curve of de Veth et al. (2004), a 2.5 g/d dose of *trans*-10, *cis*-12 CLA would cause a 20-25% reduction in milk fat yield. When considering a large dose of PHVO (e.g. 500 g/d) infused into the abomasum of lactating dairy cows, any fatty acid that comprises nearly 1% of fatty acids in the PHVO supplement would be inadvertently supplied at about 5 g/d, an amount which may be more than sufficient to elicit bioactive effects.

Trans Fatty Acids and Human Health

The numerous adverse health effects associated with industrial-derived TFA have led to a widespread discontinuation of their use as table spreads and in baking and food manufacturing processes (Willett et al., 2006). The adverse biological effects are typically attributed to the presence of *trans* 18:1 fatty acids, as they are the major fatty acid fraction in PHVO. Just as with the case for the effects of PHVO on milk fat synthesis, however, direct examinations of individual *trans* 18:1 isomers and their effects on biomarkers of chronic diseases are limited. The second study of this thesis was designed to investigate the biological effects of the two more prevalent dietary *trans* 18:1 isomers, EA and VA, with regard to biomarkers of CHD disease risk using the Golden Syrian hamster as a biomedical model (Chapter 4). It was of particular interest to investigate whether the adverse health effects associated with industrial-derived TFA are dependent on the presence of EA or VA in PHVO.

The primary objective of the second study was to examine the effects of EA and VA on the plasma cholesterol and lipoprotein profile. As expected, the PHVO supplement, serving as a positive control treatment, produced changes

in the plasma lipoprotein profile characteristic of an increased CHD risk (e.g. increased plasma total cholesterol and increased cholesterol in the atherogenic lipoprotein fractions, including very low-density lipoproteins, intermediate-density lipoproteins and low-density lipoproteins, LDL). In contrast, consumption of the EA and VA treatments caused major improvements in the lipoprotein profile, primarily resulting from decreased cholesterol in the atherogenic lipoprotein fractions. The magnitude of these effects was striking, and merited further investigation to identify the underlying cause(s) for such a drastic reduction in plasma cholesterol. Using quantitative, real time PCR analysis, we examined the expression of the hepatic low-density lipoprotein-receptor (LDL-R) which is involved in hepatic LDL uptake and clearance from plasma. Animals fed the EA treatment showed significant increases in expression of hepatic LDL-R, indicating a probable mechanism to explain the reduction in plasma cholesterol. The effects for the VA treatment, with regard to plasma cholesterol and hepatic LDL-R expression were less dramatic compared with the EA treatment. These results were unexpected as VA serves as the precursor for *cis*-9, *trans*-11 CLA (rumenic acid; RA), a fatty acid known for its antiatherogenic properties (Bauman and Lock, 2006).

A secondary objective was to examine the effects of TFA supplementation (i.e. PHVO, EA or VA treatments) on liver variables and to confirm through fatty acid analysis that the supplemental fats were absorbed and incorporated into tissue lipids. While there was little effect of treatment on liver weight or the liver content of total cholesterol and triglycerides, the effect on liver fatty acids was dramatic. Previous studies have indicated that *trans* 18:1 fatty acids are well absorbed and incorporated into tissue lipids (Emken,

1985). These results were confirmed in the present study, with differences in the liver fatty acid profiles reflecting differences in the fatty acid composition of the fat supplements. For animals consuming the VA and PHVO treatments, the liver content of RA was significantly increased, providing evidence for the endogenous conversion of VA to RA via Δ^9-desaturase. These results were expected, and have been previously demonstrated in hamsters (Meijer et al., 2001; Lock et al., 2005b), as well as other species (see review by Bauman and Lock, 2006).

With regard to effects on essential fatty acid synthesis, the EA treatment resulted in notable reductions in the liver content of several LCPUFA, including 20:4*n*6, 20:5*n*3, 22:5*n*3 and 22:6*n*3. Similar results were previously demonstrated in a study with rats that were fed dietary trieladin (Astorg and Chevalier, 1987), and suggest a potential inhibition of Δ^5- and Δ^6-desaturase enzymes common to the pathways of essential fatty acid synthesis. Feeding high levels of PHVO (20% en) to rats has also been associated essential fatty acid deficiency and a suppression of desaturase activity (Hill et al., 1982). These results were not observed for the PHVO treatment in the present study, and most likely relate to lower levels of PHVO supplementation (2.5 g/100g of diet). The observed effects of the EA treatment to lower plasma cholesterol and alter essential fatty acid synthesis suggest that *trans*-9 18:1 may be a bioactive fatty acid that produces unique biological effects which are not consistent with the effects observed for PHVO.

