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Interaction between dietary polyunsaturated fatty acids and vitamin e in body lipid composition and α-tocopherol content of broiler chickens

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BAJO LA DIRECCION DE LA Dra. María D. Baucells

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Que la memoria titulada "Interaction between dietary polyunsaturated fatty acids and vitamin E in body lipid composition and α-tocopherol content of broiler chickens" presentada por Cecilia Villaverde Haro para optar al grado de Doctor en Veterinaria, ha sido realizada bajo su dirección y, considerándola acabada, autoriza su presentación para que sea juzgada por la comisión correspondiente.

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Dra. María D. Baucells Sanchez

La realización de la presente memoria de Tesis ha sido posible gracias a la colaboración que el grupo de Nutrició Animal del Departament de Ciència Animal i dels Aliments de la Facultat de Veterinària de la Universitat Autònoma de Barcelona ha mantenido con el Avian Science Research Center de Scottish Agricultural Collage en Ayr, Escocia (UK). La presente Tesis ha sido realizada gracias a una beca de formación de personal de investigación de la Generalitat de Catalunya y gracias a la financiación de la Comisión Interministerial de Ciencia y Tecnología (CICYT).

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Finagle's Rules:

Ever since the first scientific experiment, man has been plagued by the increasing antagonism of nature. It seems only right that nature should be logical and neat, but experience has shown that this is not the case. A further series of rules has been formulated, designed to help man accept the pigheadedness of nature.

1. To study a subject best, understand it thoroughly before you start.
2. Always keep a record of data. It indicates you've been working.
3. Always draw your curves, then plot the reading.
4. In case of doubt, make it sound convincing.
5. Experiments should be reproducible. They should all fail in the same way.
6. When you don't know what you are doing, do it NEATLY.
7. Teamwork is essential; it allows you to blame someone else.
8. Always verify your witchcraft.
9. Be sure to obtain meteorological data before leaving on vacation.
10. Do not believe in miracles. Rely on them.

RESUMEN

El objetivo principal de esta memoria de tesis es el evaluar la interacción entre ácidos grasos poliinsaturados y vitamina E dietéticos sobre la deposición corporal de ácidos grasos y α-tocoferol en pollos broiler. Otro objetivo de la tesis es el comprobar la validez de la concentración hepática y plasmática de α-tocoferol como estimadores de la concentración total de α-tocoferol en pollos.

Con tal de obtener estos objetivos, se diseñaron dos experimentos. Ambos se basaron en niveles crecientes de acetato de α-tocoferol (0, 100, 200 y 400 mg/kg) y ácidos grasos poliinsaturados en los piensos. En la primera prueba, el gradiente de poliinsaturación se obtuvo al reemplazar una grasa saturada (sebo) con una mezcla de aceites insaturados (aceites de linaza y pescado), manteniendo siempre el nivel de inclusión de la grasa añadida constante (9%). En la segunda prueba, el gradiente se creó incrementando el nivel de inclusión de la grasa añadida (2, 4, 6 y 8%), utilizando una mezcla de aceites de linaza y pescado.

Se utilizaron un total de 192 broilers hembras en cada experimento. Durante el periodo experimental, se realizaron 2 balances para determinar la energía metabolizable aparente, la absorción aparente de la materia orgánica, la grasa bruta, los ácidos grasos y el α-tocoferol, y el porcentaje de hidrólisis del acetato de α-tocoferol. Al final de cada estudio (44 y 40 días de edad respectivamente), la mitad de los animales fueron sacrificados mediante inyección letal y la otra mitad se sacrificaron en un matadero comercial. Los primeros se conservaron enteros (incluyendo sangre y plumas) y los últimos se despiezaron, obteniéndose la grasa abdominal y el hígado. Se cuantificaron los ácidos grasos y el α-tocoferol en el cuerpo entero. α-tocoferol también se determine en el hígado. También se analizó la composición química y el contenido en energía bruta del pollo entero.

Composición química y contenido en energía del pollo entero

El aumento del grado de poliinsaturación dietético manteniendo el nivel de inclusión de la grasa constante (prueba 1) tuvo efecto sobre la energía metabolizable aparente de los piensos. Los animales que ingirieron el pienso más saturado tuvieron una menor absorción aparente de la materia orgánica, la grasa bruta y los ácidos grasos, disminuyendo así la energía metabolizable aparente de este pienso.

A pesar de la menor captación intestinal de la grasa y la energía del tratamiento con 9% sebo, los animales alimentados con este pienso tuvieron un mayor contenido en grasa bruta, ácidos grasos totales y energía bruta en su cuerpo. El contenido en proteína y cenizas no se vio afectado por el tratamiento. Por el contrario, incrementar la poliinsaturación dietética mediante el aumento del nivel de inclusión de grasa (prueba 2) no tuvo ningún efecto sobre la composición química ni el contenido en energía del pollo. Asimismo, el consumo de energía metabolizable tampoco fue diferente entre los tratamientos, aunque la absorción aparente de los ácidos grasos fue superior con niveles crecientes de grasa añadida. El peso de la grasa abdominal fue menor en las dietas más insaturadas comparado con las saturadas (prueba 1), pero no hubo diferencias en este parámetro según el nivel de inclusión de la grasa añadida.

Depósito de los ácidos grasos

Los ácidos grasos poliinsaturados no pueden ser sintetizados por el animal, y son depositados directamente de la dieta. El aumento de su ingestión resultó en incrementos lineales de su concentración en el cuerpo entero de los pollos en ambos experimentos. La pendiente de la ecuación de regresión entre su ingesta y su concentración en el cuerpo es menor en la segunda prueba que en la primera, sugiriendo que cuando el nivel de grasa añadida es alto y aporta ácidos grasos saturados y monoinsaturados, los poliinsaturados se depositan más directamente desde la dieta que en el caso de que el pienso no aporte altas cantidades de grasa.

Los ácidos grasos saturados y monoinsaturados en el cuerpo provienen tanto de la dieta como de la síntesis endógena. La contribución de la síntesis endógena a la concentración corporal de ácidos grasos se estimó utilizando los datos combinados de las dos pruebas. Ésta varia entre 17.7% (cuando hay un 10% de grasa en el pienso) a 35.3% (cuando el pienso no aporta ácidos grasos) en el caso de los ácidos grasos saturados; y entre un 7.0% y 52.7% en el caso de los monoinsaturados.

La inclusión de acetato de α-tocoferol hasta dosis de 400 mg/kg no tuvo efectos marcados sobre el contenido ni perfil de ácidos grasos del pollo.

Absorción aparente y depósito de α-tocoferol

Los valores de absorción aparente del α-tocoferol en broilers variaron entre 11 y alrededor de 50%, con un valor medio de 42.1% bajo nuestras condiciones experimentales. No hubo diferencias en estos valores al comparar las dos edades (20 y 39 días de edad). La inclusión de sebo al 9% en el pienso afectó negativamente a la absorción aparente del α-tocoferol y el porcentaje de hidrólisis de acetato de α-tocoferol. Por otra parte, incrementos en el nivel de inclusión de la grasa añadida rica en ácidos grasos poliinsaturados no tuvieron efecto sobre estos parámetros.

Niveles elevados de ácidos grasos poliinsaturados en la dieta no afectaron la absorción aparente de α-tocoferol, pero redujeron la concentración de α-tocoferol no esterificado en heces, sugiriéndose un gasto en el tracto gastrointestinal por proteger a los ácidos grasos poliinsaturados frente a la peroxidación lipídica.

El depósito de α-tocoferol en el hígado fue menor en los animales alimentados con los piensos más insaturados comparados con los animales alimentados con el pienso saturado (prueba 1) y a los animales alimentados con el pienso bajo en grasa, pese a la menor absorción aparente del α-tocoferol en la dieta saturada y a la falta de diferencias en este parámetro según el nivel de inclusión de grasa del pienso. Las diferencias en la concentración de α-

tocoferol sólo fueron significativas en los tratamientos con dosis elevadas de acetato de α-tocoferol (200 y 400 mg/kg).

Las ecuaciones de regresión entre la concentración de -tocoferol en el cuerpo entero frente a su concentración en hígado y plasma tuvieron correlaciones muy altas, especialmente entre el hígado y el cuerpo entero. Pese a todo, el coeficiente de variación de estas ecuaciones fue elevado, por lo que la concentración de α-tocoferol en plasma e hígado es útil para estudiar variaciones en el status de esta vitamina pero no pueden ser considerados estimadores.

SUMMARY

The main objective of this PhD dissertation is to evaluate the interaction between polyunsaturated fatty acids and vitamin E content in chicken feeds upon fatty acid and vitamin E deposition. It is also an objective of this work to assess the validity of plasmatic and hepatic α-tocopherol concentration as estimators of whole body α-tocopherol content. In order to achieve these goals, two experiments were designed.

Both experiments were based in increasing levels of α-tocopheryl acetate (0, 100, 200 and 400 mg/kg) and polyunsaturated fatty acids in the diets. In the first trial, the poyunsaturation gradient was achieved replacing a saturated fat (tallow) by a mixture of polyunsaturated oils (linseed and fish oil), keeping added fat inclusion level constant (9%). In the second trial, this gradient was created by means of increasing fat inclusion level (2, 4, 6 and 8 %), using a polyunsaturated-rich fat (a mixture of linseed and fish oil).

A total of 192 female broiler chickens were used in each experimental trial. During the experimental period, two balance studies were performed to determine apparent metabolizable energy, apparent absorption of organic matter, crude fat, fatty acids, α-tocopherol and the percentage of hydrolysis of α-tocopheryl acetate. At the ends of each study (44 and 40 days of age for the first and second trial respectively), half of the animals were killed by lethal injection and the other half was killed in a commercial slaughterhouse. The former were kept whole (including blood and feathers) and the latter were quartered to obtain the abdominal fat pad and the liver. Fatty acid quantification was measured in the whole body and α-tocopherol concentration was determined in whole body and liver. Chemical composition and gross energy content of the whole body of chickens was also determined.

Chemical composition and energy content of whole body of chickens

Increasing dietary polyunsaturation gradient keeping added fat inclusion level constant (trial 1) had effect on the apparent metabolizable energy of the diets.

The more saturated diet had lower organic matter, crude fat and total fatty acids apparent absorption, lowering the metabolizable energy content of this diet.

Despite the lower fat and energy intestinal uptake of the saturated-rich diet, the animals fed this diet had higher percentage of crude fat, total fatty acids and crude energy content in the body. Protein and ashes were not affected by treatment. On the contrary, increasing dietary polyunsaturation with increasing added fat inclusion level (trial 2) had no effect upon chemical composition and energy content of the animal. Apparent metabolizable energy intake was not different among treatments, but fatty acid apparent absorption was higher in the high-fat highly unsaturated diets. Similarly, abdominal fat pad weight was lower in the highly polyunsaturated diets compared to the saturated rich diets (trial 1), but there were no differences among treatments in the second trial.

Fatty acid deposition

Polyunsaturated fatty acids cannot be synthesized by the animal and are deposited directly from the diet. Increasing their intake resulted in a linear increase of these fatty acids in the whole body of chickens in both experiments. The slope of the regression equations is lower in trial 2 than in trial 1, suggesting that when added fat level is high, dietary PUFA are deposited more readily from the diet, whereas the efficiency of deposition is lower when added fat does not contain high amounts of saturated and monounsaturated fatty acids.

Saturated and monounsaturated fatty acids in the body come from the diet and from endogenous synthesis. The contribution of endogenous synthesis to fatty acid deposition was estimated combining the data obtained from both experiments. It increased from 17.7% (10 % dietary fat) to 35.3 % (0 % added fat) for saturated fatty acids, and from 7.01% to 52.7% for monounsaturated fatty acids.

The supplementation with α-tocopheryl acetate up to levels of 400 mg/kg had no marked effects on fatty acid content or profile of the whole chicken.

α-tocopherol apparent absorption and deposition

Apparent absorption values of α-tocopherol in broilers varied between 11 and around 50%, with an average value of 42.1% under our experimental conditions. There were no differences in the apparent absorption values of the birds when comparing the two ages (20 and 39 days). α-tocopherol apparent absorption and α-TA percentage of hydrolysis were affected negatively by tallow inclusion at a 9%. Increasing levels of PUFA-rich dietary fat did not affect this parameter.

High levels of dietary PUFA did not affect negatively α-tocopherol apparent absorption, but decreased the amount of non-esterified α-tocopherol in the excreta of birds, suggesting a gastrointestinal degradation while protecting PUFA from oxidation.

α-tocopherol concentration in the liver was lower in the animals fed the more unsaturated diets compared to the animals fed the saturated-rich ones (trial 1) and to the birds fed the low-fat ones (trial 2), despite the lower apparent absorption of α-tocopherol in the saturated diets and to the same α-tocopherol apparent absorption among different inclusion levels of dietary fat. The differences among treatments were only significant in the treatments highly supplemented with vitamin E (E200 and E400).

The regression equations between α-tocopherol concentration in whole body and in liver and plasma had very high correlations, especially between liver and whole body α-tocopherol concentration. However, the coefficient of variation in these equations was high, which is why α-tocopherol concentrations in plasma and liver are useful in order to study changes in the status of this vitamin, but cannot be considered estimators.

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ABBREVIATIONS USED

ADG: Average daily gain
ADI: Average daily intake
AFP: Abdominal fat pad
AME: Apparent metabolizable energy
ANOVA: Analysis of variance
BHT: Butylated hydroxytoluene
BW: Body weight
CF: Crude fat
CLA: Conjugated linoleic acids
CP: Crude protein
CV: Coefficient of variation
DM: Dry matter
EDTA: Ethylenediaminetetraacetic acid
EFA: Essential fatty acids
FA: Fatty acids
F:G: Food-to-gain ratio
FM: Fresh matter
GC: Gas chromatography
GE: Gross energy
HDL: High density lipoproteins
HPLC: High performance liquid chromatography
IDL: Intermediate density lipoproteins
L●: Lipid radical
LCFA: Long chain fatty acids
LDL: Low density lipoproteins
LH: Lipid (polyunsaturated fatty acid)
LOO●: Peroxyl radical
LOOH: Hydroperoxyde
LPL: Lipoprotein lipase
MCFA: Medium chain fatty acids
ME: Metabolizable energy
MUFA: Monounsaturated fatty acids
ND: Not detected
NS: Non significant
OM: Organic matter
PM: Portomicrons
PUFA: Polyunsaturated fatty acids
RC: Response coefficient
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
RSD: Residual standard deviation
SCFA: Short chain fatty acids
SFA: Saturated fatty acids
TAG: Triacylglycerol
UHP: Ultra high pressure
VLCFA: Very long chain fatty acids
VLDL: Very low density lipoproteins
2-MG: 2-monoglyceride
α-TA: α-tocopheryl acetate
α-TE: α-tocopherol equivalents

Chapter 1
Introduction

Phases of a Project:

1. Exultation.
2. Disenchantment.
3. Confusion.
4. Search for the Guilty.
5. Punishment of the Innocent.
6. Distinction for the Uninvolved.

Murphy's Law of Research
Enough research will tend to support whatever theory.

Dobie's Dogma:
If you are not thoroughly confused, you have not been thoroughly informed.

1.1. BIOLOGICAL IMPORTANCE OF LIPIDS

1.1.1. Definition of lipid

Lipids are a chemically heterogeneous group of substances that are insoluble in water but soluble in organic solvents, and are essential structural component of living cells (along with proteins and carbohydrates).

1.1.2. Classification of lipids

It is difficult to classify lipids, which is why there are so many types of classifications. For example, there are classifications depending on their chemical structure, on their polarity, on their functionality or on their ability to form soaps. The classification shown in this dissertation divides lipids depending on their functionality as described in Lehninger Principles of Biochemistry (Nelson and Cox, 2000). It divides lipids into three categories: storage lipids, membrane lipids and other lipids (signalling, cofactors and pigments).

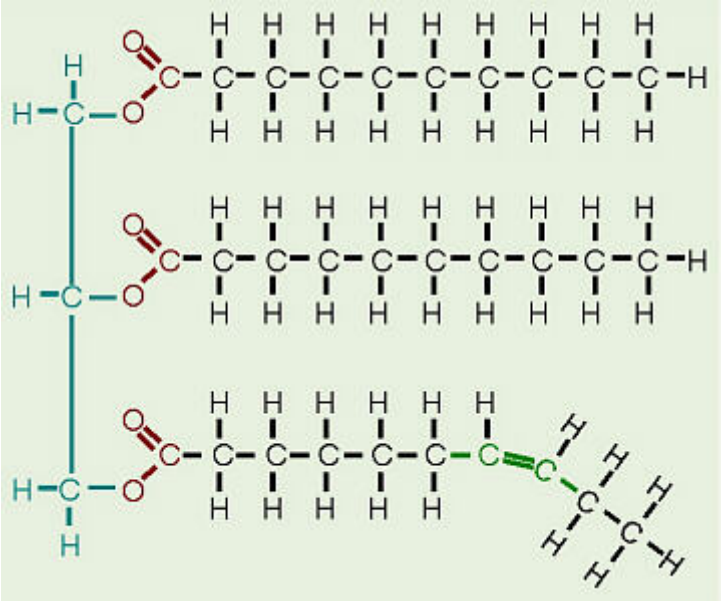
a) Storage lipids

Tryacylglycerides (TAG, figure 1) and waxes are included in this category. TAG, or neutral fat, are molecules consisting in 3 fatty acids (FA) esterified to a glycerol molecule. When TAG are rich in saturated FA, they form **fats**, and when TAG are rich in more unsaturated FA they are called **oils**. Fats and oils are the main storage energy forms in nature. The difference between them is that fats are solid and oils are liquid at room temperature. **Waxes** are formed by esters of long chain fatty alcohols with long chain fatty acids along with other lipid components and are the main energy storage in plankton.

FA are carboxylic acids with hydro carbonated chains of 4 to 36 carbon atoms. Usually, FA found in nature have an even number of carbons. FA properties (solubility and melting point) are determined by the number of carbons and by the number of double bounds. The nomenclature of FA indicates the number of carbons, the number of double bonds (if any) and also their position inside the

molecule. For example, 16:0 indicates that this fatty acid has 16 carbon atoms and no double bonds.

Figure 1. Structure of a triacylglycerol.



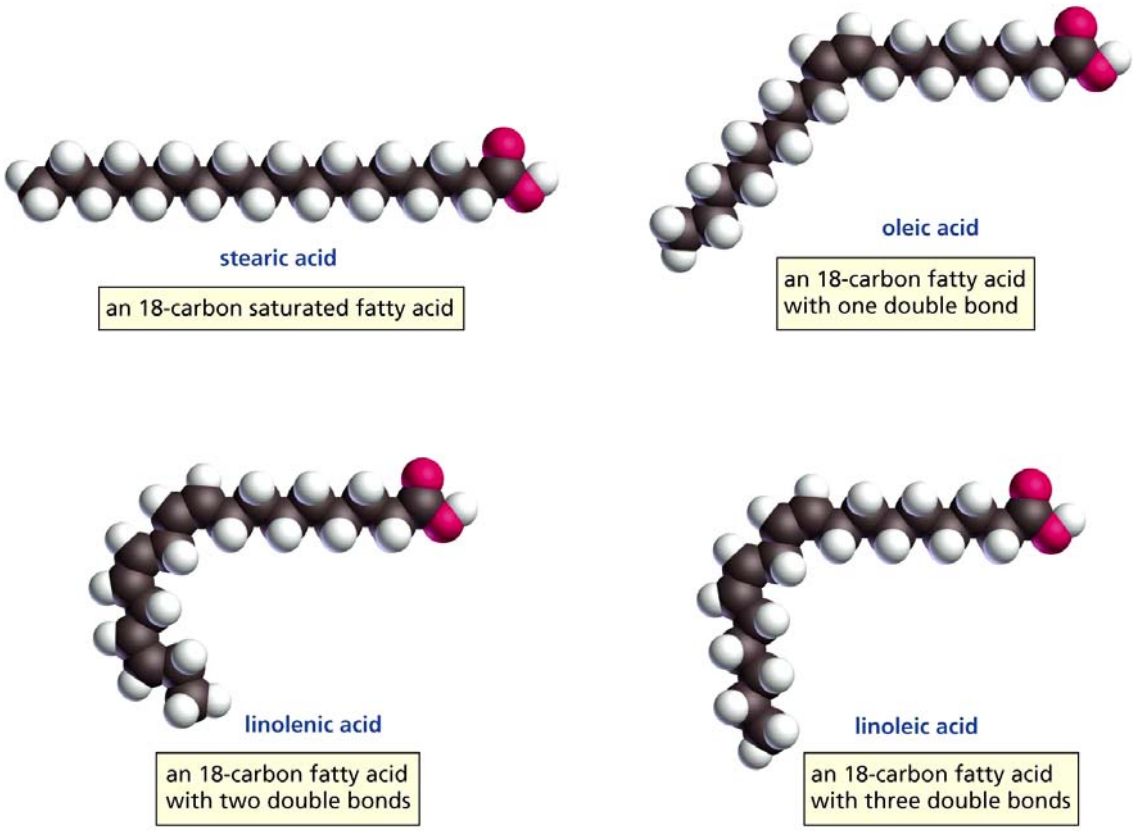
Number of carbons: FA are classified into short-chain fatty acids (SCFA, 2-4 carbons), medium-chain fatty acids (MCFA, 6-14 carbons), long-chain fatty acids (LCFA, 16-18 carbons) and very long-chain fatty acids (VLCFA, 20 or more carbons). In general, the shorter the chain, the more soluble in water is the FA. LCFA have to be included in micelles in order to blend in the hydrophilic environment of the intestine to be absorbed, whereas SCFA and MCFA can be absorbed directly (Young and Renner, 1960; Garret and Young, 1975). Regarding melting point, the longer the chain the higher the melting point, but it has to be considered that the presence of double bonds overrules the chain length effect.

Double bonds: FA can also be classified depending on the number of double bonds of the molecule. These double bonds are mainly in cis configuration. Saturated fatty acids (SFA) have none, monounsaturated fatty acids (MUFA) have one and polyunsaturated fatty acids (PUFA) have at least two (see figure 2). Double bonds give polarity to FA, hence, in FA of the same

length, unsaturated fatty acids (MUFA + PUFA) are more hydrophilic than SFA. This higher polarity makes unsaturated FA more capable to enter the micelles in the intestine, so their absorption is superior to that of SFA (Young and Garret, 1963).

The melting point of FA is also affected by the degree of unsaturation. Double bonds reduce the melting point of the FA. The carbon bonds in SFA give the FA a molecular geometry relatively linear and FA can be packaged together and create strong intermolecular interactions. The presence of double bonds bends the FA and intermolecular interactions are much weaker.

Figure 2. Structure of saturated, monounsaturated and polyunsaturated fatty acids.



There are two ways to indicate the position of the double bounds inside a FA. The Δ nomenclature counts the double bonds from the carboxyl position, and the ω nomenclature counts the double bonds from the methyl end of the FA. For example, 18:2 Δ 9, 12 is a FA with 18 carbon atoms and 2 double bonds

that are in the 9 and 12 position from the carboxyl end. These same fatty acids can be named 18:2 ω 6, 9. Thus unsaturated FA are divided in ω9, ω7, ω6, and ω3, depending on the position of their last double bound. FA of the same ω family follow the same metabolic routes.

The high melting point and the low solubility of SFA are the cause of their poor absorption in the intestine. Administering SFA and unsaturated FA together can improve SFA absorption, because unsaturated FA make micelles bigger and facilitate the pass of SFA into the micelles, thus helping in the emulsification of SFA . Krogdahl (1985) reviews the factors affecting the processes of digestion and absorption of lipids in poultry.

b) Membrane lipids:

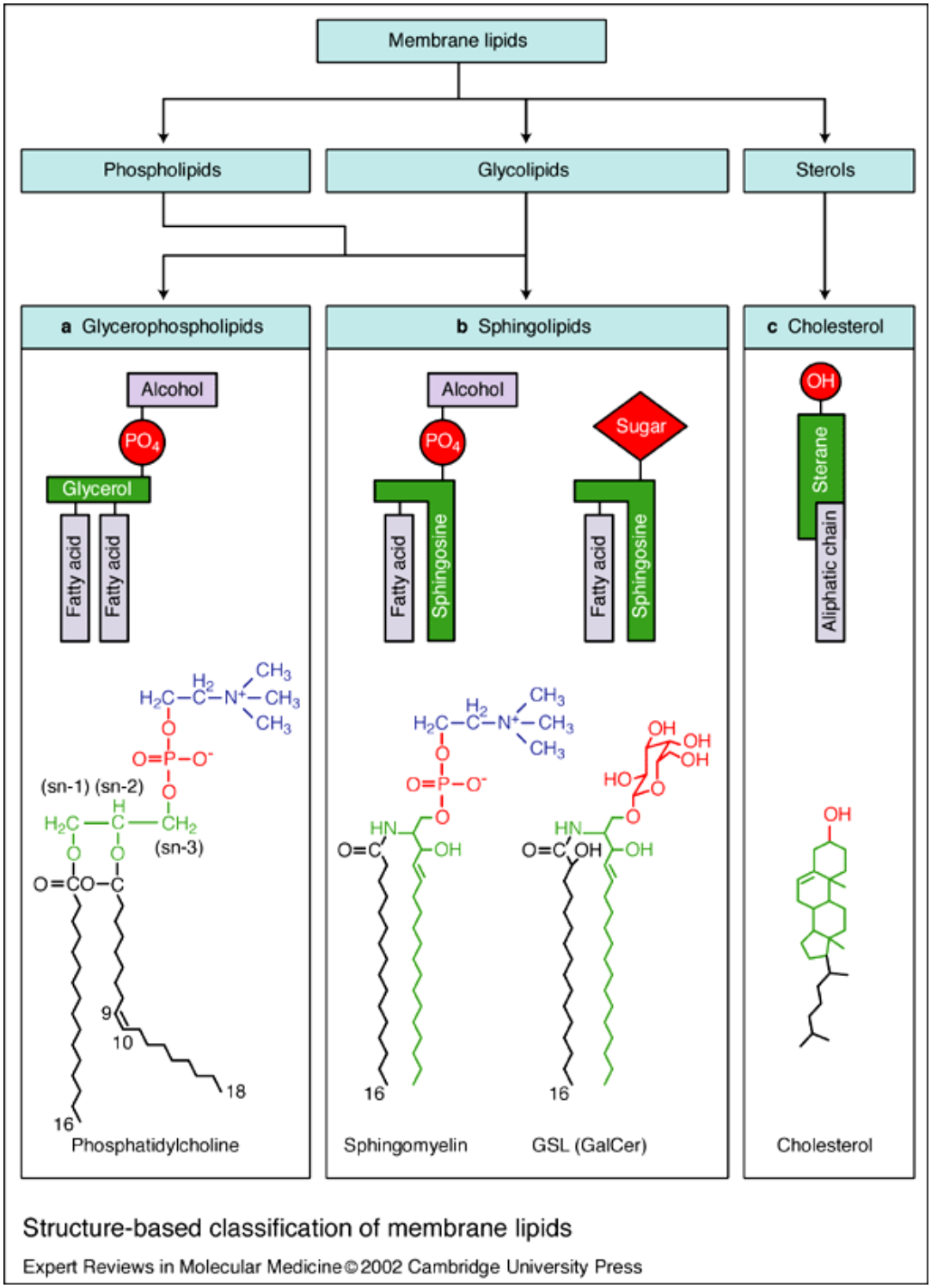
There are three types of lipids forming the lipid bilayer of biological membranes: glycerophospholipids, sphingolipids and sterols. Their structure is shown in figure 3. They are amphipatic molecules, with a polar head and a non-polar body.

Glycerophospholipids consist in 2 FA esterified to a glycerol molecule, which in turn is united to an alcohol by a phosphodiester link. Their nomenclature depends on the type of alcohol linked (etanolamine, choline, inositol…).

Sphingolipids are composed of sphingosine (a long-chain amino alcohol), one LCFA and a polar group. Depending on the nature of the polar group, sphingolipids are classified in sphingomyelins (phosphocholine or phoshoetanolamine), glucosphingolipids (one or more sugars) and gangliosides (oligosaccharides and sialic acid).

Sterols have a steroid nucleus consisting in 4 fused rings. This nucleus is flat and rigid. Cholesterol is the main sterol in animal tissues, and consists of the steroid nucleus united to a hydro carbonated lateral chain (non-polar body) and a hydroxyl group (polar head) in C3 position.

Figure 3. Membrane lipids.



c) Other lipids

There are lipids present in small amounts in the cells that play important roles in metabolism. Some participate in cell signalling, other are enzymatic cofactors, and other act as pigments (important in photosynthesis, vision, and in color production). Examples of these compounds are phosphatidyl inositol,

eicosanoids (prostaglandins, tromboxanes and leucotrienes), steroid hormones, and fat-soluble vitamins (A, D, E, K).

1.1.3. Roles of lipids in the organism

Lipids have a large variety of functions in the organism. These functions are described elsewhere (Gurr, 1984; Nelson and Cox, 2000; Stryer et al., 2002). The main functions are the following:

1. Energy storage: Animals have a limited ability to store carbohydrates (in the form of glycogen) and none to store protein. TAG are the main form of energy storage in animals. Animals store energy in adipose tissue, and FA are mobilized and oxidized when energy is needed. 1 gram of fat yields 9 calories, whereas 1 gram of carbohydrate yields 4 calories.

2. Insulation and support of vital organs: Visceral adipose tissue is located around vital organs to protect them from heat loss and temperature changes and from possible injuries.

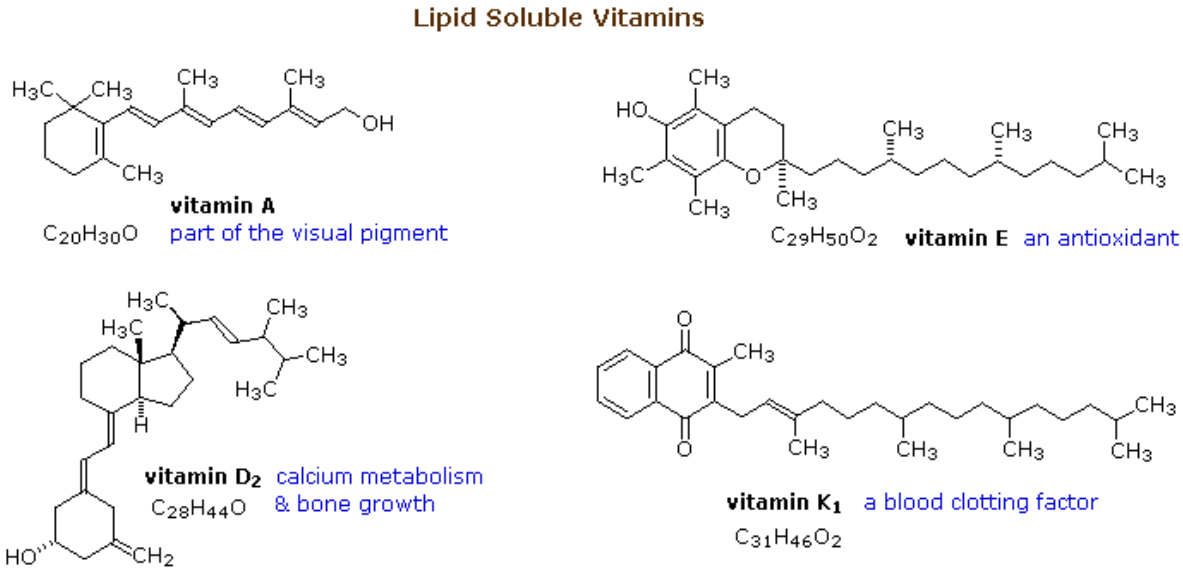
3. Protective coating to prevent loss or gain of water and infections: TAG and also waxes are used to protect the skin of mammals, feathers of birds and other species.

4. Heat generation: TAG located in the skin and in adipose tissue can be oxidized in order to generate heat when temperature of the environment drops.

5. Structural roles: phospholipids, sphingolipids, and cholesterol (see figure 3) are then main lipid components of cellular and subcellular membranes. Brain and nervous tissue, such as the myelin sheath, and retinal tissue are rich in certain sphingolipids.

6. Fat-soluble vitamins: A, D, E, K. These vitamins exert several important functions in the organism, as shown in figure 4.

Figure 4. Fat-soluble vitamins.



7. Functional roles: Among other functions, lipids are emulsifying agents (bile acids, phospholipids) and participate in intracellular and extracellular signalling. Phosphatidil inositol, a type of phospholipid, is an important second messenger. Several hormones are synthesized from cholesterol (sexual hormones, glucocorticoids) and finally VLCFA are precursors of the eicosanoids, autocrine and paracrine mediators that regulate cell and tissue functions, which control smooth muscle contraction, the aggregation of platelets and inflammatory processes, among many other functions, including reproduction. The main precursors of eicosanoids are γ-linolenic acid (18:3ω6), arachidonic acid (20:4ω6), eicosapentaenoic acid (20:5ω3), and docosahexaenoic acid (22:6ω3). Fatty acids also regulate cell function by modifying the fluidity of biological membranes

8. Hormone synthesis: Abdominal tissue synthesizes a hormone, leptin, which is related with satiety and reproduction. In poultry, the main site of the synthesis of leptin occurs in the liver, where fatty acid synthesis also occurs, even though there is some synthesis in adipose tissue. In mammals, leptin synthesis takes place in the adipose tissue.

1.1.4. Roles of lipids in animal nutrition

Adding fats or oils (neutral lipids) into poultry diets is essential in order to provide adequate amounts of essential fatty acids (EFA), which cannot be synthesized by the organism, and to act as carriers for fat-soluble vitamins. Other functions of dietary fat are to provide a concentrated source of energy, to improve the utilization of other nutrients and to improve palatability. It is also included in animal feeds to facilitate the granulation, reduce dustiness during processing and decrease the wear and tear on milling machinery in feed mills. Inclusion of fat in poultry diets improves feed to gain ratios compared to diets with no added fat (Fedde et al., 1960). Both animal and vegetable fat and oil sources can be used in poultry feeds. The typical inclusion level of added fat in broilers ranges between 2 and 6%, and it provides approximately a 6-20 % of the dietary energy.

1.2. ORIGIN OF BODY LIPIDS

Lipids existing in the body have double origin: exogenous (from the diet) or endogenous (synthesized by the organism). Excepting EFA, lipids can be synthesized as long as there are enough EFA, carbohydrate and protein precursors. Chickens, like mammals, cannot add double bonds to a FA farther than the 9 position (counting form the carboxyl end), so FA of the ω6 and ω3 family are either EFA or EFA-derivatives and must be supplied by the diet.

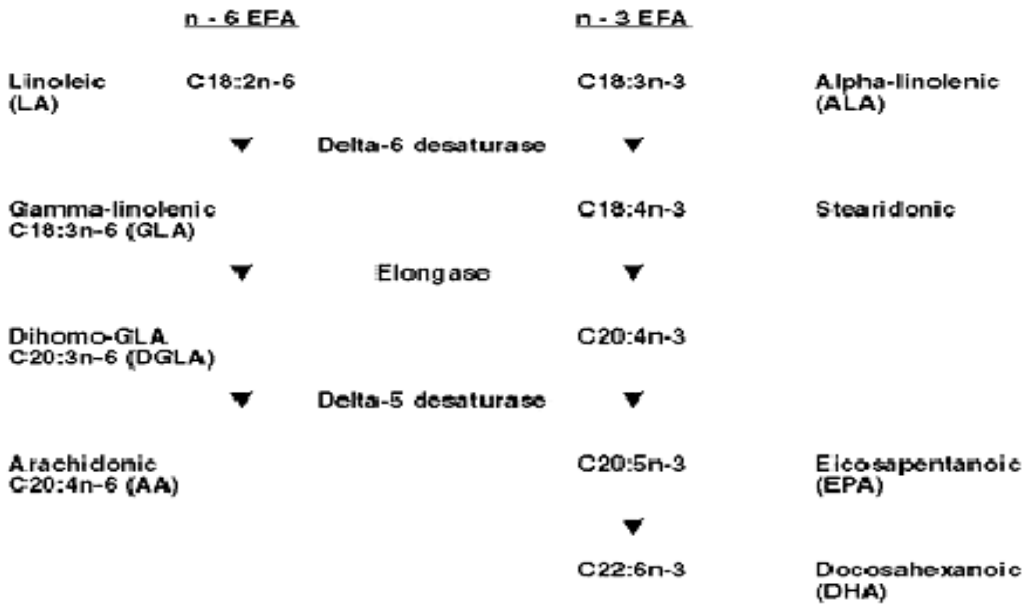
Fatty acids in avian species are synthesized mainly in the liver from acetyl-CoA, and the final product is palmitic acid (16:0). The process is catalyzed by the enzymes Acetyl-CoA Carboxylase and Fatty Acid Synthase in the cytosol. Longer FA can be obtained by elongation systems in smooth endoplasmatic reticulum and mitochondria, and double bonds are added by the enzymes desaturases.

1.2.1. Essential fatty acids

In poultry production, the NRC (1994) recommends a minimum of 1% of linoleic acid (18:2ω6). Linoleic acid is the precursor of all the ω6 PUFA (figure 5), like arachidonic acid. Linolenic acid (18:3ω3) is also considered essential although

there are no minimum recommendations established in chicken. In humans, the recommended ratio of ω6 to ω3 FA by the European Union is of 4:1-10:1 (Sanchez and Malo, 1995). Linolenic acid is the precursor of eicosapentaenoic acid (20:5ω3, EPA) and docosahexaenoic acid (22:6ω3, DHA) (see figure 5). EFA functions are related to the synthesis of long-chain PUFA that give way to eicosanoids (mainly from arachidonic and docosahexaenoic acids) and are also incorporated in cellular membranes where they modify physical properties of membranes, such as transport systems, ion channels, enzyme activities, etc. (Uauy et al., 2000). The long-chain derivatives of EFA also affect neural development and function. Brain, retina and other nervous tissues are particularly rich in long-chain PUFA, especially DHA (Uauy and Castillo, 2003). There is a tendency to maintain the amount of PUFA in the organism in an effort to maintain physical properties of the membranes, for instance, when there is a ω3 EFA deficiency, ω6 derivatives are increased in the tissues. When there is a ω3 and ω6 EFA deficiency at he same time, the concentration of FA 20:3ω9 (the longest and most unsaturated FA that can be synthesized in the organism) is increased (Holman, 1960; Levy et al., 1990). The trieneoic to tetraenoic FA ratio is a good indicator of ω6 and ω3 EFA deficiency, but not of a ω3 EFA deficiency alone (Uauy and Castillo, 2003).

Figure 5: Metabolism of essential fatty acis (linoleic and linolenic acids).

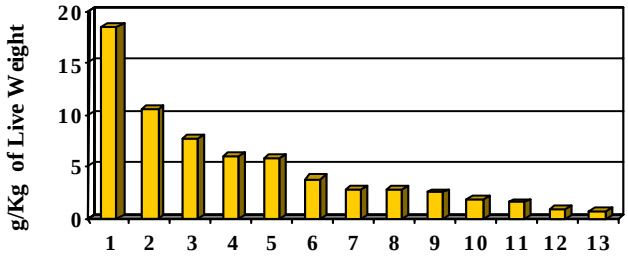


1.3. DISTRIBUTION AND COMPOSITION OF BODY LIPIDS IN BROILER CHICKENS

Given that body fat has two different origins (endogenous and exogenous), body lipid content depends on the balance between fat intake, lipid synthesis and lipid catabolism in the body. Storage fat (mainly TAG) is distributed in different parts of the animal. Evans (1977) estimated in the chicken that adipose tissue and skin can store more than 85% of the TAG. Storage TAG are localized in the following tissues: abdominal fat (from the gizzard to the cloaca), fat around the gizzard, fat located in the sartorius muscle, fat located in the neck (from the shoulder to the head) and mesenteric fat (from the pylorus to the colon) (Cahaner et al., 1986).

The same study states that adipose tissue from these 5 locations accounts for the 20% of total body lipids, the skin for the 18%, liver and feathers for a 2.5%, the skeleton for a 15% and the rest of the carcass (muscles, intestine, kidneys, lungs, connective tissue…) gets the 40%. Other authors (Nir et al., 1988) have described in more detail the fat distribution of 6-week old broilers with a weight of approximately 1.9 kg (figure 6).

Figure 6. Adipose tissue distribution in 6-week old broilers (Nir et al., 1988)*.



*1, abdominal; 2, neck; 3, thigh; 4, back; 5, cloaca; 6, mesenteric; 7, sartorius; 8, breast; 9, crop; 10, proventriculus; 11, bursa of Fabricius; 12, pericardium; 13, liver.

Ratnayake et al. (1989) studied lipid composition of thigh, breast and skin in chickens. Skin contains mainly TAG, while breast contains both TAG (43%) and phospholipids (55%). In thigh, 85% of the fat is in form of TAG and 16% of phospholipids. Cholesterol is present in higher amounts in breast (2%) than in thigh (1%) or than in skin (0%). Cholesterol and phospholipids are important compounds of cellular membranes, and these results agree with the fact that lipids found in breast are mainly from the cellular membrane whereas in thigh and skin adipocytes are major components.

The carcass fat content of broiler chickens ranges between 13 and 14.5 % (Havenstein et al., 2003). This percentage changes depending on several factors affecting fat deposition in chickens including genetics, sex, age, live weight and nutrition. Genetic selection during the years has selected broilers in favour of a higher growth rate and a higher live weight, and this indirectly has resulted in a higher fat body deposition (Havenstein et al., 1994). Nowadays, it is possible to select lines for high or low fat deposition, based on abdominal fat content or in the plasmatic concentration of very low density lipoproteins (Hermier et al., 1991).

Females are able to deposit more body fat than males (Pan et al., 1979), and old chickens have higher fat content in the carcass compared to young ones (Deaton and Lott, 1985).

Nutritional factors that affect body fat deposition are well described. Fat deposition happens when there is a positive energy balance and when the nutrients in the feeds are not balanced. In particular, the energy to protein ratio of the feeds and the type and amount of dietary fat are the most important factors affecting body fat deposition. Both energy and protein are necessary for growth, and when the energy to protein ratio is increased (due to an excess of energy or a deficiency in protein), there is an excess of energy needed for growth due to the relatively low protein content, and hence this energy is stored in form of fat (Bartov, 1987; Leeson and Summers, 1997). On the other hand, a reduction of this ratio can lead to a loss of body fat due to the lower energy intake and to the energy expenditure to metabolize and excrete the excess protein (Bartov, 1979).

The effect of dietary fat on fat deposition is controversial. Regarding fat inclusion level, several works using isocaloric and isoproteic diets with different levels of fat inclusion have been conducted. Deaton et al. (1981) found that increasing levels of animal fat caused higher abdominal fat pad weight (% of live weight) and total body fat increased. However, Donaldson (1985) using increasing levels of cottonseed oil did not found differences in fat deposition. Keren-Zvi et al. (1990) observed lower body fat deposition in chickens with increasing dietary vegetable oil levels. Vila and Esteve-Garcia (1996) found that increasing fat inclusion levels increased abdominal fat pad weight when the added oil used was tallow, but no effect was found when the added fat was sunflower. It seems that the effect of fat inclusion level is dependent on FA profile of the added fat.

For years the effect of FA composition was not considered important, since it was supposed that, once absorbed, FA behaved similarly. Currently, it is known that dietary FA composition affects fat deposition, and this will be discussed later in this review.

1.4. DIETARY MODIFICATION OF LIPID COMPOSITION IN CHICKENS

Chicken meat is considered a healthy food, due to its content of protein of high biological value, vitamins (especially the B group vitamins) and minerals (iron, phosphorus, zinc, magnesium, calcium…), and to its relatively low fat content compared to other meats. FA composition of chicken meat differs depending on the portion of the chicken. In general, when the animals are fed a standard commercial diet, the main FA present are oleic (18:1ω9), palmític (16:0) and linoleic (18:2ω6) acids (Ratnayake et al., 1989). Breast presents more PUFA, especially long-chain PUFA, and less MUFA than the thigh. Skin has higher amounts of oleic and palmitoleic compared to thigh and breast.

Animals fed diets without added fat have to synthesize FA from carbohydrate precursors in order to store the energy supplied by the diet and to have enough FA to perform all the roles already described. In animals fed this type of diets, the main FA are oleic (18:1ω9) and palmitic (16:0) acids (Crespo and Esteve-Garcia, 2002*b*, see table 1). Fat inclusion reduces FA *de novo* synthesis and fat deposition depends on a balance between direct deposition from the diet and on FA synthesis, keeping fat content of the animal relatively constant (Saadoun and Leclercq, 1987). FA synthesis is decreased because of the lower carbohydrate content of the diets (due to fat inclusion) and to a direct inhibition of lipogenic enzymes by dietary FA (Mourot and Hermier, 2001).

Table 1. Body composition in fatty acids (g/100 g of fat) of chickens fed a diet without added fat (Crespo and Esteve-Garcia, 2002*b*).

Saturated fatty acids		**Monounsaturated fatty acids**		**Polyunsaturated fatty acids**			
C 14:0	0,8	C 16:1 ω7	7,5	C 18:2 ω6	18,6	C 20:4 ω6	0,9
C 15:0	0,1	C 18:1 ω9	35,1	C 18:3 ω6	0,4	C 20:5 ω3	0,1
C 16:0	24,3	C 18:1 ω9t	0,0	C 18:3 ω3	1,3	C 22:4 ω6	0,5
C 18:0	6,5	C 18:1 ω7	2,5	C 20:2 ω6	0,2	C 22:5 ω3	0,4
C 20:0	0,1	C 20:1 ω9	0,4	C 20:3 ω3	0,0	C 22:6 ω3	0,1

The possibility of modifying FA composition in chicken tissues was discovered long ago (Marion and Woodroof, 1963; Atkinson et al., 1972). When fat

inclusion in the feeds increases, FA composition of the body is more similar to FA composition of the diets. Several authors have increased the proportion of specific FA increasing the inclusion of certain fat sources (Hulan et al., 1989; Lopez-Ferrer et al., 2001*a, b*). However, FA composition changes are lower in tissues than in the diets (Lopez-Ferrer et al., 1999*a, b*, 2001*a, b*, see table 2) and SFA are less modifiable than unsaturated FA. Moreover, MUFA vary in an inverse way to PUFA.

Table 2. Variation of the fatty acid profile (% of fatty acids) in chicken thigh depending on the variation on the diets (based on Lopez-Ferrer et al., 1999*a, b*, 2001*a, b*).

% of variation in diets		
SFA	**MUFA**	**PUFA**
76,53	54,12	63,62
% of variation in thigh		
SFA	**MUFA**	**PUFA**
24,58	28,71	38,33

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: polyunsaturated fatty acids

Chicken meat can be enriched in SFA with the use of cocoa oil (Yau et al., 1991) or palm oil (Cherian et al., 1996); in MUFA using olive oil (Lauridsen et al., 1997; Crespo and Esteve-Garcia, 2002*b*); in linoleic acid using rapeseed oil (Zollitsch et al., 1997) or sunflower oil (Lopez-Ferrer et al., 1999*b*) or soybean oil (Zollitsch et al., 1997); in linolenic acid using linseed oil (Nam et al., 1997; Crespo and Esteve-Garcia, 2002*c*); and in EPA and DHA using fish oil (Scaife et al., 1994; Lopez-Ferrer et al., 2001*a*). There are a high number of works concerning FA modification of chicken meat due to the high acceptability of this meat by the consumer and by the fast response of body FA to dietary changes.