There has been a long standing debate regarding ruminant-derived TFA and whether they pose the same adverse health effects as those observed for PHVO (Lock et al., 2004; Pfeuffer and Schrezenmeir, 2006). Evidence that the two TFA sources are not alike is based on differences

related to the *trans* 18:1 fatty acid profile and the total dietary contribution of *trans* 18:1 fatty acids by source. While the fatty acid profile of PHVO indicates a Gaussian distribution of *trans* 18:1 isomers, the TFA content of ruminant fat is largely VA (Figure 2.2). The endogenous conversion of VA to RA provides two beneficial health aspects: it reduces the total content of *trans* 18:1 fatty acids in the body and provides potential health benefits associated with the RA isomer (Lock and Bauman, 2006). Arguments for similar health effects between the two *trans* fat sources are rare; rather it is the lack of evidence for differences that is often recognized. However, as reviewed by Weggemans et al. (2004), the total dietary contribution of ruminant-derived *trans* fats may be too small to elicit the adverse effects associated with PHVO (Weggemans et al., 2004). Results from the present study (Chapter 4) demonstrate there are clear differences between VA and PHVO with regard to biomarkers of CHD risk. While we cannot assume that the biological effects attributable to VA will be entirely consistent with effects observed for ruminant fat, these data do provide evidence that VA is not an atherogenic component of PHVO.

Concluding Remarks

Perhaps well ahead of their time, Ancel Keys, D. Mark Hegsted and colleagues may have been among the first to establish a role for bioactive fatty acids relating to human heart disease. During the 1960's, these researchers recognized that some dietary saturated fatty acids (e.g. 12:0, 14:0 and 16:0) and dietary cholesterol promoted undesirable human health effects, including plasma hypercholesterolemia and hypertriglyceridemia (Keys et al., 1965; Hegsted et al., 1965). More recently, it has been suggested that over 270 known risk factors for CHD exist (Maijala, 2000), suggesting that the

relationships between CHD, genetics and environment are more complicated than previously considered. Results from the second study indicate that the adverse effects associated with the PHVO supplement are not dependent on the presence of EA or VA, and rather that other bioactive fatty acid components must have been responsible. It remains unclear, however, whether the biological effects are attributable to the presence of potent bioactive molecules found in very small amounts, whether it is the cumulative accumulation of less-bioactive fatty acids or fatty acids with unknown bioactivity over time, or some combination of the two.

All too often the biological effects of dietary fats are oversimplified and generalized without an appreciation for differences among individual fatty acid isomers. This is apparent with regard to differences in the physical structure of dietary fatty acids relative to their use in food production and with regard to differences in the biological effects and utilization of individual fatty acids in the body. Nevertheless, certain dogmas surrounding dietary fat persist; e.g. ruminant fat is bad due to its content of saturated fat and cholesterol, and PHVO is unhealthy because of its content of TFA. As we are becoming increasing aware of the roles of bioactive fatty acids to regulate metabolic processes and/or alter gene expression and through the utilization of more sophisticated analytic techniques, the biological effects of individual fatty acids, rather than dietary fats, will undoubtedly become more clear to researchers and consumers, alike.

Table 5.1: Effect of *trans* octadecenonic acid administration on milk fat yield, percent change from control[1, 2]

Reference	Isomer	Species	Dose	% Change in milk fat yield from control
Chapter 3	*trans*-9 18:1	Cow	42 g/d	-1.3
Rindsig and Schultz (1979)	*trans*-9 18:1	Cow	25 g/d	0.0
Lock et al. (2007)	*trans*-10 18:1	Cow	42 g/d	1.8
Chapter 3	*trans*-11 18:1	Cow	41 g/d	0.0
Griinari et al. (2000)	*t*11 & *t*12 (50:50 mix)	Cow	25 g/d	2.6
Shingfield et al. (2007)	*t*11 & *t*12 (50:50 mix)	Cow	7-29 g/d	-1.2
Gaynor et al. (1994)	PHVO[3]	Cow	698 g/d	-24.5
Romo et al. (1996)	PHVO	Cow	561 g/d	-13.8
Corl et al. (2001)	PHVO	Cow	250 g/d	-16.5
Teter et al. (1990)	PHVO	Mouse	5-15 %en	-17.3[4]
Anderson et al. (2005)	PHVO	Woman	38 g/d	-39.5[4]

[1]Values calculated by author based on data presented in publications.