1.4.1. PUFA enrichment

The modification of chicken FA composition can be aimed to create functional foods, enriching chicken meat with PUFA, due to the reported health benefits of these FA, especially the ω3 ones. A high intake of SFA has been related to a

higher incidence of cardiovascular diseases (Wolfram, 2003; Srinath & Katan, 2004). PUFA of the ω3 family, from vegetal origin (rich in linoleic acid) and especially from marine origin (rich in EPA, DHA) are thought to have protective effects against cancer, atherosclerosis and other diseases (as reviewed by Kinsella et al., 1990 and by Calder, 2004). Recently, the FDA has accepted the claim that ω3 enriched products have protecting effects against cardiovascular disease (Food and Drug Administration, 2004). Another PUFA, conjugated linoleic acids (CLA), which are produced in the rumen by hydrogenation processes (and are also available commercially), are believed to have, depending on the specific isomer studied, anti-carcinogenic, anti-obesity, anti-atherogenic and immunomodulatory functions (reviewed by Azain, 2003) in several species. The effects of CLA in humans are still not clearly defined (Wahle et al., 2004).

Besides the health benefit of PUFA compared to SFA, recently it has been observed in rats and in chickens that PUFA inclusion in the diets reduces abdominal fat and total body fat compared to SFA-rich diets (Shimomura et al. 1990; Sanz et al. 2000*a, b*; Crespo and Esteve-Garcia, 2001, see table 3). That is, besides the energy to protein ratio and level of inclusion of added fat, dietary FA composition affects not only the composition but also the amount of fat deposited. The better intestinal absorption of PUFA compared to SFA supposes higher metabolizable energy (ME) values for PUFA-rich diets (Blanch et al., 1995). This means that at the same inclusion level, a diet supplemented with PUFA-rich oil has a higher energy to protein ratio than a diet supplemented with SFA-rich fat. According to the well established effect of this ratio, a diet rich in PUFA should cause higher fat deposition but all the works cited have found the contrary effect.

There is no clear explanation yet, but the proposed hypothesis are that PUFA produce higher heat losses than SFA (Takeuchi et al. 1995; Mercer & Trayhurn, 1987), increase FA catabolism (Sanz et al. 2000*a*; Crespo and Esteve-Garcia,2002*c*) or decrease FA synthesis (Wilson et al. 1990, Sanz et al. 2000*a*).

There are few works designed to assess the relationship between inclusion level and type of added fat on body fattening in chickens. The work presented in this dissertation consists in two experiments where a gradient of dietary polyunsaturation is achieved by two strategies: mixing different fat sources in different proportions to have a dietary PUFA gradient with the same fat inclusion level (trial 1) and increasing fat inclusion level of PUFA-rich oil (trial 2). This design permits to study the potential of endogenous FA synthesis in different circumstances. Fat content of the birds fed these diets has been carried out using Soxhlet extraction and FA quantification. It has to be stressed that most of the works concerning fat deposition in broilers measure body fat content using the methodology described by Folch et al. (1957), which is not an official method for body fat quantification.

Table 3. Effect of added dietary fat sources differing in their saturation degree on abdominal fat pad weight and body fat content of broiler chickens.

Source	Age	Sex	Type of fat	Inclusion level	AFP[1] (g/100 g BW[2])	Body fat	
Sanz et al. 1999	52d	♀	Tallow	8%	3.46[a]	23.52[a]	intramuscular (g/100 g tissue)
		♂	Sunflower oil		2.97[b]	18.53[b]	
Sanz et al., 2000*b*	35d	♀	Tallow	8%		15.15[a]	Body fat (g/100 BW[2])
			Sunflower oil			12.91[b]	
Sanz et al., 2000*a*	53d	♀	Tallow	8%	3.03[a]		
			Sunflower oil		2.63[b]		
Crespo et al., 2001	49d	♀	Tallow	10%	3.22[a]		
			Sunflower oil		2.67[b]		
			Linseed oil		2.63[b]		
		♂	Tallow	6-10%	2.00[a]		
			Sunflower oil		1.70[b]		
			Linseed oil		1.75[b]		
Crespo et al., 2002 *a, b*	53d	♀	Tallow	10%	2.87[a]		
			Sunflower oil		2.04[b]		
			Linseed oil		2.31[b]		
Newman et al., 2002	56d	♀ ♂	Tallow	8%	3.96[a]		
			Sunflower oil		1.50[b]		
			Fish oil		1.67[b]		

[1]AFP: Abdominal fat pad
[2]BW: body weight

Currently, our team is carrying out studies regarding the effect of different fat sources (rich in SFA, MUFA, and ω6 and ω3 PUFA) on fat distribution in chicken's body (skin, edible parts, liver…) and also on activities and RNA

expression of enzymes related to fatty acid oxidation (Carnitin Palmitoil Transferase), synthesis (Fatty Acid Synthase) and tissue uptake of FA (lipoprotein lipase).

1.4.2. Problems related to PUFA enrichment

There are several problems associated with a high PUFA content of tissues. These FA are more prone to lipid peroxidation due to presence of double bonds in the molecule. This process gives raise to oxidation products highly reactive that can damage the cells and are related to the ethiopatogeny of several diseases, as commented below. Moreover, other problems also appear in chicken meat: the appearance of off-flavours and the reduction of the melting point of carcass fat.

a) Off-flavours

The use of fish oil as a source of VLCFA of the ω3 family can lead to the appearance of unacceptable flavours ("fishy") for the consumers (Edwards and May, 1965; Lopez-Ferrer et al., 1999*a*).

b) Melting point

The structure of the FA present in TAG (table 4) determines the melting point of the depot fat. Feeding animals with unsaturated oils can result in oily carcasses that are not accepted by the consumers.

The solution to both of these problems is reducing the percentage of highly unsaturated fat inclusion or substituting over 1-2 weeks before slaughter the PUFA-rich oil by another source of fat (Lopez-Ferrer et al., 1999*a*).

Table 4. Melting point of some fatty acids (adapted from Nelson and Cox, 2000)

Saturated		Unsaturated	
Name	Melting point	Name	Melting point
Lauric acid (12:0)	44ºC	Palmitoleic acid (16:1ω7)	0ºC
Myristic acid (14:0)	54ºC	Oleic acid (18:1ω9)	13ºC
Palmitic acid (16:0)	63ºC	Linoleic acid (18:2ω6)	-5ºC
Stearic acid (18:0)	70ºC	Linolenic acid (18:3ω3)	-11ºC
Arachidic acid (20:0)	76ºC	Arachidonic acid (20:4ω6)	-49ºC

c) Lipid peroxidation

Lipid peroxidation is a process that can occur in the feeds, in the living organism and in the stored meat. It reduces the nutritive value of the meat due the destruction of PUFA, fat-soluble vitamins, and other nutrients. Moreover, organoleptic characteristics are also altered, with appearance of off-flavours (Frankel, 1991; Robey and Shermer, 1994). This process gives raise to oxidation products, that, once absorbed, can be deposited in the tissues and be responsible for the appearance of several pathologies (Halliwell and Gutteridge, 1990; Emanuel et al., 1991). There are numerous reviews that explain in detail the process of lipid oxidation (Halliwell and Chirico, 1993), Halliwell et al. (1995), Addis et al. (1996), Guardiola et al. (1996) and Surai (2002).

Table 5. Some free radicals that participate in lipid peroxidation reactions (from Surai, 2002).

Radical	Name
$O_2^{-}\bullet$	Superoyde
$HO_2\bullet$	Hydroperoxyl
$HO\bullet$	Hydroxyl
H_2O_2	Hydrogen peroxyde
$LOO\bullet$	Peroxyl
LOOH	Hydroperoxide

Free radicals are atoms or molecules with at least one unpaired electron, which makes them highly reactive and unstable. These free radicals are produced as a consequence of the metabolic activity of the organism and are also used by the immune system to fight against micro organisms. Free radicals are also produced by external agents, such as smoke, irradiation, pollution, chemical reagents, etc. If these free radicals are not neutralized, they attack unsaturated FA present in cell membranes. Free radicals act as initiator agents, which take an electron from the unsaturated FA (LH), creating a lipid radical (L●) (inititation phase). In the presence of oxygen, the lipid radical is converted to a peroxyl radical (LOO●), which will react with another FA to generate a hydroperoxyde (LOOH) and another lipid radical (propagation phase). The reaction ends

(termination phase) when the radicals react among them to form non-radical compounds or when the substrate for the reaction (unsaturated FA) is all spent (see figure 7). This chain reaction affects mainly highly unsaturated PUFA, because susceptibility to peroxidation is proportional to the amount of double bonds in the molecules.

Free radicals are the most important, but there are other initiation agents. They are classified in reactive oxygen species (ROS) and reactive nitrogen species (RNS). Some of them are presented in table 5. Transition metals such as iron and copper can promote lipid peroxidation reactions (Kanner et al., 1987), not by acting directly upon the lipid but producing hydrogen peroxide, superoxyde radical and hydroxyl radical in the presence of oxygen (Halliwell and Gutteridge, 1990).

Figure 7. Scheme of lipid peroxidation chain reaction.

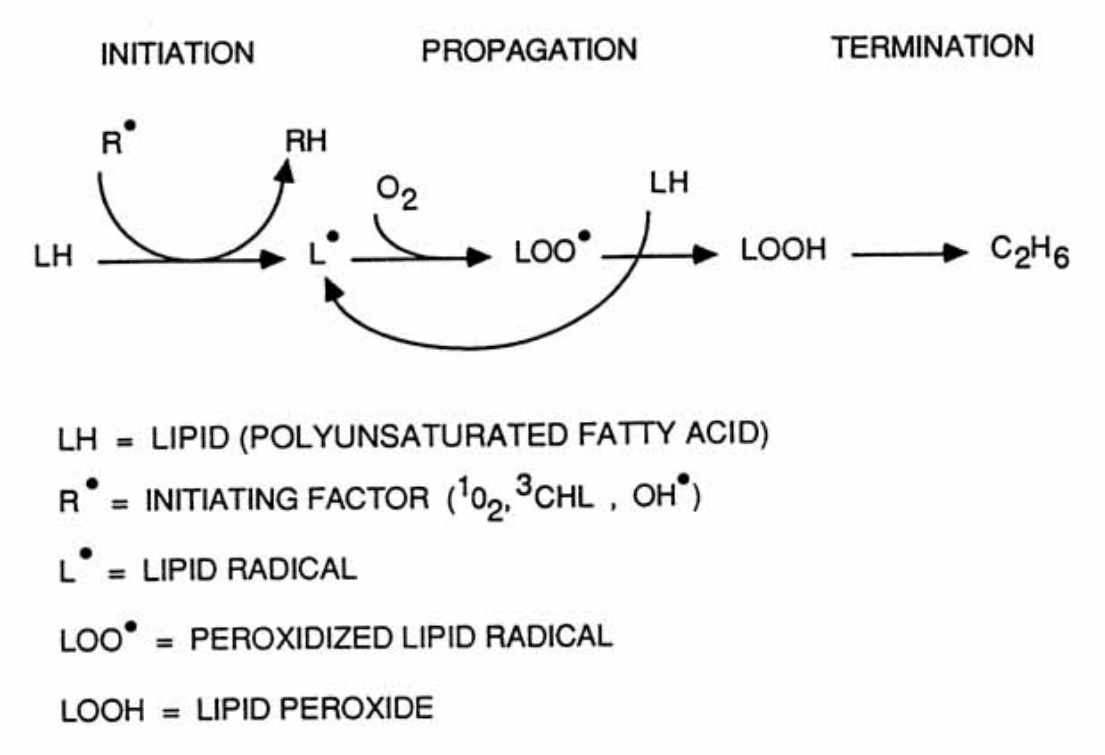


Photo-oxidation is initiated by singlet oxygen $^1O_2$, which is an excited state of molecular oxygen. Singlet oxygen does not take an electron from PUFA, but it combines directly with double bonds (Halliwell and Chirico, 1993) forming peroxides that can act as initiators of the chain reaction.

Hydroperoxides are called primary oxidation products. But their duration in time is short because they react among them to form the so-called secondary oxidation products: hydrocarbons, aldehydes, ketones, alcohols and esters. These products are responsible for the smell and taste of rancid products (Frankel, 1991).

The damaging effects of lipid peroxidation can be overcome in part by antioxidant defence systems.

1.5. ANTIOXIDANT DEFENCE

1.5.1. In the body (*in vivo*)

Surai (2002) reviewed the most important mechanisms of defence against ROS, which are divided in three levels: 1) prevention of radical formation, 2) prevention and restriction of chain formation and propagation, and 3) excision and repair of damaged molecules.

1) Antioxidant enzymes (superoxide dismutase, glutathione peroxidase, catalase), glutathione and thioredoxin systems, and metal-binding proteins remove precursors of free radicals and inactivates catalysts. Most of the enzymes are metalloproteins containing selenium, iron or copper. Vitamin E and C also scavenge free radicals.
2) Vitamins A, E, C, carotenoids, ubiquinols, glutathione and uric acid are chain-breaking water-soluble and lipid-soluble antioxidants. They scavenge peroxyl radicals intermediates in the chain reaction, thus finishing the reaction. Among them, vitamin E is the most effective. The action of these antioxidants results in an antioxidant radical (much less reactive than peroxyl radicals) that can be regenerated by redox

processes, and a lipid hydroperoxyde, that is still toxic and must be removed by glutathione peroxidase.

3) Lipases, peptidases, proteases, transferases, DNA-repair enzymes are responsible for the repair of damaged molecules and their elimination when repair is not possible.

This antioxidant system cannot be 100% effective, because most of the ROS and RNS have important metabolic functions (Halliwell and Chirico, 1993). That is, there is a balance between antioxidant defence and free radical generation (Surai, 2002). Low activity of antioxidant defence or overproduction of free radicals leads to oxidative stress that produces tissue damage and is related to various pathologic disorders such as diabetes mellitus (Robertson, 2004), atherosclerosis (Stocker and Keaney, 2004) and Alzheimer's disease (Butterfield and Boyd-Kimball, 2004).

1.5.2. In the feeds

Inclusion of antioxidants in the feeds is a normal practice in poultry production. There is a tendency towards the use of natural antioxidants in detriment of synthetic ones. Vitamins A, C and E have antioxidant properties and act once they absorbed and deposited in the tissues. Vitamin A and E are usually supplemented as esters, so they become active after hydrolysis in the intestine and are not be wasted during feed storage. Other natural antioxidants that can be used are carotenoids, flavonoids, and other substances obtained from plant extracts.

There are synthetic antioxidants like BHA that are added to the feeds or to the oils to preserve them from oxidation during processing and storage. Initially, these antioxidants are non-absorbable, so they action is limited to the feeds and possibly to the gastrointestinal tract.

Among the antioxidants that can be supplemented in the feed, this review will focus on vitamin E for two reasons. First, because it is the more potent natural chain breaking antioxidant (Parks and Traber, 2000, Surai, 2002) and one of the most utilized in practice despite its high cost. It protects from oxidation *in vivo*

and also in the processed meat (Cortinas et al., 2004*a*). Second, because it is the antioxidant used in the experimental studies presented in this dissertation.

1.5.3. Vitamin E

1.5.3.1. Definition, chemical structure and bioactivity

There are extensive reviews concerning this antioxidant vitamin, from where the information here presented has been collected: Burton and Ingold (1989), Bjørneboe et al. (1990), Burton and Traber (1990), Burton et al. (1990), Cohn (1992 and 1997), Niki (1993 and 1996), Burton (1994), Dutta-Roy et al. (1994), Kamal-Eldin and Appelqvist (1996), Scherf et al. (1996),Traber and Sies (1996), Traber and Arai (1999), Bramley et al. (2000), Herrera and Barbas (2001), Parks and Traber (2000), Surai (2002), among others.

The term Vitamin E encloses 8 molecules of vegetal origin: 4 tocopherols (α, β, γ and δ) and 4 tocotrienols (α, β, γ and δ) (figure 8). Only α-tocopherol has been demonstrated to be required by humans (Parks and Traber, 2000), and this is the isomer usually supplemented in poultry diets. They have a chromanol ring and a lateral phytil chain. This chain is saturated in tocopherols, whereas it has three double bonds in tocotrienols. The α, β, γ and δ isomers differ in the number and position of the methyl groups in the chromanol ring (table 6).

Figure 8. General structure of tocopherols and tocotrienols.

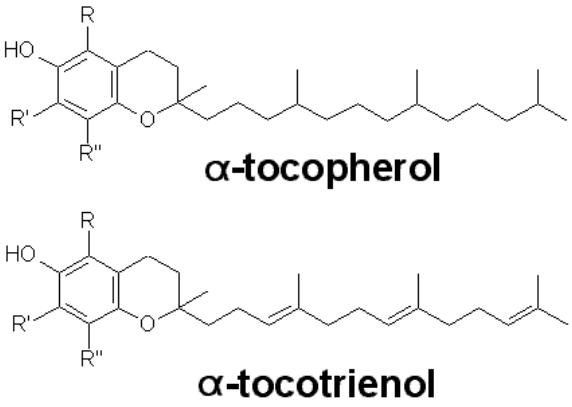


Table 6. Formula and relative bioactivity of the 4 tocopherols and tocotrienols with vitamin E activity (Machlin, 1984)

Position of the methyl groups	Tocopherols	Relative Bioactivity (%)	Tocotrienols	Relative Bioactivity (%)
5,7,8	α-tocoferol	100	α-tocotrienol	30
5,8	β-tocoferol	30	β-tocotrienol	3
7,8	γ-tocoferol	10	γ-tocotrienol	-
8	δ-tocoferol	1	δ-tocotrienol	-

The biological activity of the vitamin E compounds is not the same, α-tocopherol being the most active (table 6). 70-90% of vitamin E available in the feeds is α–tocopherol, but it is not always the most abundant: corn oil is rich in γ-tocopherol, soybean oil in γ- and δ-tocopherols and palm oil contains high amounts of γ-tocopherol and α-tocotrienol (Sheppard et al., 1993). The biological activity of the compounds with vitamin E action has been measured using different methodologies: resorption fetal test in rats, haemolysis test induced by dialuric acid and muscular dystrophy prevention. Antioxidant activity has been also measured depending on the chemical reactivity of the molecules. Antioxidant and biological activity are not always coincident (Kayden and Traber, 1993). For example, γ-tocpherol has the highest *in vitro* antioxidant activity but not *in vivo* (Dutta-Roy et al., 1994). Antioxidant protection of vitamin E in tissues and meat can also be determined by measuring the concentration of oxidation end-products such as malondialdehyde (Kubo et al., 1997), cholesterol oxidation products (Lopez-Bote et al., 1998) or lipid hydroperoxides (Grau et al, 2000).

α-tocopherol has three chiral centres in the isoprenoid chain (2, 4' and 8'). Because of this, there are 8 stereoisomers of α-tocopherol: each chiral centre can be in R (or d) conformation or S (or l). One of them, RRR-α-tocpeherol, is the major form found in nature. Synthetic vitamin E is an equimolar mixture of all stereoisomers (*all-rac*-α-tocpherol or dl-α-tocpherol). The bioactivity of the

stereoisomers is not the same among them, and some studies with rats and humans suggest that this difference is due to a selective uptake of the forms with R conformation in the 2 position (2R forms) (Ingold et al., 1987; Scherf et al., 1996) and not to a different antioxidant activity. Recently, a work in broiler chickens has been published (Cortinas et al., 2004*b*) where it is shown that chicken liver also discriminates in favour of the 2R forms.

The usual way to supply α-tocopherol in poultry feeds is in the form of *all-rac*-α-tocopheryl acetate, the synthetic form, where a molecule of acetate is esterified in the position 6 of the ring. In the intestine, the ester is hydrolyzed and α-tocopherol becomes active. This way, α-tocopherol is protected from oxidation during processing and storage of the feeds. Humidity is the main factor that can decrease α-tocopherol stability, especially in the presence of iron and copper (Surai, 1999). 1 mg of *all-rac*-α-tocopheryl acetate is defined as international unit (IU) and the other forms are expressed in UI with respect to *all-rac*-α-tocopheryl acetate (see table 7), to supply depending on the bioactivity independently of the vitamin E source used.

Table 7. Vitamin E equivalencies (Machlin, 1984).

Source	Vitamin E activity	
	UI	% of 2R
all-rac-α-tocoferyl acetate	1.00	67
RRR-α-tocoferyl acetate	1.36	91
all-rac-α-tocopherol	1.10	74
RRR-α-tocopherol	1.49	100

Currently, vitamin E is expressed in α-tocopherol equivalents (α-TE) rather than in IU, where 1 mg of α-TE is 1 mg of RRR-α-tocopherol or 1.49 mg of *all-rac*-α-tocopheryl acetate (Morrissey and Sheehy, 1999). This seems logic, given that the 2R forms are more bio-available for the animal.

1.5.3.2. Absorption and metabolism

Data regarding vitamin E absorption and metabolism processes in birds are scarce but there is a high amount of information in mammals (Ingold et al.,

1987; Cohn, 1997; Scherf et al., 1996; Traber and Sies, 1996; Traber and Arai, 1999; Brigelius-Flohe and Traber, 1999, Herrera and Barbas, 2001, Mardones and Rigotti, 2004). There are two reviews by Surai (1999 and 2002) that collect the information obtained from mammalian studies and adapt it to the physiological characteristics of the avian species.

It is commonly accepted that vitamin E intestinal uptake occurs following the processes necessary for digestion and absorption of dietary fats. Hence, bile (Gallo-Torres, 1970) and pancreatic secretions are necessary for a correct digestion and absorption of vitamin E. Therefore, the type and amount of dietary fat will likely have an important effect on vitamin E uptake. In a study with rats, Gallo-Torres et al. (1971) have stated that PUFA interfere somehow with α-tocopherol in the gastrointestinal tract, reducing its absorption. Recently, some studies have questioned this theory (Tijburg et al. 1997).

Hence, vitamin E absorption and deposition can be affected by different dietary factors, such as the amount of fat, the degree of polyunsaturation of the diets, the presence of pro-oxidant substances (oxidized fat, iron, copper, etc.), the dose of vitamin E inclusion and the form of supplementation (for instance, Jensen et al. (1999) showed that α-tocopheryl acetate is more available for broiler chickens than α-tocopheryl succinate). There is great complexity in the relationship involving the PUFA source, the amount of dietary fat, the dietary ratio ω3:ω6 PUFA , the form of vitamin E consumed and the tissues examined (Center, 2004). The age and sex of the animal are non-nutritional factors that could also affect vitamin E uptake.

In general, there is not much information in birds concerning the factors affecting vitamin E uptake and deposition, and some of these factors will be the object of study of the experimental work in this dissertation.

In short, the gastrointestinal events are (Cohn et al., 1992; Surai, 1999):

1. Emulsification: It is a physical process that, by the means of gastrointestinal motility, breaks down dietary fat globules into smaller

lipid droplets. This way, the contact surface of dietary fat with the intestinal medium is greatly increased. This process starts in the gizzard and is accelerated in the small intestine, where bile salts stabilize the lipid droplets.

2. Solubilization: Pancreatic lipase, with the help of colipase, hydrolyzes dietary TAG into free FA and 2-monoglycerides (2-MG) (figure 9). Phospholipases break down dietary phospholipids into a free FA and a lysophospholipid. Pancreatic esterase (or carboxyl ester hydrolase) hydrolizes cholesterol, retinol and α-tocopherol esters. This step is important because α-tocopherol is absorbed mainly in its free form. The products of dietary fat hydrolysis then combine with bile salts and biliary phospholipids to form mixed micelles (figure 10). This step is called micellar solubilization. The more polar hydrolysis products (2-MG, unsaturated FA, phospholipids) expand the micelle and thus facilitate the incorporation of the more hydrophobic compounds (long-chain SFA, cholesterol, cholesterol esters, fat-soluble vitamins) (Krogdahl, 1985).
3. Difusion across the unstirred water layer: Mixed micelles are water soluble and can cross the unstirred water layer to reach the brush border membrane of enterocytes.

Figure 9. Hydrolysis of dietary triacylglycerols by pancreatic lipase.

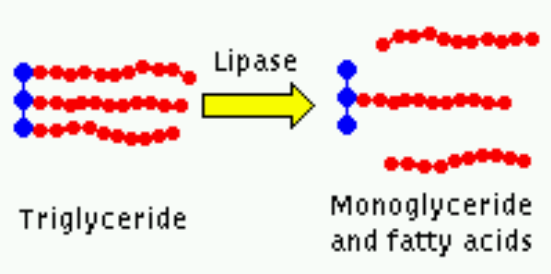
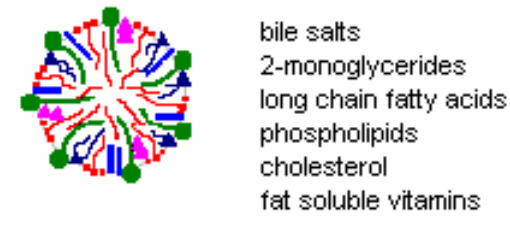


Figure 10. Mixed micelle.



4. Permeation through the membrane: Once the unstirred water layer has been crossed, it seems that the reduced pH of the microenvironment causes disruption of the micelle (Besnard et al., 1996) and the hydrolysis products are released. α-tocopherol diffuses passively into the enterocytes.
5. Incorporation into lipoproteins: The intracellular trafficking of tocopherols is not well-known, not even in mammals. It is unknown if there is a cytosolic tocopherol binding protein that transports tocopherols until the Golgi apparatus to be incorporated into nascent lipoproteins.
6. Release into circulation: Avian enterocytes secrete mainly portomicrons (PM) and some very low density lipoproteins (VLDL). Since avian species do not have lymphatic system, lipoproteins are secreted into the portal vein. There are no specific plasma proteins to carry vitamin E.

Behrens and Madère (1983) and Ingold et al. (1987) state that there is some biodiscrimination between α-tocopherol stereoisomers in the intestinal uptake in favor of 2R forms. However, Traber et al. (1990) affirm that there is no discrimination during this process.

PM in portal vein are carried directly to liver but are not metabolized because they are too big to cross between the cells of the capillary vessels of the liver (Hermier, 1997). The TAG in the PM are hydrolyzed by lipoprotein lipase (LPL), an enzyme expressed by some tissues (adipose tissue, muscle) in the endothelium of the capillaries. These tissues thus obtain FA and glycerol from PM, and they can also obtain dietary tocopherols. It seems that LPL acts as a "bridge" between PM and tissues in order to facilitate tocopherol transfer to tissues (Mardones and Rigotti, 2004)

As TG (core material of PM) is taken up by tissues from PM, there is an excess surface material in this lipoproteins (including vitamin E). This excess surface is readily transferred to other circulating lipoproteins, mainly high density lipoproteins (HDL). The metabolized PM are taken up by the liver (PM remnants).

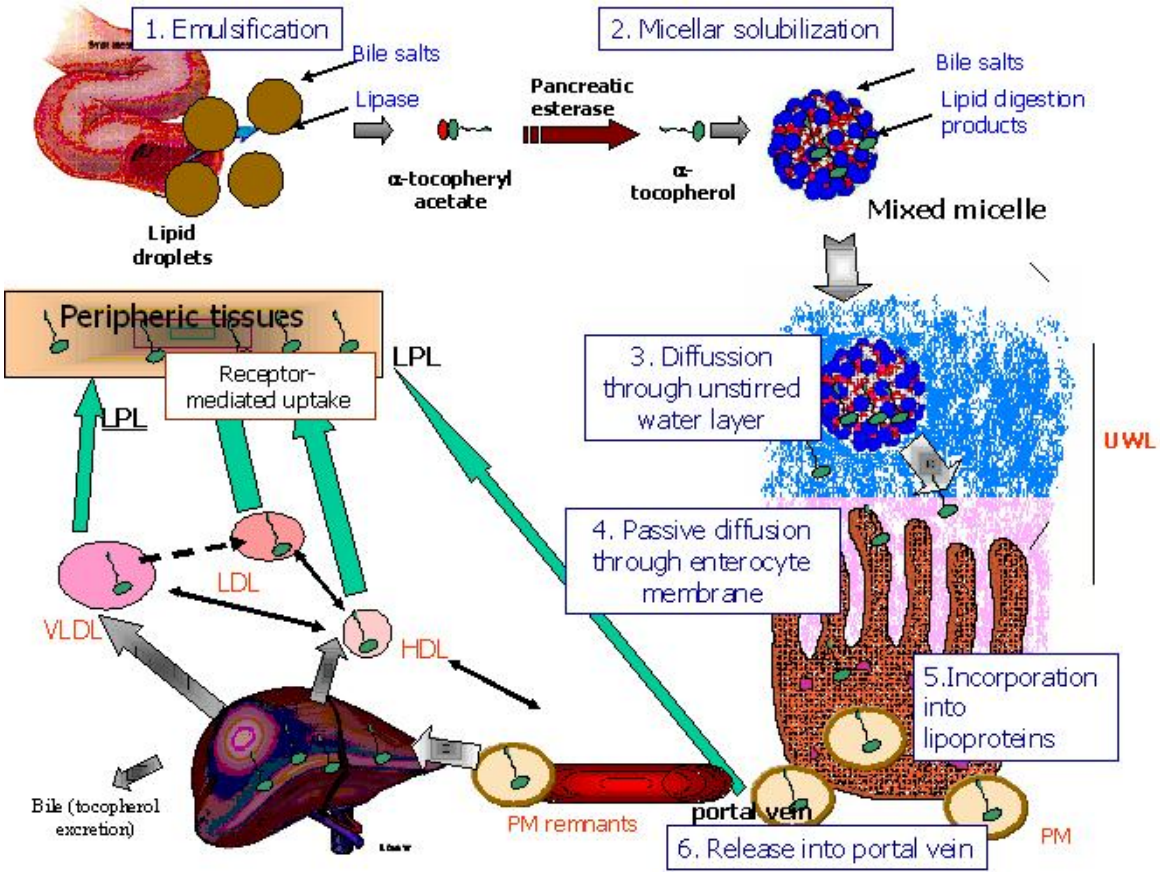
The liver is the main site for α-tocopherol discrimination. An α-tocopherol binding protein has been described in rats and humans, but not yet in chickens, and several tissues, mainly liver and brain, as reviewed by Blatt et al. (2001). The mechanism by which it works is not perfectly understood, but the result is that hepatic VLDL are preferentially enriched in the 2R forms of α-tocopherol. Other vitamin E isomers and α-tocopherol stereosiomers are excreted in the bile (Traber et al., 1990).

VLDL is metabolized in the circulation by LPL, similarly to PM. As VLDL loose TAG, they are converted into intermediate density lipoproteins (IDL) and low density lipoproteins (LDL). LDL transfer α-tocopherol to HDL. This way, all circulating lipoproteins have α-tocopherol.

Hence, the cells can obtain their α-tocopherol by different mechanisms (Mardones and Rigotti, 2004): uptake facilitated by lipid transfer proteins (phospholipids transfer protein) and lipases (LPL); receptor-mediated lipoprotein endocytosis (LDL and HDL) and by selective uptake (from HDL, without net uptake of the lipoprotein). This redundancy makes sure that tissues receive vitamin E when one of the mechanisms fails.

Surai and Sparks (2000) measured α-tocopherol concentration in different tissues of chicken supplemented with 40 mg/kg of α-tocopheryl acetate. The greatest concentration is found in internal fat, liver and after in heart, pancreas, lung, spleen, muscle, testes, kidney, brain and finally cerebellum. Many works use α-tocopherol concentration in plasma, although it is regulated by the liver, because it responds to dosage increases to a certain extent and it is in equilibrium with the tissular concentration (Ingold et al., 1987). All tissues in general respond to vitamin E supplementation but the turnover rates differ greatly among them. Lung, liver and small intestine have the greatest turnover rates and muscle, brain and especially spinal chord the lowest (Ingold et al., 1987). Excretion of vitamin E is mainly via bile and some part is excreted in the urine.

Figure 11. Summary of α-tocopherol absorption and metabolism.



1.5.3.3. Functions

It is believed that α-tocopherol main function is to protect PUFA present in biological membranes from lipid peroxidation (Burton, 1994). α-tocopherol is positioned in the biological membranes among the phospholipids as shown by figure 12. There is 1 molecule of α-tocopherol for 2000-3000 phospholipids and it is considered that 1 molecule can protect 20,000 FA (Surai, 1999). However, vitamin E is not randomly distributed in the phospholipids bilayer, there are domains enriched with vitamin E (Quinn, 2004).

Vitamin E donates its phenolic hydrogen (in the position 6 of the chromanol ring) to lipid free radicals, turning into a tocopheroxyl radical, much less reactive than lipid hydroperoxides:

LOO● + Toc-OH ----------→ LOOH + Toc-O●

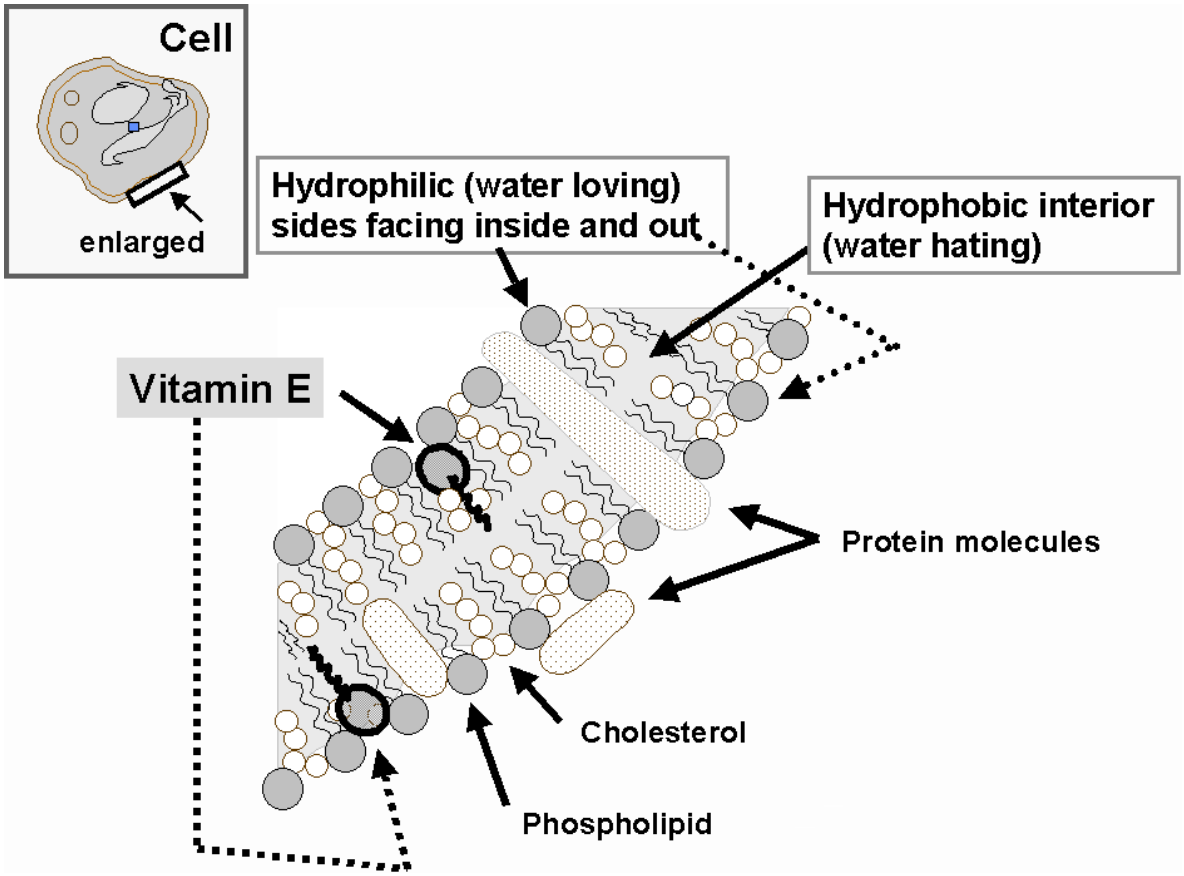
(LOO● : peroxyl radical; Toc-OH: tocopherol; LOOH: hydroperoxide; Toc-O●: tocopheroxyl radical)

α-tocopherol scavenges the peroxyl radical 10 times faster than lipid reacts with the radical (Niki, 1996). It is considered that α-tocopherol can be recycled from the tocopheroxyl radical by molecules with redox activity such as ascorbate, glutathione, carotenoids or ubiquinol, but this has not yet been demonstrated *in vivo* (Surai, 1999).

α-tocopherol can also quench singlet oxygen, which can initiate the lipid oxidation chain reaction.

There are plenty of works showing that PUFA oxidation during meat storage or heat processing of broiler meat can be attenuated by vitamin E supplementation in the diets (Jensen et al., 1998; Ruiz et al., 1999; Bou et al., 2001, Cortinas et al., 2005).

Figure 12. Positioning of α-tocopherol inside biological membranes.



Although α-tocopherol has received most of the attention, recently the study of other vitamin E forms is increasing. For example, it seems that γ-tocopherol can

have important protective functions against cardiovascular disease due to its preferential reaction with reactive nitrogen species (Hensley et al., 2004).

Besides its antioxidant function, α-tocopherol has other effects. It has been described a membrane stabilizing effect, by forming complexes with hydrolysis products of membrane lipids (such as free fatty acids), which have destabilising effects on the bilayer structure of the membrane (Erin et al., 1984).
Other non-antioxidant functions of vitamin E are reviewed by Azzi et al. (2002) and Rimbach et al. (2002) among others. These functions include inhibition of smooth muscle proliferation via inhibition of protein kinase C, up-regulation of α-tropomyosin expression, modulation of α-TTP expression, down-regulation of oxidized LDL scavenger receptor, inhibition of monocyte-endothelial adhesion, inhibition of platelet adhesion and aggregation, hypotensive effect, etc. These effects are mainly protein kinase C mediated or modulated by changes in gene expression. The importance of these effects on the physiology of the organism is still not clearly defined.

For all the above mentioned, there is an increasing interest in creating functional foods enriched in this nutrient. However, the inclusion of high doses of vitamin E in human clinical studies is not achieving the expected results despite the epidemiological relationship between high plasma α-tocopherol concentration and a lower incidence of heart disease (Gey et al., 1991). A recent meta-analysis (Miller et al., 2005) has even found that high-dosage vitamin E (α-tocopherol) supplementation may increase all-cause mortality. It has to be considered that vitamin E compounds (and, in general, all compounds with antioxidant activity) have redox activity, and these substances can be antioxidant or pro-oxidant depending on the environment, for example, the presence of other antioxidants (Stocker, 1999). More research is necessary in order to determine the best vitamin E intake in order to get the expected additional benefits of this vitamin (and of antioxidant supplements) against disease.

Chapter 2

Objectives

Rule of Accuracy
When working toward the solution of a problem, it always helps if you know the answer.
Corollary
Provided, of course, that you know there is a problem.

Nonreciprocal Laws of Expectations:

1. Negative expectations yield negative results.
2. Positive expectations yield negative results.

Hein's Law
Problems worthy of attack prove their worth by hitting back.

Persig's Postulate
The number of rational hypotheses that can explain any given phenomenon is infinite.

The European Union legislation (CE 198/2002) states the clear relationship between animal and human nutrition. In the introduction it has been shown that feeding polyunsaturated fatty acids to poultry is an effective means to create PUFA-enriched chicken tissues and it is used to nutritionally improve poultry meat. On the other hand, the problem of the bovine spongiphorm encephalopathy has caused a rejection to the use of fats of animal origin (such as tallow and lard), and their substitution for vegetable oils, mainly insaturated, in the formulation of feeds. It has also been commented in the introduction that the increase in the unsaturation level of the feeds can have deleterious effects. Some of these effects can be overcome by the use of antioxidants, being vitamin E one of the most utilized.

There are not many studies that deepen the relationship between the two nutrients in poultry. The global objective of this PhD dissertation is to evaluate the **interaction between polyunsaturated fatty acids and vitamin E content in chicken feeds upon different factors affecting fatty acid and vitamin E deposition**.

This objective can be divided in partial objectives

Effect of the intake of PUFA-rich diets on:

1. chemical composition (mainly lipid deposition and FA profile) and energy content of the whole body of chickens.
2. the potential of endogenous fatty acid synthesis of the organism
3. vitamin E apparent absorption
4. and vitamin E deposition.

Effect of vitamin E supplementation on:

5. PUFA aparent absorption and deposition in the whole body
6. and vitamin E apparent absorption and deposition.

7. It is also an objective of this work to assess the validity of plasmatic and hepatic α-tocopherol concentration as estimators of whole body α-tocopherol content.

In order to achieve these objectives, two experiments have been designed, described in detail in chapter 3, based in increasing inclusion levels of PUFA and α-TA:

In trial 1, the polyunsaturation gradient was created by replacing a saturated source of fat (tallow) by a mixture of polyunsaturated oils (linseed and fish oils), keeping added fat constant (9%). This first trial allowed the comparison of different fat sources upon chemical composition and fatty acid and α-tocopherol deposition. It also permitted the adjustment of the different techniques used during the development of this thesis.

In trial 2, the PUFA gradient was achieved by increasing added fat inclusion level (2, 4, 6 and 8%). Added fat was a mixture of linseed and fish oil like the one used in trial 1. This design permitted us to determine the potential of endogenous fatty acid synthesis and to assess the effect of increasing dietary fat levels upon chemical composition and both fatty acid and α–tocopherol deposition.

Chapter 3

Materials and Methods

Nothing is as easy as it looks. Everything takes longer than you think. Everything takes longer than it takes.

Turnauckas's Observation
To err is human; to really foul things up takes a computer.

Behind every little problem there's a larger problem, waiting for the little problem to get out of the way.

Williams and Holland's Law
If enough data is collected, anything may be proven by statistical methods.

Ordering Principle
Those supplies necessary for yesterday's experiment must be ordered no later than tomorrow noon.

Ashley-Perry Statistical Axioms

1. Numbers are tools, not rules.
2. Numbers are symbols for things; the number and the thing are not the same.
3. Skill in manipulating numbers is a talent, not evidence of divine guidance.
4. Like other occult techniques of divination, the statistical method has a private jargon deliberately contrived to obscure its methods from nonpractitioners.
5. The product of an arithmetical computation is the answer to an equation; it is not the solution to a problem.
6. Arithmetical proofs of theorems that do not have arithmetical bases prove nothing.

3.1. ANIMALS AND HOUSING

Two experimental studies with broiler chickens were designed in order to achieve the proposed objectives. Both studies were carried out in the experimental farms of the Veterinary School of the Universitat Autònoma de Barcelona. The experimental protocol was approved by the Ethics Committee of the Universitat Autònoma de Barcelona. All animals housing conformed to the European Union guidelines.

A total of 192 one-day female broiler Ross strain 308 chickens were used in each of the trials[1]. The animals were randomly distributed in 48 cages (distributed in 3 floors) and kept under controlled conditions of temperature, humidity and ventilation. Food and water were provided *ad libitum*. Controls of temperature, ventilation, health status and casualties were carried out daily.

In both trials the animals were randomly assigned to one of the 16 experimental treatments, with 3 replicates of 4 birds each. The animals arrived to the facilities with one day of age and were kept in floor pens until the beginning of the trials. The experimental period took place from day 8 to day 44 (trial 1) and from day 6 to day 40 of age (trial 2).

3.2. DIETS

The experimental diets were formulated to meet or exceed the requirements indicated by the NRC (1994). The diets were produced in the feeds manufacturing plant of IRTA[2] (Institut de Recerca i Tecnologia agroalimentàries). The composition of the diets was as follows:

3.2.1. Trial 1

The basal diet was composed of wheat, soybean and barley, to which different types of fat were added (table 8). The experimental treatments resulted from a 4x4 factorial arrangement, where the input factors were the following:

[1] Granja Solé, Tarragona, Spain (trial 1) and Terra-Avant, S.A., Anglés, Girona, Spain (trial 2).
[2] Mas Bové , Reus, Tarragona, Spain

- Polyunsaturation degree: The polyunsaturation degree was achieved by replacing tallow[3], a saturated fat, by a mixture of linseed[3] and fish oil[4], polyunsaturated-rich oils in different proportions, keeping the percentage of added fat constant (9%). The achieved PUFA content of the diets was 15, 34, 45 and 61 g/kg (table 9) (treatments PU15, PU34, PU45 and PU61).

- Level of supplementation with α-tocopheryl acetate (table 10).

  - *Not supplemented*: 0 mg/kg (treatment E0)
  - *Supplemented*: 100, 200 and 400 mg/kg of α-tocopheryl acetate[5] (treatments E100, E200 and E400).

Table 8. Composition and chemical analysis of the diets. Trial 1[1].

Ingredients	%	Chemical analysis	%
Wheat	39,30	Dry matter	90,78
Soybean 48	34,09	Crude protein	22,98
Barley	13,39	Crude fat	10,17
Added fat	9,00	Crude fibre	3,47
Bicalcium phosphate	2,17	Ashes	6,08
Calcium carbonate	0,98	AME PU15[3]	2718
Salt	0,45	AME PU34[3]	3040
Vitamin-mineral mix[2]	0,40	AME PU45[3]	3168
DL-Methionine	0,28	AME PU61[3]	3168
L-Lysine	0,04		

[1] Values are the average of 16 determinations.
[2]Vitamin and mineral mix per kg of feed: Vitamin A: 12000 UI; Vitamin D3: 2400 UI; Vitamin K3: 3 mg; Vitamin B1: 2.2 mg; Vitamin B2: 8 mg; Vitamin B6: 5 mg; Vitamin B12: 11 μg; Folic acid: 1.5 mg; Biotin: 150 μg; Calcium pantotenate: 25 mg; Nicotinic acid : 65 mg; Mn: 60 mg; Zn: 40 mg; I: 0.33 mg; Fe: 80 mg; Cu: 8 mg; Se: 0.15 mg.
[3]Apparent metabolizable energy, measured experimentally (kcal/kg).

[3] Cailà I Parés, Barcelona, Spain
[4] Agrupación de Fabricantes de Aceites Marinos, Vigo, Spain
[5] Rovimix® E-50 Adsorbate, F. Hoffmann-La Roche Ltd., Basel, Switzerland

Table 9. Inclusion levels (g/kg of feed) of the different fat sources and polyunsaturation level of the diets (g PUFA/kg feed). Trial 1[1].

	Added fat			Dietary polyunsaturation
	Tallow	Linseed oil	Fish oil	
	g/kg feed			g PUFA/kg
PU15	90	0	0	15
PU34	55	30	5	34
PU45	35	45	10	45
PU61	0	70	20	61

[1]PU15: 15 g PUFA/kg; PU34: 34 g PUFA/kg; PU45: 45 g PUFA/kg; PU61: 61 g PUFA/kg.

Table 10. Measured values α-tocopherol in the diets (mg/kg). Trial 1[1].

Dietary polyunsaturation[2]	Dietary supplementation with α-tocopheryl acetate[3]			
	E0	E100	E200	E400
PU15	5 ± 0.3	135 ± 7.3	252 ± 13.3	441 ± 32.4
PU34	6 ± 0.7	136 ± 3.7	243 ± 15.3	452 ± 12.3
PU45	6 ± 0.8	138 ± 11.2	232 ± 5.7	477 ± 25.0
PU61	5 ± 0.6	135 ± 6.3	219 ± 7.9	436 ± 47.1

[1] These values are means ± SE.
[2] PU15: 15 g polyunsaturated fatty acids /kg of feed; PU34: 34 g polyunsaturated fatty acids /kg of feed; PU45: 45 g polyunsaturated fatty acids /kg of feed; PU61: 61 g polyunsaturated fatty acids /kg of feed.
[3]E0: without supplementation with α-tocopheryl acetate; E1: supplemented with 100 mg/kg α-tocopheryl acetate; E2: supplemented with 200 mg/kg α-tocopheryl acetate; E4: supplemented with 400 mg/kg α-tocopheryl acetate.

3.2.2. Trial 2

The main ingredients were maize and soybean (table 11). The experimental treatments resulted from a 4x4 factorial arrangement, where the input factors were the following:

- Polyunsaturation degree: The polyunsaturation degree was achieved by increasing the inclusion level of the added fat, a mixture of linseed[3] and fish oil[4] in a ratio of 4 to 1. The addition of 2, 4, 6 and 8% of this oil (treatments O2, O4. O6 and O8) resulted in PUFA contents of 27, 38, 48 and 59 g /kg. In order to

achieve isoenergetic diets, almond husk was added as energy diluter. Chroumium oxide[6] (Cr_2O_3) was included at 0.18% as a digestibility marker.