[2]*Trans* fatty acids administered either through abomasal infusion to dairy cows or fed to monogastrics.

[3]PHVO, partially hydrogenated vegetable oil.

[4]Data calculated using change in milk fat content.

APPENDIX I

Supplemental Table 1. Composition of Rat and Mouse No. 3 Breeding Diet[1, 2]

Nutrient	Unit	Total calculated value	Supplementation[2]
Fraction			
Crude Oil	%	4.3	
Crude protein	%	22.3	
Crude Fiber	%	4.5	
Ash	%	7.7	1.9
Carbohydrate	%	51.2	
Dig. Crude Oil	%	4.0	
Dig. Crude protein	%	20.5	
Tot. Dietary Fiber	%	12.2	
Pectin	%	1.3	
Hemicellulose	%	6.5	
Cellulose	%	3.5	
Lignin	%	0.9	
Starches	%	35.8	
Sugars	%	7.7	
Energy			
Gross Energy	MJ/kg	15.3	
Dig. Energy	MJ/kg	12.8	
Met. Energy	MJ/kg	11.5	
Fatty Acids			
Myristoleic Acid	%	0.02	
Palmitoleic Acid	%	0.18	
Oleic Acid	%	1.04	
Linoleic Acid	%	1.25	
Linolenic Acid	%	0.19	
Arachidonic Acid	%	0.24	
Clupanodonic Acid	%	0.06	
Lauric Acid	%	0.05	
Myristic Acid	%	0.20	
Palmitic Acid	%	0.35	
Stearic Acid	%	0.10	
Amino Acids			
Arginine	%	1.56	
Lysine	%	1.41	0.10
Methionine	%	0.47	0.10
Cystine	%	0.36	

Tyrptophan	%	0.28	
Histidine	%	0.58	
Threonine	%	0.92	
Isoleucine	%	1.05	
Leucine	%	1.78	
Phenylalanine	%	1.07	
Valine	%	1.16	
Tyrosine	%	0.84	
Glycine	%	1.97	
Aspartic Acid	%	1.58	
Glutamic Acid	%	4.14	
Proline	%	1.42	
Serine	%	1.03	
Hydroxyproline	%	0.08	
Alanine	%	0.12	
Major Minerals			
Calcium	%	1.22	0.52
Total Phosphorus	%	0.82	0.10
Phytate Phosphorus	%	0.20	
Available Phosphorus	%	0.62	0.10
Sodium	%	0.32	0.20
Chlorine	%	0.52	0.30
Magnesium	%	0.24	0.04
Potassium	%	0.76	
Trace Minerals			
Iron	mg/kg	136.0	25.0
Copper	mg/kg	19.0	10.0
Manganese	mg/kg	93.0	60.0
Zinc	mg/kg	35.0	10.0
Cobalt	mg/kg	561.0	500.0
Iodine	mg/kg	1317.0	750.0
Selenium	mg/kg	285.0	
Fluorine	mg/kg	24.0	10.0
Vitamins			
Retinol	mg/kg	5835.0	5540.0
Vitamin A	IU/kg	19256.0	18282.0
Cholecalciferol	mg/kg	106.0	75.0
Vitamin D3	IU/kg	4250.0	3000.0
α-Tocopherol	mg/kg	107.0	90.0
Vitamin E	mg/kg	117.7	99.0
Vitamin B1	mg/kg	25.7	20.0
Vitamin B2	mg/kg	10.9	8.0

Vitamin B6	mg/kg	19.3	15.1
Vitamin B12	mg/kg	28.2	18.0
Vitamin C	mg/kg	8.0	
Vitamin K3	mg/kg	4.3	4.0
Folic Acid	mg/kg	2.7	0.5
Nicotinic Acid	mg/kg	81.5	20.0
Pantothenic Acid	mg/kg	39.0	25.2
Choline	mg/kg	1882.0	400.0
Inositol	mg/kg	2064.0	
Biotin	mg/kg	302.0	
β-Carotene	mg/kg	0.2	

[1]Data supplied by Special Diet Supplies, Chelmsford, Essex, UK.