- Level of supplementation with α-tocopheryl acetate (table 12).
    - *Not supplemented*: 0 mg/kg
    - *Supplemented*: 100, 200 and 400 mg/kg of α-tocopheryl acetate[5] (treatments E100, E200 and E400).

In both trials, oils were sampled before feed manufacturing. Feeds were also sampled during manufacturing and in different moments during the experimental period. Samples were pooled and kept at -20ºC until analysis.

Table 11. Composition and chemical analysis of the diets. Trial 2[1].

Ingredients (%)	**Added oil level**			
	2%	**4%**	**6%**	**8%**
Corn	58,49	52,68	46,86	41,02
Soybean 48	35,49	36,42	37,34	38,24
Almond husk	0,00	2,92	5,84	8,76
Added fat	2,00	4,00	6,00	8,00
Bicalcium phosphate	1,75	1,76	1,76	1,77
Calcium carbonate	1,08	1,07	1,06	1,05
Salt	0,57	0,57	0,57	0,57
Mineral-vitamin mix[2]	0,40	0,40	0,40	0,40
DL-Methionine	0,18	0,19	0,19	0,20
L-Lysine	0,05	0,03	0,02	0,00
Chemical analysis (%)				
Dry matter	88,26	89,08	89,42	90,28
Crude protein	21,31	21,82	21,48	21,85
Crude fat	4,45	6,30	8,40	9,91
Crude fibre	3,22	5,45	7,93	9,86
Ashes	5,79	6,10	5,85	6,20
AME[3]	3246	3282	3228	3261

[1] Values are the means of 4 determinations.
[2]Vitamin and mineral mix per kg of feed: Vitamin A: 12000 UI; Vitamin D_3: 2400 UI; Vitamin K_3: 3 mg; Vitamin B_1: 2,2 mg; Vitamin B_2: 8 mg; Vitamin B_6: 5 mg; Vitamin B_{12}: 11 µg; Folic acid: 1,5 mg; Biotin: 150 µg; Calcium pantotenate: 25 mg; Nicotinic acid: 65 mg; Mn: 60 mg; Zn: 40 mg; I: 0,33 mg; Fe: 80 mg; Cu: 8 mg; Se: 0,15 mg.
[3]Apparent metabolizable energy, measured experimentally (kcal/kg).

[6] Sigma-Aldrich Chemical Co., St Louis, MO.

Table 12. Measured values of α-tocopherol in the diets (mg/kg). Trial 2[1].

Level of added oil[2]	Dietary supplementation with α-tocopheryl acetate[3]			
	E0	**E1**	**E2**	**E4**
O2	4 ± 0.2	105 ± 8.1	173 ± 2.2	351 ± 7.6
O4	3 ± 0.2	98 ± 3.0	200 ± 7.6	354 ± 7.1
O6	3 ± 0.3	96 ± 1.2	198 ± 0.7	336 ± 16.5
O8	3 ± 0.2	96 ± 6.9	191 ± 14.1	316 ± 21.2

[1] These values are means ± SE.
[2] O2, O4, O6, and O8: 2%, 4%, 6%, and 8% of added oil (65.2% PUFA), respectively.
[3]E0: without supplementation with α-tocopheryl acetate; E1: supplemented with 100 mg/kg α-tocopheryl acetate; E2: supplemented with 200 mg/kg α-tocopheryl acetate; E4: supplemented with 400 mg/kg α-tocopheryl acetate.

3.3. PERFORMANCE CONTROLS

Food consumption and weight were controlled three times during the trials: at 8, 23 and 44 days (trial 1) and at 6, 26 and 40 days of age (trial 2). Average feed intake, average daily gain and food to gain ratio were determined from these data.

3.4. BALANCE STUDIES

Two balance studies were carried out in both trials to determine apparent absorption of organic matter, fat, fatty acids and α-tocopherol and to determine apparent metabolizable energy (AME).

3.4.1. Trial 1

The two balances were carried out between days 19 and 23 and between days 37 and 39. Due to water loss problems in the second balance, data from this balance is not available. The procedure followed was the total excreta collection.

3.4.2. Trial 2

The two balances were carried out between days 19 and 21 and on day 39. In the first balance both the total excreta collection and the inert digestibility marker (chromium oxide) methods were used, in order to compare them. Due to

the similarity of results obtained by the two methods, in the second balance only the chromium oxide method was used.

3.5. SAMPLING

1. Feed samples were obtained during the performance controls: at 8, 23 and 44 days (trial 1) and at 6, 26 and 40 days of age (trial 2).

2. Excreta samples were obtained during the balance studies.
    a) Total excreta collection method: The excreta from each cage were sampled after weighing every day and samples from different days belonging to the same cage were pooled.
    b) Inert digestibility marker method: The excreta from each cage were sampled at day 20 and 39 of age.

3. At the end of the trials (44 days for trial 1 and 40 days for trial 2) half of the animals were killed in a commercial slaughterhouse and thigh and breast portions were destined to a parallel experiment focused on lipid oxidation (PhD thesis of L. Cortinas, 2004). Abdominal fat pad and liver were weighted and obtained from these animals. The other half of the birds (96) was killed by lethal injection with sodium pentobarbitate (200 mg/kg). The animals were frozen in plastic bags and kept at -20ºC.
4. In trial 2, 5 mL of blood were obtained in EDTA-coated tubes from 48 animals (3 per treatment).

3.6. PROCESSING AND STORAGE OF THE SAMPLES

Feed samples

Feed samples taken during different times of the trial were homogenized, milled and divided in two bags for each sample. One of the bags was kept at -20ºC and the other at -80 ºC (for α-tocopherol analysis).

Excreta

Excreta samples were homogenized into a single sample for each cage. They were freeze-dried[7], ground and divided in two bags for each sample. One of the bags was kept at -20ºC and the other at -80 ºC (for α-tocopherol analysis). In trial 2, an antioxidant solution (1 mg/kg of EDTA plus 1 mg/kg of pyrogallol) was added to prevent oxidation during storage. Also, these samples were vacuum packed just after freeze-drying them.

Liver

Liver samples were homogenized, freeze-dried, ground and kept at -80ºC.

Whole chicken

The whole chicken was minced frozen with a cutter[8] until its complete homogenization. (c.a. 5 minutes). A representative sample was taken, freeze-dried, ground and divided in two bags for each sample. One of the bags was kept at -20ºC and the other at -80 ºC (for α-tocopherol analysis). In trial 2, an antioxidant solution (1 mg/kg of EDTA plus 1 mg/kg of pyrogallol) was added to prevent oxidation during storage. Also, these samples were vacuum packed just after freeze-drying them.

Blood

Blood tubes were centrifuged 5 minutes at 4000 rpm. The plasma samples were kept in aliquots at -80ºC.

3.7. ANALITICAL DETERMINATIONS

All analytical determinations were carried out in the laboratories of the Animal Nutrition unit of the Veterinary School of the UAB, except for α-tocopherol concentration of the whole body in trial 2, which was determined in the Avian Science Research Centre of the Scottish Agricultural College (UK).

The analytical determinations are shown in table 13. All determinations from excreta and plasma were carried out in 3 samples per treatment (n=48, one

[7] FTS Systems: condenser Dura-Dry™ model FD2055D0T000
[8] Tec Maq model cut-20, INTEFISA

sample per each cage). The analytical determinations from liver and whole body were made in 6 samples per treatment (n=96). Fatty acid and α-tocopherol content of the feeds was made in one pooled sample per treatment (n=16).

Table 13. Analytical determinations of the experimental samples.

Feed	Excreta	Liver	Whole body	Plasma
Weende	Dry matter Ashes Hydrolized Crude fat	Dry matter	Dry matter Ashes Crude protein Hydrolized Crude fat	
Gross Energy	Gross Energy		Gross Energy	
α-tocopherol	α-tocopherol	α-tocopherol	α-tocopherol*	α-tocopherol*
α-tocopheryl acetate	α-tocopheryl acetate			
Fatty acids	Fatty acids		Fatty acids	
Chromium	Chromium			

*Only in the second trial

3.7.1. Analytical determinations of the experimental oils

Oils and fats used in both trials were analyzed for moisture, impurities, unsaponifiable matter, acidity and peroxide value (table 14). Fatty acid profile and α-tocopherol content was also measured (table 15).

Table 14. Analytical measurements of the fats and oils used in the experimental trials[1].

		Moisture (%)	Impurities (%)	Unsaponifiable matter (%)	Acidity (g oleic/100g)	Peroxide value (mEq O_2/kg)
Trial 1	Tallow	0,05	0,09	0,25	0,31	3,40
	Linseed oil	ND	ND	0,57	0,28	4,14
	Fish oil	ND	ND	0,54	0,32	4,51
Trial 2	Linseed oil	0,06	0,45	0,50	1,78	6,12
	Fish oil	ND	0,26	0,90	3,74	23,58

[1]ND: Not detected.

Table 15. Fatty acid profile (direct area normalization, % of total fat) and α-tocoferol (mg/kg) of the experimental oils[1].

	Trial 1			Trial 2	
	Tallow	Linseed oil	Fish oil	Linseed oil	Fish oil
SFA	46,78	8,88	39,91	9,21	36,72
C14:0	3,08	0,04	8,33	0,05	8,61
C15:0	0,45	ND	0,36	0,02	0,58
C16:0	25,18	5,27	19,49	5,49	20,99
C17:0	1,24	0,07	1,87	0,07	0,44
C18:0	16,65	3,25	6,69	3,39	3,78
C20:0	0,14	0,13	2,48	0,18	0,29
MUFA	48,47	18,31	27,30	18,68	25,80
C16:1 ω7t	0,16	0,01	0,24	0,03	0,27
C16:1 ω7	2,68	0,09	8,37	0,07	11,47
C18:1 ω9	44,76	17,02	13,99	17,88	8,97
C18:1 ω7	0,20	0,88	2,90	0,68	3,84
C20:1 ω9	0,23	0,14	0,55	ND	0,20
C21:1 ω9	ND	0,14	0,80	ND	0,35
C24:1 ω9	ND	0,02	0,19	0,02	0,48
PUFA	4,75	72,81	32,79	72,11	37,48
C18:2 ω6	2,99	14,98	0,81	14,90	0,08
C18:3 ω3	0,36	56,38	0,49	56,79	1,22
C20:2 ω6	0,07	0,05	0,29	ND	0,23
C20:3 ω3	0,05	0,05	0,09	ND	ND
C20:4 ω6	0,08	ND	0,90	ND	0,18
C20:5 ω3	0,04	0,34	20,26	0,13	25,17
C22:4 ω6	0,02	0,07	0,14	0,10	0,11
C22:6 ω3	0,11	0,10	7,28	0,18	9,32
α-Tocopherol	ND	10,7	18,7	10,6	40,5

[1]SFA: Saturated fatty acids, MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.
ND: Not detected

3.7.2. Chemical composition of feed, excreta, whole body and liver

Weende analysis of the feeds was carried out following the methodology described in the AOAC (1995). Ash and hydrolyzed crude fat content of the faeces, and ash, crude protein and hydrolyzed crude fat content of the whole body was also determined (AOAC, 1995). Dry matter content of faeces, liver and whole body was determined as indicated by the Journal Official des Communités Européenes L279/9.

3.7.3. Chromium

Chromium concentration was analyzed in feed and excreta samples following the methodology described by Williams et al. (1962). Briefly, 0.6 (excreta) or 1 g

(feeds) of sample was incinerated 4 hours at 550ºC. The ashes were digested with acid and analyzed by atomic absorption.

3.7.3. Gross Energy content

Gross energy (GE) was determined in feed, excreta and whole body samples by the means of an adiabatic bomb calorimeter[9].

3.7.4. Fatty acid content

FA were determined following the methodology described by Sukhija and Palmquist (1988) for feed (table 16) and excreta samples, and the one described by Carrapiso et al. (2000) for the rest of the samples. Briefly, these techniques consist in a direct transesterification: lipid extraction and fatty acid methylation is achieved in only one step. The sample is incubated at 70ºC with metanolic chloride. After that, the organic layer is extracted with toluene. Nonadecanoic acid (C_{19})[6] was added at the beginning of the procedure as an internal standard.

The heptane extracts were injected in a Gas-Chromatograph[10] of the Servei d'Analisi Quimica of the UAB. The method conditions were the following:

Capillary column: Hewlett Packard HP-23 (cis/trans FAME column) 60 m $\times$ 0,25 mm internal diameter with a thickness of stationary phase film of 0,25 µm (USA).

Carrier gas: Helium.

Flow: 1,3 mL/min.

Detector: Flame ionization detector (FID).

Temperature program of the oven:

From 140°C to 160°C:1,50°C/min.

From 160°C to 180°C: 0,50°C/min.

From 180°C to 230°C: 2,50°C/min.

Injector and detector temperature: 280°C.

Injection volume: 1 µL.

[9] IKA-calorimeter C4000 adiabatic

[10] HP6890, Agilent, Waldbronn, Germany.

Peak areas were integrated and converted to concentrations with comparison with the internal standard peak area.

Concentration FA_x = (Area FA_x/Area C_{19}) × (µg C_{19} / (RC × simple weight), where RC is the response coefficient.

Identification of fatty acids was made by comparison between retention times of the simple peaks with the retention time of the standards[6] and also with a mass spectrometer[11]

[11] HP5973, Agilent, Waldbronn, Germany.

Table 16. Fatty acid composition of the experimental diets[1], expressed as g per kg.

	Trial 1[2]				Trial 2[3]			
	PU15	**PU34**	**PU45**	**PU61**	**O2**	**O4**	**O6**	**O8**
% Added oil	9	9	9	9	2	4	6	8
g PUFA/kg	15	34	45	61	28	38	48	59
Total FA	100.45	98.81	99.57	96.89	45.37	60.29	75.70	91.63
Fatty Acid								
SFA	43.75	32.38	26.22	15.74	7.89	9.79	11.65	13.66
C 10:0	0.05	0.03	0.02	0.00	0.08	0.08	0.08	0.08
C 14:0	2.72	2.01	1.79	1.45	0.30	0.53	0.76	0.99
C 15:0	0.44	0.30	0.23	0.11	0.03	0.05	0.07	0.09
C 16:0	23.80	18.15	15.25	10.31	5.82	6.84	7.84	8.96
C 17:0	1.19	0.77	0.53	0.14	0.06	0.08	0.09	0.11
C 18:0	14.64	10.23	7.68	3.33	1.39	1.92	2.44	2.98
C 20:0	0.12	0.16	0.17	0.17	0.16	0.19	0.22	0.26
MUFA	41.30	32.55	28.32	20.31	9.91	12.83	15.90	19.07
C 16:1t	0.20	0.15	0.12	0.07	0.02	0.03	0.03	0.04
C 16:1	2.25	1.73	1.65	1.52	0.40	0.71	1.01	1.32
C 18:1 ω9[5]	35.62	27.76	23.59	15.69	8.67	10.85	13.21	15.67
C 18:1 ω7t	1.60	1.37	1.29	1.12	0.49	0.67	0.86	1.05
C 20:1	0.28	0.29	0.31	0.35	0.15	0.23	0.30	0.38
C 24:1	0.09	0.46	0.81	1.46	0.10	0.11	0.12	0.14
PUFA	15.40	33.77	45.03	60.84	27.60	37.66	48.08	58.79
C 18:2 ω6	13.16	16.23	17.98	20.17	17.79	18.72	19.87	21.31
C 18:3 ω3	1.55	16.45	24.62	36.27	8.57	16.65	24.63	32.69
C 18:4 ω3	0.27	0.11	0.23	0.43	0.14	0.27	0.41	0.53
C 20:4 ω6	ND	ND	0.13	0.19	ND	ND	0.12	0.14
C 20:5 ω3	ND	0.81	1.77	3.35	0.72	1.38	2.07	2.75
C 22:6 ω3	ND	0.07	0.18	0.33	0.28	0.53	0.76	1.01
PUFA:SFA	0.35	1.04	1.72	3.87	3.50	3.85	4.13	4.30

[1] Values are means of 4 dietary treatments with different level of supplementation with α-TA: 0, 100, 200 and 400 mg/kg.
[2]PU15: 15 g polyunsaturated fatty acids /kg of feed; PU34: 34 g/kg dietary polyunsaturated fatty acids; PU45: 45 g/kg dietary polyunsaturated fatty acids; PU61: 61 g/kg dietary polyunsaturated fatty acids.
[3] O2: 2% of added oil; O4: 4% of added oil; O6: 6% of added oil; O8: 8% of added oil.
[4] Total FA: total fatty acids; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
[5] C 18:1 ω9 includes sum of cis and trans forms.
ND: Not detected.

3.7.5. α-tocopherol content

Feeds, liver and plasma

α-tocopherol from feed, liver and plasma samples was analyzed following the methodology described by Jensen et al. (1999), with some modifications. In trial 1, 100 mg of liver were weighed in a 10-mL tube. 2.0 mL of ethanol, 0.5 mL of methanol, 1 mL of an ascorbic acid aqueous solution (20%) and 0,3 mL of a potassium hydroxide aqueous solution (1:1 w/w) were added to the sample. After shaking the samples 10 seconds in the vortex, the tubes were saponified in a water bath at 70ºC during 20 minutes. The tubes were cooled under tap water during 20 minutes. All the processes described above were carried out under red light to avoid oxidation of α-tocopherol. Afterwards, 5 mL of heptane were added and the tubes were shook in the vortex during 30 seconds. After centrifugation (10 minutes at 1500 g) the organic layer was transferred to another 10 mL tube. The aqueous phase was re-extracted with 5 mL of heptane. After shaking with vortex and centrifugation, the organic layer was collected together with the first one and the resulting solution was injected directly into the HPLC[12].

Feed samples were processed in the same way but using more solvent volumes. 2 g of samples were weighed in a 250 mL flask, where 70 mL of ethanol, 30 mL of methanol, 30 mL of ascorbic acid aqueous solution (20%) and 20 mL of potassium hydroxide aqueous solution (1:1 w/w) were added. Once saponified, 2.0 mL of the samples were transferred to a 10 mL tube with 1,0 mL of distilled water. Like in liver samples, α-tocopherol was extracted with a total of 10 mL of heptane in two steps.

In liver, plasma and feed samples from trial 2, α-tocopherol was extracted in a similar way (from 100 mg of liver sample, 0.5 mL of plasma and 2.0 g of feed) but BHT was added to reduce α-tocopherol oxidation during the extraction process. BHT was included in the methanol in a 0.02 % (w/v). This modification resulted in better recovery values of the method.

[12] Agilent 1100 series, Waldbronn, Germany.

The chromatographic conditions were the ones described by Jensen et al. (1999): Column: Perkin Elmer HS-5-Silica, 125 × 4 mm internal diameter[13].

Eluent: heptan modified with 0,5% of 2-propanol.

Flow: 1,3 mL/min.

Detector: Fluorescende detector (290 nm excitation and 327 nm emission).

Injection volume: 100 μL.

Identification of α-tocopherol was made by comparison with the retention time of the external standard[6].

Feeds and excreta (balance studies)

α-tocoferol in its free form and α-tocopheryl acetate (α-TA) in feeds and faeces were analyzed following the method described by Lee et al. (1999), with some modifications. The extraction procedure was carried out under red light to minimize sample oxidation. 100 mg of sample were weighed in a 10 mL tube. 250 μg approximately of phenyl-dodecane[6] in 2-propanol were added as an internal standard (Ruperez *et al*., 1999). 400μL of UHP water at 80ºC 80ºC, 1 mL of 2-propanol, 0.5 g of anhidrous sodium sufate and 2.5 mL of extraction solvent (85% hexane (v/v):15% ethyl-acetate (v/v): 0.05 BHT (w/v)) were added to the tubes. The tube contents were homogenized during 1 minute[14] and centrifuged at 1500 g during 5 minutes. The organic layer was transfered to 10 mL glass tubes. The organic solvent was dryed under a stream of nitrogen and the pellet was rediluted in 500 μL of methanol. This was injected directly in the HPLC.

The chromatographic conditions were the following (Rupérez *et al*., 1999):

Reverse phase column Waters Spherisorb® 5μm ODS2 of 250 x 4.6 mm of internal diameter.

Eluent: methanol

Flow: 1.3 mL/min

Detectors:

[13] Perkin-Elmer, Überlingen, Alemania.
[14] Homogenizer Omni 2000, from Omni International

1) Fluorescence detector[15]. The excitation wavelength was 260 nm and the emission wavelength was 600 nm the first 8 minutes, to detect the internal standard (phenyl-dodecane). From 8 to 15 minutes, the excitation and emission wavelengths were 295 nm and 330 nm to detect α-toc.

2) Ultraviolet detector[12]. The first 8 minutes the wavelength was 260 nm to detect phenyl-dodecane. Afterwards, it was 284 to detect α-TA.

Injection volume: 20 μL.

α-tocopherol and α-TA were identified by comparing the retention times with the external standards[6]. Quantification was made by the means of the internal standard.

Whole body

The technique of Jensen et al. (1999), used for α–tocopherol determinations in liver and plasma samples was tried in the whole chicken samples, but the recovery percentages were extremely low. Several techniques suitable for meat samples were tried, but in the meantime the samples from trial 1 were not reliable and are not available. Maybe the presence of minerals promoted excessive oxidation of the sample during the extraction procedures. Finally, a methodology adapted from Surai et al. (1996) was used for α–tocopherol determination in whole body of samples in trial 2 in the Avian Science research Centre of the Scottish Agricultural College (UK) laboratories. Also the extraction procedure was carried out under red light. Briefly, 500 mg of sample were weighed in 25 mL glass tubes. 5 mL of ethanol with 10% pyrogallol and 1.25 mL of potassium hydroxyde (60% in water) were added to the tubes. After vortex shaking, N_2 was added to the tubes and they were capped. The samples were saponified in a water bath at 70ºC for 30 minutes. They were cooled under tap water. After cooling, 7 mL of sodium chloride and 5 mL of hexane were added and the tubes were vortex shaken again. The tubes were placed in a box with ice during 30 minutes in the dark. Afterwards, the organic layer was collected in a glass vial. A second extraction with 5 mL of hexane was performed and both extractions were collected in the same glass vial. Hexane was then evaporated

[15] Hewlett-Packard 1100 series, Agilent, Waldbronn, Germany

under nitrogen and the pellet re-suspended in 200 μL of dichloromethane and 200 μL of methanol. This was injected in the HPLC after centrifugation.

The chromatographic conditions were the following:

Reverse phase column: waters spherisorb 5 μ ODS2 4.6 x 150 mm Analytical Cartridge

Flow: 1.05 mL/min

Injection volume: 50 μL

Detector: Fluorescence detector. The excitation and emission wavelengths were 295 and 330 nm.

α-tocopherol was identified by comparing the retention times with the external standard[6].

3.8. CALCULATIONS

Apparent absorption of organic matter, crude fat (only in trial 1) **and fatty acids**

It was calculated from the data obtained in the balance studies. It has to be considered that chickens excrete faeces and urine together and the term digestibility is not correct, that is why we use "apparent absorption".

For total excreta collection method, the following formula was used

% Apparent absorption of X = [(X intake-X excretion)/(X intake)]*100

where X is organic matter, crude fat or fatty acids.

For the inert marker method, the following formula was used:

% Apparent aborption of X = {1-[(Cr_2O_3 in feed/Cr_2O_3 in excreta)/(X in excreta/X in feed)]}*100

where Cr_2O_3 is the concentration of the inert marker and X is the concentration of organic matter or fatty acids.

Apparent absorption of α-tocopherol

It was calculated from the data obtained in the balance studies.

To calculate apparent absorption of α-tocopherol, α-tocopherol equivalents (α-TE) of feed and excreta were calclulated considering that 1 mg of α-tocopheryl acetate is 0.67 mg of α-tocopherol equivalents (Weiser and Vecchi, 1982).