[2]Additional supplementation by manufacturer to meet estimated dietary requirements of the animals.

APPENDIX II

Removal of Cholesterol from a Mixture of Cholesterol and Fatty Acid Methyl Esters (FAME) using Solid-phase Extraction with Aminopropyl Columns

For use following acid methylation of cholesterol esters; optimized for bovine plasma for gas chromatography analysis of FAME using DANN.M. Method adapted from Agren et al. (1992).

Reagents:

Hexane

Acetone:water: 7:1 (v/v)

Hexane:diethyl ether:acetic acid: 40:10:1 (v/v)

Hexane:chloroform:ethyl acetate: 100:5:5 (v/v)

Hexane:diethyl ether: 1:1 (v/v)

Columns:

HF Mega Bond Elut NH2, 1 g, 6 ml, 30/pk, Varian # 1425-6012 – upon opening columns, store in desiccator and use within a maximum of ~2 wk.

All solvents are eluted gravimetrically and the column should never be allowed to dry out (stop elution of solvent when last bit enters the solid phase).

1) Treat column with 0.7 ml acetone-water (7:1).
2) Wash column with 2 1-ml portions of hexane into waste tube or tray.

3) Resolubilize FAME sample in 200 µl of hexane:diethyl ether:acetic acid (40:10:1) and apply to column. Wash sample container with 100 µl of solvent and apply to column.
4) Elute FAME with 12 ml hexane:chloroform:ethyl acetate (100:5:5) into clean extraction tube.
5) Dry down FAME under N_2 at 40°C and transfer to conical GC vial.
6) Resolubilize FAME in 200 µl hexane and run on GC with DANN.M using 3 µl injection volume.

References:

Agren, J. J., A. Julkunen, and I. Penttila. 1992. Rapid separation of serum lipids for fatty acid analysis by a single aminopropyl column. J. Lipid Res. 33:1871-1876.

The Lipid Library. http://www.lipidlibrary.co.uk.

Notes and Validations:

11/29/07 – This protocol has been developed based on Agren et al. (1992) and optimized for plasma CE fraction from bovine plasma.

- Validation was carried out using TLC in a hexane:diethyl ether:acetic acid (40:10:1) developing solvent.
- Test lanes (TLC)
    1. Hexane
    2. Hexane:diethyl ether (95:5)
    3. Hexane:chloroform:ethyl acetate (100:5:5)
    4. chloroform:methanol:acetic acid (100:2:2)

- All hexane-based solvents adequately eluted FAME, with hexane:chloroform:ethyl acetate (100:5:5) enabling greatest recovery.
- Chloroform:methanol:acetic acid (100:2:2) was determined not an appropriate elution solvent, as it initially reacted with the FAME, turning the solution a light blue color, and did not recover FAME.
- Based on TLC validation, it was determined that 8 ml of elution solvent was not sufficient for recovering all FAME, and elution solvent quantity was increased to 12 ml.

12/03/07 – An alternate protocol was developed for elution of FAME from methylated hamster liver lipids, starting with FAME that were still suspended in 5 ml hexane. FAME-hexane mixture was added directly to column following priming wash, and eluted. Column was washed with an additional 6 ml of hexane:chloroform:ethyl acetate (100:5:5), and eluted into the "sample" vial.

12/04/07 – Columns are regenerated by eluting free cholesterol from the column after the elution of FAME, and have been validated for one regeneration of the column. Following elution of FAME, sample collection tubes were removed and replaced with waste tray. Columns were washed with 12 ml of hexane:diethyl ether (1:1). Prior to adding the second sample, column required priming, using 0.7 ml acetone:water (7:1) and 2-1ml portions of hexane.

- Validation was carried out using TLC in a hexane:diethyl ether:acetic acid (40:10:1) developing solvent.
- Test lanes (TLC)

1. The first sample run contained only FAME and no free cholesterol.
2. The hexane:diethyl ether (1:1) wash contained no FAME and only cholesterol.
3. The second sample run contained only FAME and no free cholesterol.
4. The priming wash preceding the second sample contained no FAME or free cholesterol.

- These tests indicate that the quantity of elution solvents were sufficient for eluting all FAME and free cholesterol in respective washes, and that the columns may be regenerated.
- Regeneration protocol adapted from http://www.lipidlibrary.co.uk based on polarity of free cholesterol.

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