Hence:

mg α-TE = mg α-tocopherol + mg α-tocopheryl acetate x 0.67

Once the α-TEs were obtained, apparent absorption was calculated as follows for the total excreta collection method data:

% α-TE apparent absorption= ((α-TE intake - α-TE excretion)/(α-TE intake))*100

For the inert marker method data, the formula was the following:

% α-TE apparent absorption = {1-[(Cr_2O_3 in feed/Cr_2O_3 in excreta)/(α-TE in excreta/α-TE in feed)]}*100

Hydrolysis of α-TA

It was calculated from the data obtained in the balance studies as the percentage of dietary α-TA that disappeared between ingestion and excretion and was calculated as follows for the total excreta collection method:

% α-TA hydrolysis= ((α-TA intake - α-TA excretion)/(α-TA intake))*100

For the inert marker method data, the formula was the following:

% α-TA hydrolysis = {1-[(Cr_2O_3 in feed/Cr_2O_3 in excreta)/(α-TA in excreta/α-TA in feed)]}*100

Apparent metabolizable energy intake

It was calculated using the first balances of both trials and with the total excreta collection method data. First, the percentage of AME of the diets was calculated:

% AME = ((GE intake – GE excretion)/(GE intake))*100

AME intake = %AME x GE of feed (kcal/kg) x Average daily intake (kg/animal/day).

3.9. STATISTICAL ANALYSIS

The SAS statistical package (SAS® Institute, 2002) was used for the analysis.

In all cases, values of $P \leq 0.05$ were considered significant.

ANOVA

Analysis of variance was carried out to assess whether the dietary factors (polyunsaturation and supplementation with α-tocopheryl acetate) had any effect upon the studied parameters: apparent absorption of organic matter, crude fat, fatty acids and α-TE, α-TA percentage of hydrolysis, AME intake, chemical (water, ashes, crude fat, crude protein, total fatty acids) and energy composition of the whole body, fatty acid composition of the whole body and α-tocopherol content of liver, plasma and whole body of chickens. The experimental unit for the apparent absorption coefficients and the AME intake was the cage (3 replicates per treatment, 4 birds per cage) whereas for the rest of parameters the experimental unit was the chicken (6 birds per treatment). Differences between treatment means were tested using Tukey's correction for multiple comparisons

In all cases, the data were analyzed through the GLM procedure of SAS and the level of significance was pre-set at $p<0.05$.

Regresion analyses

Various linear regression analyses were carried out with the obtained experimental data with the use of the REG procedure of SAS (SAS Institute, 2002). These analyses are described in the results section, at the beginning of each chapter.

ADDENDUM

Two more experiments were carried out by the author during the development of the present experimental study. These experiments are not going to be presented in the results section of this thesis, but the results obtained have been used in the discussion section.

One of these experimental works was carried out during winter of 2002 in the experimental farms of the Veterinary School of the Universitat Autònoma de Barcelona, with the collaboration of the Nutrition and Bromatology Department of the Pharmacy Faculty (Universitat de Barcelona). We studied the apparent absorption and deposition of α-tocopherol in the presence of oxidized dietary sunflower oil. The second experiment took place in the experimental station of Unterer Lindenhof (germany) in spring of 2003 in collaboration with the Institut für Tierzuchtung of Hohenheim University (Stutgart, Germany). In this experiment, apparent absorption and deposition of α-tocopherol was assessed depending on the type of added fat (10%): cocoa (MCFA), palm oil (SFA), olive oil (MUFA), soybean oil (ω6 PUFA) and linseed oil (ω3 PUFA).

The Harvard Principle
Under the most rigorously controlled conditions of pressure, temperature, volume, humidity, and other variables the organism will do as it damn well pleases.

Finagle's Creed
Science is Truth. Don't be misled by fact.
Finagle's First Law
If an experiment works, something has gone wrong.
Finagle's Second Law
No matter what result is anticipated, there will always be someone eager to (a) misinterpret it, (b) fake it, or (c) believe it happened according to his own pet theory.
Finagle's Third Law
In any collection of data, the figure most obviously correct, beyond all need of checking, is the mistake.
Corollaries
No one whom you ask for help will see it.
Everyone who stops by with unsought advice will see it immediately.

Grossman's Misquote
Complex problems have simple, easy to understand wrong answers.

Horngren's Observation: (generalized)
The real world is a special case.

Maier's Law:
If the facts do not conform to the theory, they must be disposed of.
Corollaries
The bigger the theory, the better.
The experiment may be considered a success if no more than 50% of the observed measurements must be discarded to obtain a correspondence with the theory. (Compensation Corollary).

Chapter 4

Effect of the dietary degree of poliunsaturation on abdominal fat pad weight, chemical composition and gross energy content of the whole body in broiler chickens

4.1. Summary

Two experiments were carried out to study the effect of a dietary gradient of polyunsaturated fatty acids (PUFA) on abdominal fat pad weight and chemical composition of the whole body of chickens. The PUFA gradient was achieved following two strategies. In trial 1, different fat sources (tallow, linseed oil and fish oil) were blended in different ratios keeping the added fat level constant (9%). The four dietary treatments had 15, 34, 45 and 61 g PUFA/kg of diet. In experiment 2, the gradient was obtained by increasing the level of inclusion (2, 4, 6 and 8 %) of PUFA-rich oil (a mixture of linseed and fish oil). The dietary treatments had 28, 38, 48 and 59 g PUFA/kg of diet. Apparent metabolizable energy ingestion was similar in both experiments, except for PU15, where it was lower. In trial 1, abdominal fat pad weight, total body fat and body energy was lower in the animals on the high PUFA diets ($p<0.05$) compared to the animals on the saturated-rich ones. Water, crude protein and ash content were not changed with increasing dietary polyunsaturation. In trial 2 a similar body fat content was obtained with increasing added PUFA-rich oil inclusion level.

4.2. Statistics

The α-TA dosage level was excluded from the statistical model once checked that it had indeed no effect on any of the studied parameters ($p>0.05$ in all cases). This lack of effect was expected, because α-TA was included in the experimental design in order to assess its effect upon fatty acid profile and α-tocopherol uptake and deposition, but not upon chemical and energy composition in the chicken.

Statistical analysis was carried out separately for each trial through ANOVA. The input factors were dietary PUFA in trial 1 and dietary fat level in trial 2. Data were treated using the GLM procedure of SAS® package (SAS Institute, 2002). The experimental unit was the chicken (24 animals per treatment) except for AME intake, where the experimental unit was the cage (12 cages per treatment). Differences between treatment means were tested using Tukey's correction for multiple comparisons. The correlation of gross energy content and gross energy estimations was assessed by regression analysis using the REG

procedure of SAS package. In all cases, P values ≤ 0.05 were considered significant.

4.3 Results

TRIAL 1

Performance parameters: Average daily gain, average daily intake, feed-to-gain ratio and final body weight are presented in table 17. There were no differences among treatments regarding average daily gain and final body weight, but the animals fed the more saturated had a higher average daily intake ($p<0.05$). Therefore, the feed-to-gain ratio was worse for the PU15 treatment ($p<0.005$).

Table 17. Performance parameters. Trial 1[1].

	PU15	**PU34**	**PU45**	**PU61**	
% Added oil	**9**	**9**	**9**	**9**	RSD[3]
g PUFA[2]/kg	**15**	**34**	**45**	**61**	
ADG[2]	59.5	61.0	60.0	59.7	2.98
ADI[2]	111.7[a]	108.3[ab]	105.8[b]	104.2[b]	6.07
F:G[2]	1.9[a]	1.8[b]	1.8[b]	1.7[b]	0.07
Final BW[2]	2297.9	2351.2	2319.0	2304.1	108.65

[1]Values are least square means obtained from ANOVA (n=12). Means in a row not sharing a superscript letter differ ($p<0.05$).
[2]PUFA, polyunsaturated fatty acids; ADG; average daily gain (g/animal/day); ADI, average daily intake (g/animal/day); F:G. feed-to-gain ratio (g/g); BW, body weight (g).
[3]RSD, residual standard deviation.

Aparent Metabolizable Energy Intake: AME intake values (kcal/animal/day) are shown in table 18. The animals consuming the more saturated diet had a lower ME intake compared to the other dietary treatments.

Abdominal fat weight. Abdominal fat pat (AFP) weight, expressed as grams per 100 grams of live weight, decreased gradually with increasing dietary PUFA (table 18).

Table 18. Effect of dietary polyunsaturation on metabolizable energy intake and abdominal fat pad weight. Trial 1[1].

	PU15	PU34	PU45	PU61	RSD
% Added oil	**9**	**9**	**9**	**9**	
g PUFA/kg	**15**	**34**	**45**	**61**	
ME intake	299[b]	333[a]	338[a]	334[a]	20.2
AFP weight	1.17[a]	1.01[ab]	1.10[ab]	0.76[b]	0.469

[1]Values are least square means obtained from ANOVA (n=12). Means in a row not sharing a superscript letter differ (p<0.05).
[2]PUFA, polyunsaturated fatty acids; ME, metabolizable energy (kcal/animal/day); AFP, abdominal fat pad (g/100 g of live weight).
[3]RSD, residual standard deviation.

Chemical composition and Gross Energy content. Chemical composition and Gross Energy (GE) content of the whole chicken are shown in table 19. Dietary polyunsaturation did not significantly affect crude protein (CP) and ash content, although there was a tendency (p<0.10) towards a higher value in these two measures with high levels of dietary PUFA.

As to hydrolyzed Crude Fat (CF), increasing polyunsaturation of the diets led to a dramatic decrease (more than 50% between PU15 and PU61) of CF. Dietary PUFA caused also a significant decrease (p<0.05) in GE between PU15 and PU61, but of considerably less magnitude (10%). Given that protein content was not different between treatments, GE differences should reflect basically body fat variations and thus, both CF and GE should respond to the dietary treatments in a similar proportion. This incongruence between CF and GE leads one to think that there may be a problem with hydrolyzed CF determination. To check the reliability of CF, the sum of CP, ash and CF (in a dry matter (DM) basis) was calculated. The sum should be practically 100% of the DM of the whole body (given that carbohydrate content is negligible) but moves away from 100 (99.7, 99.3, 98.2 and 92.1% for PU15, PU34, PU45 and PU61 respectively) as dietary PUFA increase. In the face of the doubts raised by CF, Substracted Fat (SF) and Total Fatty Acid content (TFA) were considered as possible estimators of body fat content. SF was calculated as fresh matter (FM) minus water, ash and CP (considering carbohydrate content negligible). TFA is the

sum of all individual fatty acids quantified in the chicken. Both SF and TFA values are shown in table 5. The sum of CP, ash and TFA is more constant than when using CF instead and slightly lower than 100, because TFA does not account for the glycerol moiety of triglycerides and phospholipids (98.6, 98.1, 98.8 and 97.2 % for PU15, PU34, PU45 and PU61 respectively).

Table 19. Effect of dietary polyunsaturation on chemical composition (% fresh matter) and gross energy content of the whole body. Trial 1[1].

	PU15	**PU34**	**PU45**	**PU61**	RSD[4]
% Added oil	**9**	**9**	**9**	**9**	
g PUFA/kg	**15**	**34**	**45**	**61**	
Water	62.31	62.57	63.27	62.72	1.807
CP[2]	19.42	19.77	19.84	20.04	0.812
CF[2]	15.48[a]	14.49[a]	12.52[b]	6.73[c]	2.226
Ash	2.48	2.48	2.61	2.66	0.270
GE[2]	2526[a]	2438[ab]	2391[ab]	2260[b]	181.9
SF[3]	15.65	15.24	14.80	14.14	2.261
TFA[2]	14.37[a]	13.24[ab]	13.10[ab]	11.78[b]	2.292

*Values are least square means obtained from ANOVA (n=12). Means in a row not sharing a superscript letter differ ($p<0.05$).
[2]CP, crude protein; CF, crude fat; GE, gross energy (kcal/kg); TFA, total fatty acids.
[3]Substracted Fat, calculated as follows: Fresh matter – water – ash - CP, considering negligible the carbohydrate content of the body.
[4]RSD, residual standard deviation.

To further check if SF and TFA are adequate estimators of body fat, gross energy estimations were made using the following formula (Agricultural Research Council, 1980):

Estimated GE (kcal) = CP kg x 5.64 kcal/kg + (CF or SF or TFA) kg x 9.39 kcal/kg

Regression equations were determined between real GE (X) (measured by calorimetric bomb) and estimated GE (Y) (table 20). It can be observed that the worse estimation is from CF measurements (R^2=0.68) and both SF and TFA are acceptable indicators of body fattening (R^2=0.86 and 0.87 respectively).

Table 20. Regression equations between gross energy (GE) content of the whole body of chickens (X, kcal) and gross energy estimations (Y, kcal). Trial 1.

Dependent variable (Y)[1]	Equation	P value	R^2	CV [5](%)
GE1[2]	Y = -1870 + 1.26*X	<0.001	0.68	10.78
GE2[3]	Y = 602 + 0.91*X	<0.001	0.86	3.68
GE3[4]	Y = 0.94*X	<0.001	0.87	4.38

[1]Estimated GE (kcal) = CP kg x 5.64 kcal/kg + CF/SF/TFA kg x 9.39 kcal/kg (Agricultural research Council, 1980).
[2]Estimated from CF (hydrolyzed crude fat) (Association of Analytical Chemists, 1995).
[3]Estimated from SF (subtracted fat) (SF=Fresh matter – ashes – crude protein – water; considering carbohydrate content negligible)
[4]Estimated from TFA (total fatty acids content) without considering the glycerol moiety.
[5]Coefficient of variation.

All this suggests that there may be a problem with Soxhlet fat extraction with highly unsaturated samples, during the previous hydrolization with hydrochloric acid or during the ether extraction, both procedures involving high temperatures.

SF is not affected by dietary PUFA. However, TFA content decreases (around an 18%) when dietary polyunsaturation increases ($p<0.001$) from 15 to 61 g PUFA/kg, similarly to GE content. Hence, it has to be considered that in this experiment SF was unresponsive to dietary PUFA contrary to GE and TFA. SF can accumulate all the errors of the analytical determinations of CP and ashes and have somewhat a lower sensitivity, but it may be useful if TFA are not determined. Due to these findings, we decided not to measure CF in trial 2 and use TFA as an estimator of body fat content.

TRIAL 2

Performance parameters: There were no differences among treatments regarding average daily gain (61.8 ± 0.42 g/animal/day), average daily intake (106.5 ± 0.74 g/animal/day), feed-to-gain ratio (1.7 ± 0.01 g/g) and final body weight (2240.3 ± 14.61 g).

Aparent Metabolizable Energy Intake. ME intake values (kcal/animal/day) are shown in table 21. There were no differences between treatments regarding this parameter.

Abdominal fat weight. Abdominal fat pat (AFP) weight, expressed as grams per 100 grams of live weight, was not affected by fat inclusion level (table 21).

Table 21. Effect of inclusion level of added oil on metabolizable energy intake and abdominal fat pad weight. Trial 2[1].

	O2	**O4**	**O6**	**O8**	
% Added oil	**2**	**4**	**6**	**8**	RSD[3]
g PUFA[2]/kg	**28**	**38**	**48**	**59**	
ME[2] intake	329	348	340	347	19.2
AFP[2] weight	0.66	0.66	0.77	0.57	0.25

[1]Values are least square means obtained from ANOVA (n=12). Means in a row not sharing a superscript letter differ ($p<0.05$).
[2]PUFA, polyunsaturated fatty acids; ME, metabolizable energy (kcal/animal/day); AFP, abdominal fat pad (g/100 g live weight).
[3]RSD, residual standard deviation.

Chemical composition and Gross Energy content. Chemical composition and Gross Energy (GE) content of the whole chicken are shown in table 22. Given the results obtained in trial 1, CF of whole chicken was not determined and in the table we show the values of TFA as a measure of body fat. Water, CP, ash and TFA content of the whole chicken were not affected by varying inclusion levels of added polyunsaturated fat ($p>0.05$). Expectedly, GE content of the animal was not affected by treatment either ($p>0.05$).

TABLE 22. Effect of inclusion level of added oil on chemical composition (% FM) and gross energy content of the whole body. Trial 2[1].

	O2	**O4**	**O6**	**O8**	RSD[3]
% Added oil	**2**	**4**	**6**	**8**	
g PUFA[2]/kg	**28**	**38**	**48**	**59**	
Water	67.64	68.06	68.12	67.75	1.101
CP[2]	20.31	20.38	20.51	20.35	0.776
Ash	2.43	2.48	2.49	2.61	0.277
GE[2]	2272	2271	2253	2245	164.9
TFA[2]	10.49	10.19	10.51	11.08	1.683

[1]Values are least square means obtained from ANOVA (n=12). Means in a row not sharing a superscript letter differ ($p<0.05$).
[2]PUFA, polyunsaturated fatty acids; CP, crude protein; GE, gross energy (kcal/kg); TFA, total fatty acids.
[3]Substracted Fat, calculated as follows: Fresh matter – water – ash - CP, considering negligible the carbohydrate content of the body.
[4]RSD, residual standard deviation.

Chapter 5

Effect of the dietary degree of poliunsaturation and vitamin E supplementation on fatty acid composition and potential of endogenous fatty acid synthesis in the whole body of broiler chickens

5.1. Summary

Two experiments were performed to assess the effect of a dietary gradient of polyunsaturated fatty acids (PUFA) on fatty acid composition of the whole body of chickens. In trial 1, different fat sources were blended in different ratios allowing a gradient of dietary PUFA, keeping added fat level constant (9%). In trial 2, PUFA-rich oil was added in increasing inclusion levels (2, 4, 6 and 8 %). The contribution of endogenous fatty acid synthesis to fatty acid profile of the body was estimated. Vitamin E was added (0, 100, 200 and 400 mg/kg) in both trials to assess its effect upon fatty acid profile, but it had no significant effect, except for some very long chain fatty acids of the ω6 family. Increasing dietary PUFA inclusion resulted in an increase of PUFA deposition, with higher efficiency when dietary fat also provided saturated (SFA) and monounsaturated (MUFA) fatty acids (trial 1). Increasing dietary PUFA in both trials resulted in a decrease of SFA and MUFA concentration in the body. The estimated deposition of fatty acids from endogenous synthesis is reduced when dietary fat increases from 0 to 10%, varying between 35.34 % and 17.66 % for SFA; and between 52.70 % and 7.01 % for MUFA in the whole body. The higher variation range for the MUFA supports the existence of a mechanism maintaining the SFA: (MUFA+PUFA) ratio inside a specific range in biological membranes.

5.2. Statistics

Statistical analysis of the fatty acid profile of the whole body was carried out separately for each trial by analysis of variance, where the input class factors were dietary PUFA and α-TA (trial 1) and dietary fat level and α-TA (trial 2). Data were treated using the GLM procedure of SAS package (SAS® Institute, 2002). Differences between treatment means were tested using Tukey's correction for multiple comparisons. The experimental unit was the chicken (6 animals per treatment).

To compare saturated, monounsaturated and polyunsaturated concentrations (% of total fatty acids) between the two trials, regression analyses were performed

using the REG procedure of SAS between PUFA intake (g/animal/day) and SFA, MUFA and PUFA content in the tissues (% of total fatty acids). To estimate the potential of endogenous synthesis in both trials linear regression analyses were performed using the REG procedure of SAS between the SFA and MUFA intakes (g/animal/day) and their respective concentration in the tissue (% of total fatty acids). In both cases, the experimental unit was the cage, because daily intake was measured per cage and not individually.

Also, as established by Crespo & Esteve-Garcia (2002*c*), the ratio between SFA, MUFA and PUFA of the whole chicken and SFA, MUFA and PUFA of the feeds (% of total fatty acids) was calculated as an indicator of endogenous synthesis. Values above 1 indicate net fatty acid synthesis and below 1 net fatty acid beta-oxidation. Data were treated using the GLM procedure of SAS package (SAS® Institute, 2002). α-TA was excluded from the model once checked it did not affect this parameter, hence the input factor was PUFA gradient (trial 1) or added fat level (trial 2). Differences between treatment means were tested using Tukey's correction for multiple comparisons. The experimental unit was the chicken (24 animals per treatment).

5.3. Results

Effect of dietary α-TA. Fatty acid profile of the whole body of chickens (tables 23 and 24) was not affected by α-TA inclusion, with the exception of 20:3 ω6 (0.11 vs 0.13% for the E0 and the E200, E400 treatments respectively, $p<0.05$), 20:4 ω6 (0.38 vs 0.45% for the E0 and the E200, E400 treatments respectively, $p<0.05$) and 20:5 ω3 (0.96 vs 1.12% E0 and the E200, E400 treatments respectively, $p<0.05$) in trial 1 and 20:4 ω6 (0.54 vs 0.61% for the E0 and the E400 treatments respectively, $p<0.05$) in trial 2.

Table 23. Effect of dietary polyunsaturation and α-tocopheryl acetate (α -TA) supplementation on fatty acid concentration (g/100g of total fatty acids) of the whole body. Trial 1[1].

Fatty acid	Polyunsaturation level				Significance		
	PU15	**PU34**	**PU45**	**PU61**	**Dietary PUFA[2]**	**α -TA[2]**	***RSD[3]***
% Added oil	**9**	**9**	**9**	**9**			
g PUFA/kg diet	**15**	**34**	**45**	**61**			
SFA[2]	33.02[a]	29.04[b]	27.24[c]	22.40[d]	<0.001	NS	1.229
14:0	2.07[a]	1.67[b]	1.49[c]	1.31[d]	<0.001	NS	0.064
15:0	0.52[a]	0.43[b]	0.34[c]	0.21[d]	<0.001	NS	0.025
16:0	21.08[a]	18.79[b]	17.20[c]	14.52[d]	<0.001	NS	0.782
17:0	1.17[a]	0.94[b]	0.73[c]	0.40[d]	<0.001	NS	0.077
18:0	7.92[a]	7.68[ab]	7.29[b]	5.98[c]	<0.001	NS	0.555
MUFA[2]	54.81[a]	43.41[b]	37.57[c]	28.75[d]	<0.001	NS	1.354
14:1	0.51[a]	0.35[b]	0.26[c]	0.13[d]	<0.001	NS	0.025
16:1	4.89[a]	3.91[b]	3.46[c]	3.09[d]	<0.001	NS	0.381
17:1	0.70[a]	0.57[b]	0.47[c]	0.31[d]	<0.001	NS	0.029
18:1 ω9	44.98[a]	35.56[b]	30.33[c]	22.17[d]	<0.001	NS	1.088
18:1 ω7	2.56[a]	1.87[b]	1.58[c]	1.39[d]	<0.001	NS	0.138
20:1	0.39[a]	0.28[b]	0.26[bc]	0.27[b]	<0.001	NS	0.020
PUFA[2]	12.15[d]	27.54[c]	35.19[b]	48.85[a]	<0.001	NS	1.393
16:2	0.05[b]	0.08[a]	0.10[a]	0.06[b]	<0.001	NS	0.023
18:2tt	0.18[a]	0.12[b]	0.08[c]	0.06[d]	<0.001	NS	0.011
18:2 ω6	9.44[c]	12.46[c]	13.87[b]	16.32[a]	<0.001	NS	0.670
18:3 ω6	0.08[a]	0.07[b]	0.07[b]	0.08[a]	<0.001	NS	0.014
18:3 ω3	1.07[d]	12.60[c]	18.34[b]	28.03[a]	<0.001	NS	0.721
18:4 ω3	0.34[b]	0.21[c]	0.15[d]	0.48[a]	<0.001	NS	0.045
20:2 ω6	0.09[b]	0.10[b]	0.10[b]	0.11[a]	<0.001	NS	0.011
20:3 ω6	0.13	0.12	0.12	0.12	NS	0.018	0.015
20:4 ω6	0.52[a]	0.39[b]	0.38[b]	0.41[b]	<0.001	0.009	0.076
20:5 ω3	0.05[d]	0.75[c]	1.24[b]	2.23[a]	<0.001	0.012	0.181
22:4 ω6	0.10[a]	0.07[b]	0.04[c]	0.05[c]	<0.001	NS	0.023
22:6 ω3	0.10[d]	0.54[c]	0.72[B]	0.92[A]	<0.001	NS	0.090
PUFA:SFA	0.37[d]	0.95[c]	1.29[b]	2.19[a]	<0.001	NS	0.125

[1]Values are least-squares means obtained from ANOVA (n=6). Given that the interaction was non significant, it is not included in the table. Means in a row not sharing a superscript letter differ ($p<0.05$).
[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; α-TA, α-Tocopheryl acetate.
[3]RSD, residual standard error.

Table 24. Effect of dietary added oil and α-tocopheryl acetate (α -TA) supplementation on fatty acid concentration (g/100g of total fatty acids) of the whole body. Trial 2[1].

Fatty acid	Polyunsaturation level				Significance		
% Added oil	**O2**	**O4**	**O6**	**O8**	**Added oil**	**α -TA[2]**	***RSD[3]***
g PUFA[2]/kg diet	**2**	**4**	**6**	**8**			
	28	**38**	**48**	**59**			
SFA[2]	27.54[a]	24.97[b]	22.16[c]	20.23[d]	<0.001	NS	1.068
14:0	0.07[d]	0.08[c]	0.09[b]	0.10[a]	<0.001	NS	0.006
15:0	0.12[b]	0.14[b]	0.15[a]	0.16[a]	<0.001	NS	0.018
16:0	20.47[a]	17.84[b]	15.16[c]	13.73[d]	<0.001	NS	0.946
17:0	0.09[d]	0.21[c]	0.29[b]	0.38[a]	<0.001	NS	0.082
18:0	6.76[a]	6.69[a]	6.45[a]	6.05[b]	<0.001	NS	0.500
20:0	0.20	0.21	0.20	0.19	NS	NS	0.038
MUFA[2]	42.15[a]	34.52[b]	29.35[c]	27.20[d]	<0.001	NS	2.085
14:1	0.14[a]	0.11[b]	0.09[c]	0.08[c]	<0.001	NS	0.016
16:1	4.71[a]	3.46[b]	2.84[c]	2.58[c]	<0.001	NS	0.429
17:1	0.04[c]	0.05[bc]	0.06[ab]	0.07[a]	<0.001	NS	0.015
18:1 ω9	33.56[a]	27.47[b]	23.19[c]	21.74[d]	<0.001	NS	1.765
18:1 ω7	1.76[a]	1.46[b]	1.34[c]	1.33[c]	<0.001	NS	0.078
20:1	0.31	0.30	0.30	0.31	NS	NS	0.016
PUFA[2]	30.58[d]	40.51[c]	48.49[b]	52.57[a]	<0.001	NS	2.200
16:2	0.13	0.14	0.14	0.14	NS	NS	0.013
18:2tt	0.05	0.04	0.04	0.05	NS	NS	0.008
18:2 ω6	18.20[b]	19.47[a]	20.02[a]	19.46[a]	<0.001	NS	1.139
18:3 ω6	0.14[a]	0.11[b]	0.0.10[c]	0.09[c]	<0.001	NS	0.014
18:3 ω3	9.03[d]	16.96[c]	23.71[b]	28.26[a]	<0.001	NS	1.021
18:4 ω3	0.27[d]	0.41[c]	0.50[b]	0.57[a]	<0.001	NS	0.045
20:2 ω6	0.25[a]	0.21[b]	0.19[c]	0.16[d]	<0.001	NS	0.024
20:3 ω6	0.10[c]	0.17[b]	0.23[a]	0.23[a]	<0.001	NS	0.025
20:4 ω6	0.70[a]	0.59[b]	0.57[b]	0.47[c]	<0.001	0.010	0.084
20:5 ω3	0.92[d]	1.52[c]	1.93[b]	2.18[a]	<0.001	NS	0.188
22:4 ω6	0.11[a]	0.08[b]	0.06[c]	0.05[d]	<0.001	NS	0.015
22:6 ω3	0.64[c]	0.90[b]	1.01[a]	0.92[b]	<0.001	NS	0.105
PUFA:SFA	1.13[d]	1.63[c]	2.20[b]	2.61[a]	<0.001	NS	0.164

[1]Values are least-squares means obtained from ANOVA (n=6). Given that the interaction was non significant, it is not included in the table. Means in a row not sharing a superscript letter differ (p<0.05).
[2]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; α-TA, α-Tocopheryl acetate.
[3]RSD, residual standard error.

Effect of dietary polyunsaturation on lipid composition

Increasing dietary PUFA inclusion, either at the expense of dietary SFA and MUFA (trial 1) or not (trial 2), had a great influence in fatty acid composition of the whole chicken (tables 23 and 24). In trial 1, SFA concentration in the chicken decreased with higher PUFA intakes. Particularly, C16:0, the main SFA, decreased in approximately the same percentage as the total SFA. Total MUFA also decreased, mainly due to the decrease in C18:1n9, which

comprises about 80% of total MUFA. As to PUFA, increasing its dietary concentration caused higher body deposition of these fatty acids. The concentration of 18:2n6 (linoleic acid) and all fatty acids from the n3 series increased ($p<0.001$), whereas the rest of the fatty acids of the n6 family (20:4n6 and 22:4n6) decreased ($p<0.001$) with higher dietary polyunsaturation.

In trial 2, the higher PUFA intake was accompanied by a higher SFA and MUFA intake as well. In spite of the higher SFA intake, SFA content of the chicken was reduced when dietary added oil level increased, and the same happened with MUFA. Considering PUFA, their concentration increased with higher PUFA intakes, similarly to the first trial. All n3 fatty acids were found in higher concentrations and very long chain n6 (20:2n6, 20:4n6 and 22:4n6) in lower concentrations in the high-fat treatments than in the low-fat ones.

Potential endogenous synthesis of fatty acids

In table 25, the ratios between SFA, MUFA and PUFA concentrations in whole animal and their concentration in the diets are shown. As established by Crespo and Esteve-Garcia (2002*c*), values above 1 indicate net fatty acid synthesis and values lower than 1 show net fatty acid beta-oxidation. Regarding SFA, in the first trial the ratio is lower than 1 in the PU15 and PU34 treatments, 1 in the PU45 and higher than 1 in the PU61. This suggests that in the latter case dietary SFA had to be synthesized. In the second trial the ratio decreases with increasing added oil but never reaches values lower than 1; this means that in trial 2 there is always a net synthesis of SFA, even in animals fed the diets with a high percentage of added fat. In the case of MUFA, there is a net synthesis for all treatments in both trials. Whereas in the first trial the ratio is relatively constant among treatments, in the second trial the ratio decreases as dietary oil increases, similarly to SFA ratio. PUFA ratio is always lower than one, because PUFA are not synthesized by the animal. In trial 1, the ratio is not different among treatments, but in trial 2 this ratio increases with added oil.

Table 25. Body-to-dietary fatty acid ratio[1] of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in response to increasing levels of polyunsaturation[2].

	Trial 1				
% Added fat	**9**	**9**	**9**	**9**	RSD[3]
PUFA (g/kg)	**15**	**34**	**45**	**61**	
SFA	0.76^{d}	0.89^{c}	1.03^{b}	1.39^{a}	0.041
MUFA	1.33^{b}	1.32^{b}	1.32^{b}	1.37^{a}	0.046
PUFA	0.79	0.80	0.78	0.78	0.040
	Trial 2				
% Added fat	**2**	**4**	**6**	**8**	RSD[3]
PUFA (g/kg)	**27**	**38**	**48**	**59**	
SFA	1.58^{a}	1.54^{a}	1.44^{b}	1.36^{c}	0.068
MUFA	1.91^{a}	1.62^{b}	1.40^{c}	1.29^{d}	0.086
PUFA	0.50^{d}	0.65^{c}	0.76^{b}	0.82^{a}	0.035

[1]Calculated as the ratio of SFA, MUFA and PUFA concentration in whole body (g/100 g of total fatty acids) between their respective concentrations in the diet (g/100 g of total fatty acids). Values are the means of 24 determinations.
[2]Values are least-squares means obtained from ANOVA (n=24). Means in a row not sharing a superscript letter differ ($p<0.05$).
[3]RSD, residual standard error.

Regression analyses were performed between SFA and MUFA intakes (g/animal/day) and their respective content (% of total fatty acids) in the whole body (Table 26) in order to estimate the endogenous synthesis potential. The intercept of the estimated equations from the first trial show the theoretical endogenous synthesis of SFA or MUFA when there is no SFA or MUFA intake but dietary fat level is high (approximately 10%). In the second trial, it represents the SFA or MUFA synthesis when no fat is added to the diets. For the whole body, the endogenous synthesis potential of SFA is 17.66% (confidence interval: from 16.86 to 18.45) for the first trial and 35.34% (confidence interval: from 33.94 to 36.74) for the second one. Concerning MUFA, the endogenous synthesis potential seen in the whole body in trial 1 was 7.01% (confidence interval: from 3.98 to 10.03) and 52.70% in trial 2 (confidence interval: from 49.38 to 56.03).

Table 26: Regression equations between saturated and monounsaturated fatty acids intakes (x, g/animal/day) and their concentration (y, g/100g of total fatty acids) in the whole body.

Trial		Equation	P value	R^2	CV^2 (%)
1	SFA[1]	y = 17.66 + 3.20·x	<0.001	0,94	3,43
2	SFA[1]	y = 35.34 - 10.18·x	<0.001	0,87	4,10
1	MUFA[1]	y = 7.01 + 10.25·x	<0.001	0,98	3,54
2	MUFA[1]	y = 52.70 - 12.62·x	<0.001	0,84	6,84

[1]SFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

[2]Coefficient of variation.

Chapter 6

Effect of the dietary degree of poliunsaturation and vitamin E supplementation on α-tocopherol apparent absorption and tissular deposition

6.1. Effect of dietary polyunsaturation on α-tocopherol apparent absorption and hepatic deposition in chickens

6.1.1. Summary

Vitamin E requirements are linked to dietary polyunsaturated fatty acids (PUFA) content due to the protective effect of vitamin E from lipid peroxidation. On the other hand, it has been suggested that dietary PUFA interfere with vitamin E absorption. A 4 x 4 factorial study was planned to asses the effect of dietary vitamin E inclusion level (0, 100, 200 and 400 mg/kg) and degree of polyunsaturation (15, 34, 45 and 61 g PUFA/kg) on vitamin E apparent absorption and tissue deposition in poultry. A total of 192 female broiler chickens were used. A digestibility balance was carried out between 19 and 23 days of age to calculate apparent absorption of fat and vitamin E. The livers of 96 animals were obtained at 44 days of age for vitamin E determination. The more saturated diet reduced fat and vitamin E apparent absorption while PUFA levels from 34 to 61 g/kg did not modify this parameter but reduced free α-tocopherol concentration in the excreta and reduced the hepatic vitamin E concentration, suggesting a greater gastrointestinal and systemic use of this vitamin. These results suggest that PUFA do not limit vitamin E absorption, although they may increase its degradation in the gastrointestinal tract and in the tissues.

6.1.2. Statistics

Statistical analysis was carried out through GLM procedure of SAS statistical package (SAS institute, 2002), where the input class factors were dietary PUFA and α-TA. The interaction was also included in the model. Differences between treatment means were tested using Tukey's correction for multiple comparisons. The experimental unit for apparent absorption, α-TA hydrolysis and α-tocopherol concentration in the excreta data was the cage (n=3 replicates of 4 animals), and for hepatic α-tocopherol concentration was the animal (n=6).

6.1.3. Results

Apparent absorption of organic matter, crude fat and total fatty acids

The apparent absorption coefficients of organic matter, crude fat and total fatty are shown in table 27. Organic matter digestibility tended to be lower for the animals fed the more saturated diet (PU15, p=0.059). Crude fat and total fatty apparent absorption values were higher in the PU45 and PU61, and the lower values corresponded to the PU15 treatment (p<0.001). The inclusion of different levels of α-tocopheryl acetate in the diets had no significant effect on these parameters (data not shown).

Table 27: Effect of dietary polyunsaturation and α-tocopheryl acetate inclusion levels on organic matter (OM), crude fat (CF) and total fatty acids (TFA) apparent absorption[1].

	Dietary polyunsaturation level					P values		
% apparent absorption	**PU15**	**PU34**	**PU45**	**PU61**	RSD[3]	**PU[2]**	**α-TA[2]**	**Interaction**
OM[2]	66.1	67.2	68.8	68.1	1.97	0.059	NS	NS
TFA[2]	65.0[c]	77.3[b]	83.0[a]	85.7[a]	1.55	<0.001	NS	NS
CF[2]	60.7[c]	71.8[b]	77.0[a]	79.1[a]	1.13	<0.001	NS	NS

[1]Values are least-squares means obtained from ANOVA (n=3). Means in a row not sharing a superscript letter differ (p<0.05).
[2]PU, dietary polyunsaturation; α-TA, α-tocopheryl acetate dosage ; OM, organic matter ; TFA, total fatty acids ; CF, crude fat.
[3]RSD, residual standard deviation.

Apparent absorption of α-tocopherol equivalents, α-tocopheryl acetate hydrolysis and free tocopherol concentration in faeces

The apparent absorption values of α-TE are shown in table 28. These values are, in general, quite low (always below 50%) and present a high variability. Due to the very low amounts of α-tocopherol found in the faeces of the animals of the E0 treatments, these data were not considered accurate and hence are not presented in this table.

There was a significant interaction between the two experimental factors (P<0.05). Dietary polyunsaturation had a significant effect (P<0.001) on the apparent absorption of α-tocopherol equivalents, but the differences were only between the more saturated diet (PU15), where apparent absorption of α-TE was markedly lower, and the rest of the treatments (p<0.001). As to the level of supplementation of α-tocopheryl acetate in the

diet, it had no effect upon apparent absorption values except for the PU15 treatment, where higher vitamin E supplementation (E400) resulted in a lower apparent absorption of α-TE.

Table 28: Effect of dietary polyunsaturation and α-tocopheryl acetate inclusion levels on α-tocopherol equivalents apparent absorption values (%)[1].

Dietary α-TA[2] (mg/kg)	Dietary polyunsaturation level				
	PU15	**PU34**	**PU45**	**PU61**	Mean values
100	20.06^{b}	46.00^{a}	54.02^{a}	46.83^{a}	41.73^{x}
200	34.23^{ab}	36.73^{ab}	35.64^{ab}	42.74^{a}	37.34^{xy}
400	10.73^{c}	38.90^{ab}	36.26^{ab}	37.47^{a}	30.84^{y}
Mean values	21.67^{y}	40.54^{x}	41.97^{x}	42.35^{x}	
RSD[3]	*7.595*				
P values:	*PU[2]*	*<0.001*			
	α-TA[2]	*0.007*			
	Interaction	*0.024*			

[1]Values are least-squares means obtained from ANOVA (n=3). $^{a, b, c}$ Lsmeans values with different superscript letters were significantly different (P<0.05). $^{x, y}$Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.05).
[2]PU, dietary polyunsaturation; α-TA, α-tocopheryl acetate.
[3]RSD, residual standard deviation.

α–TA has to be previously hydrolyzed to obtain free α-tocopherol, which can then be absorbed. In order to establish if the effects of dietary fat (type or level) and α-TA were due to a lower α-TA hydrolysis, we calculated the percentage of α-TA hydrolysis (intake of α-TA minus excretion expressed as a percentage of the intake).

Hydrolysis of α-TA values are presented in table 29. The interaction between the two dietary factors was significant (p<0.01). In the PU45 and PU61 treatments, increasing α-TA supplementation doses resulted in lower α-TA hydrolyzed. However, no dose effect was observed in the PU34 treatment. In the PU15 treatment, the lower α-TA hydrolysis value corresponded to the E400 treatment, followed by the E100; E200 having the higher value.

Table 29: Effect of dietary polyunsaturation level and α-tocopheryl acetate inclusion levels on the percentage of α-tocopheryl acetate hydrolysis[1].

Dietary α-TA[2] (mg/kg)	Dietary polyunsaturation level				
	PU15	**PU34**	**PU45**	**PU61**	Mean values
100	34.50^{d}	49.50^{abc}	56.67^{a}	53.50^{ab}	48.54^{x}
200	51.50^{abc}	43.67^{bcd}	45.33^{bcd}	39.00^{cde}	44.87^{xy}
400	32.00^{e}	48.00^{abc}	40.00^{cde}	39.67^{cde}	39.92^{y}
Mean values	39.33	47.06	47.33	44.06	
RSD[3]	*6.227*				
P values:	*PU[2]*	*0.082*			
	α-TA[2]	*0.018*			
	Interaction	*0.015*			

[1]Values are least-squares means obtained from ANOVA (n=3). $^{a, b, c, d, e}$ Lsmeans values with different superscript letters were significantly different (P<0.05). $^{x, y}$Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.05).
[2]PU, dietary polyunsaturation; α-TA, α-tocopheryl acetate.
[3]RSD, residual standard deviation.

We also measured the concentration of free (non esterified) α-tocopherol in the excreta because it can be an indicator of gastrointestinal degradation. Regarding free α-tocopherol concentration in excreta, results are shown in table 30. This free α-tocopherol comes from the non-absorbed α-tocopherol present in the ingredients of the diets and from the hydrolyzed and non-absorbed α-TA. Increasing levels of dietary α-TA resulted in a higher free α-tocopherol concentration in excreta, but only at high levels of dietary α-TA supplementation (E200 and E400) there were statistical differences depending on dietary polyunsaturation. In these treatments, higher dietary PUFA levels, resulted in lower concentration of free α-tocopherol excreted.

Liver α-tocopherol concentration

The hepatic α-tocopherol concentrations are shown in table 31. There was a significant interaction between the two experimental factors (P<0.005). The lower concentrations were found in the treatments with lower α-TA supplementation, but also in those treatments with high dietary PUFA level. The interaction between the two factors

(p<0.001) indicates that the differences in deposition due to α-TA dietary supplementation are reduced in those animals fed the highly unsaturated diets (PU45 and PU61). Independently of the dietary α-tocopheryl acetate inclusion level, the hepatic concentration of α-tocopherol in the animals fed the more saturated diet is clearly higher than in the rest.

Table 30: Effect of dietary polyunsaturation and α-tocopheryl acetate inclusion levels on the concentration of non-esterified α-tocopherol in the excreta (mg/kg of dry matter)[1].

Dietary α-TA[2] (mg/kg)	Dietary polyunsaturation level				
	PU15	**PU34**	**PU45**	**PU61**	Mean values
0	5.50^{de}	1.44^{e}	0.49^{e}	0.26^{e}	1.92^{z}
100	17.36^{de}	16.78^{de}	9.36^{de}	5.79^{de}	12.32^{z}
200	87.46^{b}	30.05^{de}	41.65^{cd}	15.01^{de}	43.54^{y}
400	143.74^{a}	70.40^{bc}	33.13^{cde}	19.89^{de}	66.79^{x}
Mean values	63.51^{x}	29.67^{y}	21.16^{yz}	10.24^{z}	
RSD[3]	*13.446*				
P values:	*PU[2]*	*<0.001*			
	α-TA[2]	*<0.001*			
	Interaction	*<0.001*			

[1]Values are least-squares means obtained from ANOVA (n=3). [a, b, c, d, e]Lsmeans values with different superscript letters were significantly different (P<0.001). [w, x, y, z]Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.001).
[2]PU, dietary polyunsaturation; α-TA, α-tocopheryl acetate.
[3]RSD, residual standard deviation.

Table 31: Effect of dietary polyunsaturation and α-tocopheryl acetate inclusion levels on α-tocopherol concentration in liver of chickens (mg/kg of fresh matter)[1].

Dietary α-TA[2] (mg/kg)	Dietary polyunsaturation level				
	PU15	**PU34**	**PU45**	**PU61**	Mean values
0	1.21^{de}	0.47^{e}	0.57^{e}	0.24^{e}	0.62^{z}
100	18.26^{cd}	6.00^{de}	12.53^{de}	5.54^{de}	10.58^{y}
200	36.22^{b}	17.50^{cd}	18.11^{cd}	10.29^{de}	20.53^{x}
400	55.95^{a}	29.92^{bc}	15.26^{cde}	16.95^{cd}	29.52^{w}
Mean values	27.91^{x}	13.47^{y}	11.62^{y}	8.25^{y}	
RSD[3]	*7.901*				
P values:	*PU[2]*	*<0.001*			
	α-TA[2]	*<0.001*			
	Interaction	*<0.001*			

[1]Values are least-squares means obtained from ANOVA (n=3). [a, b, c, d, e]Lsmeans values with different superscript letters were significantly different (P<0.001). [w, x, y, z]Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.001).
[2]PU, dietary polyunsaturation; α-TA, α-tocopheryl acetate.
[3]RSD, residual standard deviation.

6.2. Effect of dietary fat inclusion level on α-tocopherol apparent absorption and concentration in whole body, liver and plasma of chickens

6.2.1. Summary

An experiment was designed to assess the effect of dietary fat inclusion level on α-tocopherol apparent absorption and deposition in broiler chickens at two ages (20 and 39 days). The dietary fat was a mixture of linseed and fish oil, rich in polyunsaturated fatty acids (PUFA). The experimental treatments were the result of four levels of supplementation with α-tocopheryl acetate (0, 100, 200 and 400 mg/kg; E0, E100, E200 and E400 treatments respectively) and 4 dietary oil inclusion levels (2, 4, 6 and 8 %; O2, O4, O6 and O8 treatments respectively). Almond husk was used as an energy dilutor in the high fat diets. Apparent absorption of total fatty acids was very high in all treatments, and was higher with high fat dietary inclusion level. Apparent absorption of α-tocopherol was similar in both ages (47.53 ± 1.25 %) and was not affected by fat inclusion level. The concentration of free α-tocopherol in the excreta suggested that, at high α-tocopherol doses, high-fat high-PUFA diets increase the destruction of α-tocopherol by lipid oxidation in the gastrointestinal tract. Despite the lack of differences in apparent absorption among treatments, total and hepatic α-tocopherol deposition is lower in the animals fed high PUFA diets in the highly vitamin E supplemented animals, possibly due to a destruction of vitamin E when protecting these PUFA from lipid peroxidation. α-tocopherol concentration in liver and, to a lesser extent, in plasma is a useful indicator of the degree of response of this vitamin to different factors that can affect its bioavailability.

6.2.2. Statistics

Statistical analysis was carried out through GLM procedure of SAS statistical package (SAS institute, 2002), where the input class factors were dietary PUFA and α-TA. The interaction was also included in the model. Differences between treatment means were tested using Tukey's correction for multiple comparisons. The experimental unit for apparent absorption, α-TA hydrolysis and α-tocopherol concentration in the excreta and in plasma data was the cage. Given that there were no statistical differences between

the two ages (20 and 39 days), the data from both balance studies were pooled (n=6 replicates of 4 animals). The experimental unit for hepatic and total body α-tocopherol concentration it was the animal (n=6). Regression analyses between α-tocopherol concentration in whole body, plasma and liver were performed using the REG procedure of SAS (SAS institute, 2002).

6.2.3. Results

Apparent absorption of organic matter and total fatty acids

Apparent absorption values of organic matter and TFA from the two balance studies pooled are shown in table 32. Neither the the organic matter nor the TFA apparent absorption coefficients were affected by the inclusion level of α-TA (70.03 ± 0.33% and 87.7±0.31% respectively, p>0.05). Organic matter apparent absorption was lower in the high-fat high-PUFA diets, that had also a high percentage of crude fibre (p<0.001). In all treatments, TFA apparent absorption was high (above 80%) and was increased with increasing oil inclusion level (p<0.001). There were no differences between the two ages neither in organic matter nor in TFA apparent absorption values (p>0.05).

Table 32: Effect of dietary oil and α-tocopheryl acetate inclusion levels on organic matter (OM) and total fatty acids (TFA) apparent absorption values[1].

	Dietary added oil (%)				RSD[3]	P values		
	2	**4**	**6**	**8**		**Added oil**	**α-TA[2]**	**Interaction**
OM	73.67[a]	70.58[b]	68.88[c]	67.00[d]	1.594	<0.001	NS	NS
TFA	82.93[d]	86.76[c]	89.53[b]	91.65[a]	1.494	<0.001	NS	NS

[1]Values are least-squares means obtained from ANOVA (n=6). Means in a row not sharing a superscript letter differ (p<0.05).
[2]α-TA, α-tocopheryl acetate; OM, organic matter ; TFA, total fatty acids.
[3]RSD, residual standard deviation.

Apparent absorption of α-tocopherol equivalents, α-tocopheryl acetate hydrolysis and free tocopherol concentration in faeces

Results of α-TE apparent absorption coefficients are shown in tables 33 and 34. Due to the very low amounts of α-tocopherol found in the faeces of the animals of the E0 treatments, these data were not considered accurate and hence are not presented in this table There were no differences in the apparent absorption values of the birds when comparing the two ages (20 and 39 days) (48.5 ± 1.16 % vs 46.7 ± 1.25 %, p>0.05), which is why the data are presented pooled. Neither dietary added fat nor dietary α-TA affected apparent absorption values.

Also, hydrolysis of α-TA values are presented in tables 33 and 34, and has an average value of 67.39 ± 3.00 %. Neither dietary added PUFA-rich oil nor α-TA inclusion level (p>0.05) had an effect upon the percentage of α-TA hydrolysis.

Table 33: Effect of dietary oil on α-tocopherol equivalents apparent absorption values (%) and on the percentage of hydrolysis of α-tocopheryl acetate (%)[1].

	Dietary added oil (%)				RSD[3]
	2	**4**	**6**	**8**	
α-TE[2] apparent absorption	49.56	47.37	45.80	47.63	5.757
α-TA[2] hydrolysis	69.04	67.47	65.42	67.63	4.762

[1]Values are least-squares means obtained from ANOVA (n=6).
[2]α-TE, α-tocopherol equivalents; TA, α-tocopheryl acetate
[3]RSD, residual standard deviation.

Table 34: Effect of α-tocopheryl acetate inclusion level on α-tocopherol equivalents apparent absorption values (%) and on the percentage of hydrolysis of α-tocopheryl acetate (%)[1].

	Dietary α-TA[2]			RSD[3]
	100	**200**	**400**	
α-TE[2] apparent absorption	45.49	49.84	47.43	5.757
α-TA[2] hydrolysis	65.42	68.52	68.22	4.762

[1]Values are least-squares means obtained from ANOVA (n=6).
[2]α-TE, α-tocopherol equivalents; TA, α-tocopheryl acetate
[3]RSD, residual standard deviation.

As to non-esterified (free) α-tocopherol concentration in excreta, results are shown in table 35. There were no differences between both balance studies, hence the data are presented pooled. The interaction between both dietary factors was significant ($p<0.05$). In all cases, increasing levels of dietary α-TA resulted in a higher free α-tocopherol concentration in excreta, but only at high levels of dietary α-TA supplementation (E200 and E400) there were statistical differences depending on the level of added fat. In these treatments, higher fat inclusion levels (paralleled by higher PUFA inclusion levels), resulted in lower concentration of free α-tocopherol excreted, and this happened in both ages.

Table 35: Effect of dietary oil and α-tocopheryl acetate inclusion levels on the concentration of non-esterified α-tocopherol in the excreta (mg/kg of dry matter)[1].

Dietary α-TA[2] (mg/kg)	Dietary added oil (%)				
	2	4	6	8	Mean values
0	24.60[f]	15.48[f]	13.03[f]	13.02[f]	16.53[z]
100	66.89[e]	61.62[e]	53.31[e]	54.33[e]	59.04[y]
200	113.75[c]	104.30[cd]	91.80[d]	86.89[d]	99.18[x]
400	208.00[a]	211.91[a]	167.12[b]	164.51[b]	187.89[w]
Mean values	103.3[x]	98.33[x]	81.31[y]	79.69[y]	
RSD[3]	*14.005*				
P values:	*Added oil*	*<0.001*			
	Dietary α-TA[2]	*<0.001*			
	Interaction	*<0.01*			

[1]Values are least-squares means obtained from ANOVA (n=3). [a, b, c, d, e, f] Lsmeans values with different superscript letters were significantly different (P<0.05). [w, x, y, z]Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.05).
[2]α-TA, α-tocopheryl acetate.
[3]RSD, residual standard deviation.

Tissue α-tocopherol concentration

The concentration of α-tocopherol in the whole body of the animals is presented in table 36. Even though there were practically no differences among treatments in α-TE apparent absorption vales, there are clear differences in α-tocopherol deposition in the whole body of chickens. The lower concentrations were found in the treatments with lower α-TA supplementation, but also in those treatments with high inclusion levels of PUFA-rich oil. The interaction between the two factors (p<0.001) indicates that the differences in deposition due to α-TA dietary supplementation are reduced in those animals fed high levels of added fat. Results concerning α-tocopherol concentration in plasma and liver followed the same tendency as whole body α-tocopherol (tables 37 and 38). Regression equations between α-tocopherol concentrations in whole body, liver and plasma are presented in table 39, and present correlation coefficients above 0.85.

Table 36: Effect of dietary oil and α-tocopheryl acetate inclusion levels on α-tocopherol concentration in whole body of chickens (mg/kg of fresh matter)[1].

Dietary α-TA[2] (mg/kg)	Dietary added oil (%)				
	2	4	6	8	Mean values
0	0.86^{f}	0.65^{f}	0.52^{f}	0.46^{f}	0.62^{z}
100	6.63^{ef}	6.14^{ef}	5.45^{ef}	5.90^{ef}	6.03^{y}
200	16.89^{cd}	11.77^{de}	12.80^{de}	8.97^{def}	12.61^{x}
400	33.40^{a}	25.85^{ab}	22.85^{bc}	17.59^{bcd}	24.92^{w}
Mean values	14.44^{x}	11.10^{y}	10.40^{y}	8.23^{y}	
RSD[3]	*4.034*				
P values:	*Added oil*	*<0.001*			
	α-TA[2]	*<0.001*			
	Interaction	*<0.001*			

[1]Values are least-squares means obtained from ANOVA (n=3). [a, b, c, d, e]Lsmeans values with different superscript letters were significantly different (P<0.05). [x, y, z]Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.05).
[2]α-TA, α-tocopheryl acetate dosage.
[3]RSD, residual standard deviation.

Table 37: Effect of dietary oil and α-tocopheryl acetate inclusion levels on α-tocopherol concentration in liver of chickens (mg/kg of fresh matter)[1].

Dietary α-TA[2] (mg/kg)	Dietary added oil (%)				
	2	4	6	8	Mean values
0	2.00^{h}	1.90^{h}	0.70^{h}	1.94^{h}	1.63^{z}
100	18.47^{efgh}	17.94^{efgh}	11.85^{gh}	7.19^{gh}	13.86^{y}
200	36.15^{cd}	39.69^{bc}	20.25^{defg}	12.91^{gh}	27.25^{x}
400	64.68^{a}	54.63^{ab}	35.33^{cde}	20.16^{defgh}	43.70^{w}
Mean values	30.32^{x}	28.54^{x}	17.03^{y}	10.55^{y}	
RSD[3]	*8.305*				
P values:	*Added oil*	*<0.001*			
	α-TA[2]	*<0.001*			
	Interaction	*<0.001*			

[1]Values are least-squares means obtained from ANOVA (n=3). $^{a, b, c, d, e, f, g, h}$Lsmeans values with different superscript letters were significantly different (P<0.05). $^{x, y, z}$Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.05).
[2]α-TA, α-tocopheryl acetate dosage.
[3]RSD, residual standard deviation.

Table 38: Effect of dietary oil and α-tocopheryl acetate inclusion levels on α-tocopherol concentration in plasma of chickens (μg/mL)[1].

Dietary α-TA[2] (mg/kg)	Dietary added oil (%)				
	2	4	6	8	Mean values
0	0.96^{c}	1.41^{c}	1.21^{c}	1.08^{c}	1.16^{z}
100	10.25^{bc}	9.57^{bc}	6.09^{c}	9.60^{bc}	8.88^{z}
200	23.52^{bc}	22.12^{bc}	20.08^{bc}	15.81^{bc}	20.38^{y}
400	57.74^{a}	37.39^{ab}	15.60^{bc}	15.14^{bc}	31.47^{x}
Mean values	23.12^{x}	17.62^{xy}	10.74^{y}	10.41^{y}	
RSD[3]	*8.535*				
P values:	*Added oil*	*<0.05*			
	α-TA[2]	*<0.001*			
	Interaction	*<0.05*			

[1]Values are least-squares means obtained from ANOVA (n=3). $^{a, b, c,}$Lsmeans values with different superscript letters were significantly different (P<0.05). $^{x, y, z}$Lsmeans values with different superscripts letters within a row or within a column were significantly different (P<0.05).
[2]α-TA, α-tocopheryl acetate dosage.
[3]RSD, residual standard deviation.

Table 39: Regression equations between α-tocopherol concentrations in whole body, plasma and liver.

Dependent variable (y)	Independent variable (x)	equation	r	CV[1] (%)
Body α-tocopherol	Hepatic α-tocopherol	Y = 0.39·X	0.93	34.26
Body α-tocopherol	Plasmatic α-tocopherol	Y = 1.80·X	0.91	33.97
Hepatic α-tocopherol	Plasmatic α-tocopherol	Y = 4.25·X	0.87	38.21

[1]Coefficient of variation.

Chapter 7

Discussion

Great ideas are never remembered and dumb statements are never forgotten.

Uffelman's Razor
[Given Murphy's law, ...] One should not attribute to evil design any unfortunate result which can be attributed to error. A mistake (or series of mistakes) is the simpler and more likely explanation.

Research Law
No matter how clever and complete your research is, there is always someone who knows more.

Churchill's Commentary on Man
Man will occasionally stumble over the truth, but most of the time he will pick himself up and continue on as though nothing has happened.

Price's Law of Science
Scientists who dislike the restraints of highly organized research like to remark that a truly great research worker needs only three pieces of equipment -- a pencil, a piece of paper, and a brain. But they quote this maxim more often at academic banquets than at budget hearings.

7.1. Chemical composition and energy content of whole body

When the dietary PUFA variation was achieved keeping added fat constant, abdominal fat pad weight, body fat and body energy contents where lower in the high-PUFA diets compared to the SFA-rich ones. The substitution of saturated fats for unsaturated oils in this experiment led to an increase in the energy to protein ratio, due to the higher metabolizable energy supplied by unsaturated fatty acids (Young and Garret, 1963). A higher energy to protein ratio is one of the main causes of body fattening (McLeod, 1982). But the results presented concerning body fat are against this principle, given that the unsaturated diets (with a higher energy to protein ratio) have caused lower fat deposition. Although some experiments have not found clear differences between types of fat used (Zollitsch *et al.* 1997; Pinchasov and Nir, 1992), more recent studies have observed a lower fat content in animal tissues fed diets rich in unsaturated oils in whole chicken using sunflower oil (Sanz et al. 1999) and in chicken thigh using linseed and fish oil (Cortinas et al. 2004*a*). Also, as previously mentioned, lower abdominal fat pad weights have been observed in animals fed linseed oil enriched diets (Crespo and Esteve-Garcia, 2001, 2002*a*).

This situation has been observed not only in chickens but also in rats. A high number of studies addressing the problem of “cafeteria” diets (rich in saturated fats) have been performed. For example, Shimomura et al. (1990) compared body fat accumulation between rats fed tallow and safflower oil. The rats fed safflower had significantly less body fat than the rats fed tallow. Mercer and Trayhurn (1987) and Takeuchi et al. (1995) also have found higher body fattening in rats fed beef tallow and lard respectively against more unsaturated fat sources.

Explanations have been given but they are still not clear and sometimes are contradictory. Some studies suggest that PUFA decrease hepatic lipogenesis (Wilson et al. 1990, Sanz et al. 2000*a*), others propose that PUFA cause a higher oxidation of fats (Sanz et al. 2000*a*; Crespo and Esteve-Garcia,2002*c*). Another possible explanation is that unsaturated fat (maize oil, safflower oil,

linseed oil) compared to tallow or lard provokes higher diet-induced thermogenesis (Takeuchi et al. 1995; Mercer and Trayhurn, 1987).

It seems clear that the degree of saturation of fatty acids influences their metabolic utilization in some way. As already mentioned in the materials and methods section, half of the animals from this study were destined to a parallel experiment where TFA were measured in breast and in thigh with skin (Cortinas et al. 2004*a*). Interestingly, the chicken thigh had the same evolution as the whole chicken whereas breast fat content was unaffected by the dietary PUFA gradient. The thigh with skin has intermuscular, intramuscular and depot fat (subcutaneous) and thus resembles the whole chicken, where all types of fat are included. On the other hand, breast fat represents intramuscular fat. This suggests that the effect of high PUFA consumption upon body fat deposition can be more marked on fat depots than in intramuscular fat. It would be desirable to achieve chickens with less abdominal and subcutaneous fat, which are rejected by the consumers and cause carcass losses in the slaughterhouse, but with no prejudice to the organoleptical benefits of intramuscular fat.

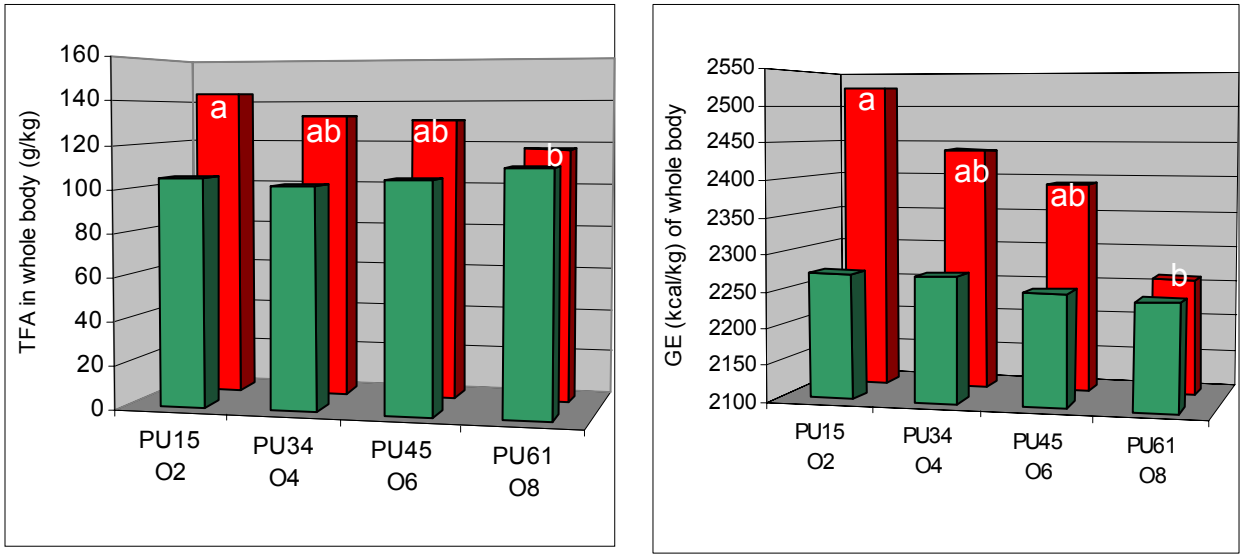
On the other hand, increasing levels of PUFA-rich fat added to feed had no effect upon abdominal fat pat weight, chemical composition (including TFA) and gross energy content of the whole animal. As mentioned previously, the energy to protein ratio is one of the most important dietary factors affecting fat deposition. In this second trial, it was the same among the experimental diets. However, it is believed that fat inclusion has an “extra-caloric” effect, because it improves nutrient utilization by the animals (Mateos and Sell, 1980) so more energy would be available. It could be expected that this extra energy coming from increasing fat inclusion would increase body fat deposition, but this was not the case in our experimental trial. Results concerning the effect of fat inclusion level upon body fat content have been so far controversial. Deaton et al. (1981) fed isocaloric and isoproteic diets to broilers with increasing added animal fat levels (4, 7 and 10%) and both abdominal and body fat were higher in the animals fed the high-fat diet than the ones fed the low-fat one. On the other hand, Donaldson (1985) used increasing amounts of cottonseed oil (from 2 to 8.6%) and found no differences in body fat content of broilers. Keren-Zvi et al.

(1990) found that increasing dietary vegetable oil inclusion caused a decrease in abdominal fat pad in broilers selected for high abdominal fat. Vilà and Esteve-Garcia (1996) used different inclusion levels (from 0 to 12%) of acid fats, tallow and sunflower, and showed that abdominal fat pad increased with fat inclusion but only in the case of feeding tallow acid oil. There were no differences on this parameter in the animals receiving sunflower acid oil, even though the energy to protein ratio increased. It seems that increasing dietary levels of saturated fat increases fat deposition whereas the use of more unsaturated oils does not have this effect.

In trial 1 we showed that increasing dietary PUFA but keeping added oil constant reduced body fat and energy content. However, there were no differences in body fat and energy deposition among treatments when the PUFA gradient was achieved by increasing the amount of PUFA-rich oil added to the feed (see figure 13). Due to the low PUFA content of the O2 diet, it could be expected a higher body fat content, similarly to PU15 in trial 1. This difference can be due to the difference of 4 days between the animals (they were killed at 44 and 40 days of age in trial 1 and trial 2 respectively). However, despite the 4-days difference, the animals had similar final weights (2318±16.1 and 2240±14.6 kg in trial 1 and trial 2 respectively) and had similar average daily gain (60±0.4 and 62±0.4 g/animal/day in trial 1 and trial 2 respectively). Another plausible reason, given that AME (kcal/animal/day) and PUFA (g/animal/day) intakes were similar in both experiments, could be the presence or absence of dietary SFA and monounsaturated fatty acids (MUFA). The ratio of PUFA to SFA in the diets in trial 1 was lower than 2 in the PU15, PU34 and PU45 treatments and higher than 3 in PU61 (trial 1) and in all treatments of trial 2. In trial 1, dietary PUFA were increased at the expense of SFA and MUFA, and the effect of high dietary PUFA could also be interpreted as the effect of reduced dietary SFA, MUFA or both. The small amount of these fatty acids in all experimental feeds of the second trial (15-16% of SFA and 21-22% of MUFA) could account for the lack of effect of increasing dietary PUFA in lowering body fat. In fact, PU61 (trial 1) and O8 (trial 2) treatments have a very similar dietary FA profile and they result in similar TFA deposition.

It has also to be considered the different contribution of *de novo* fatty acid synthesis between both trials. Fatty acid synthesis is kept to a minimum when adding 9% of fat to the diets in trial 1 whereas in trial 2 this process has much more importance. The animals on the low fat diets will synthesize more fatty acids (SFA and MUFA) than the ones in the high fat diets, but only to reach a minimum level, because it is energy-consuming. This situation can account for the similar body fat content among treatments. Furthermore, the energy used for fatty acid synthesis in trial 2 can counteract the expected higher fat and energy deposition in the low-PUFA treatments.

Figure 13. Total fatty acid (TFA) and gross energy (GE) content of the whole chicken depending on the polyunsaturation level of the diets, achieved by varying fat source (red) or by increasing added fat inclusion level (green).



7.2. Fatty acid deposition and endogenous fatty acid synthesis potential estimation

7.2.1. Effect of dietary α-TA.

Fatty acid content of the whole body of chickens was not affected by α-TA except for very long chain FA of the ω6 family, which have a biological importance as precursors of eicosanoids and components of cellular membranes. This finding can be due to the fact that one metabolite of vitamin E, α-tocopheryl quinine, is a cofactor for 20:4n6 fatty acid synthesis (Infante,

1999). Despite the statistical significance, the differences between the E0 treatments against the supplemented treatments in these fatty acids are not important in magnitude.

Given that α-TA main role is to protect PUFA from oxidation, it could be expected that its inclusion in the diet would result in a higher PUFA deposition in the body. Results found in the literature concerning effect of dietary vitamin E upon fatty acid composition in chicken tissues are controversial and the expression of fatty acid concentration as percentages (area normalization) makes comparison difficult. Although our results are presented as percentages, fatty acids were first quantified (g/kg of whole body) and there were no differences among the treatments either. Several studies have not found any effect of dietary α-TA on FA composition of chicken tissues (Lin et al., 1989; Cortinas et al., 2004*a*; Bou et al., 2004). However, Surai and Sparks (2000) fed chickens with tuna oil and found a higher 22:6 ω3 concentration in the tissues of animals supplemented with 160 mg/kg of α-TA compared to the ones supplemented with 40 mg/kg.

It can be concluded that α-TA inclusion up to levels of 400 mg/kg did not affect significantly fatty acid deposition in chicken, not even in dietary PUFA levels of 61g/kg, except for very long chain ω6 FA. It is known that α-TA supplementation can affect fatty acid concentrations in chicken meat subject to processing, such as cooking or storage (Lopez-Ferrer et al., 1999*b*; Cortinas et al., 2004*a*) but its effect upon this parameter *in vivo* is still not clear. It is possible that recycling mechanisms of vitamin E and other antioxidants can protect the animal from fatty acid losses (Surai, 2002).

7.2.2. Effect of dietary polyunsaturation on lipid composition

Fatty acid composition in chicken tissues is a combination of endogenous synthesis of fatty acids, from carbohydrate and protein precursors, and direct deposition from the diet. SFA and MUFA have this double origin, whilst PUFA deposition depends exclusively on dietary supplementation in normal

conditions. The main fatty acids resulting from hepatic lipogenesis are 16:0, 18:0, 18:1n9 and 16:1n7 (Bartov, 1979; Crespo and Esteve-Garcia, 2002*b*).

In both trials, PUFA deposition increased with increasing dietary PUFA inclusion. The reduction in SFA and MUFA concentration is due to the inverse relationship between PUFA and SFA and MUFA deposition, already described in the literature (Ajuyah et al., 1991; Lopez-Ferrer et al., 2001*a, b*). In trial 1 this reduction can be attributed to the lower intakes of SFA and MUFA when dietary PUFA increase. In trial 2 endogenous synthesis of SFA and MUFA plays an important role when animals consume a low fat diet (O2, O4), but when fat consumption increases, endogenous fatty acid synthesis decreases (Donaldson, 1985), even if the added fat is rich in PUFA and low in SFA and MUFA. The decrease in fatty acid synthesis is possibly due to a lower availability of carbohydrate precursors as dietary fat increases and to an inhibition of lipogenic enzymes by dietary fatty acids (Mourot and Hermier, 2001). For this reason, SFA and MUFA concentrations in the body in trial 2 are lower when added fat inclusion increases, in spite of higher SFA and MUFA intakes.

In table 40, linear regression equations between PUFA intakes (g/animal/day) and SFA, MUFA and PUFA deposition (% of total fatty acids) shown the reduction of SFA and MUFA when PUFA intake increases. In both trials, the slopes of SFA and MUFA equations are negative, and the slope of the MUFA equation is bigger than the SFA one. This suggests that, in the case of high PUFA intakes, SFA are preferred to MUFA for deposition in order to maintain a relatively constant unsaturated (MUFA + PUFA) to saturated fatty acids ratio in cellular membranes (Asghar et al., 1990; Bou et al., 2004). The fact that MUFA and PUFA slopes are lower in trial 2 ($p<0.05$), whereas the slopes of SFA equations between the two trials do not statistically differ ($p>0.05$), further supports the idea that MUFA are exchanged for PUFA when necessary and SFA deposition is more independent of the effect of high PUFA intakes. Other authors have reported the lower manipulation of SFA compared to MUFA and PUFA in broiler meat (Lopez-Ferrer et al., 1999*a, b*) and in eggs (Baucells et al., 2000).

Table 40. Regression equations between polyunsaturated fatty acid intake (g/animal/day, X) and the content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids (g/100g of total fatty acids, Y) in the whole body.

		Dependent variable (Y)	Equation	P value	R^2	CV[1] (%)
Trial 1	Whole body	% SFA	Y = 36.95 – 2.19·X	<0.001	0.94	3.51
		% MUFA	Y = 63.68 – 5.44·X	<0.001	0.96	4.81
		% PUFA	Y = 7.54·X	<0.001	0.96	8.02
Trial 2	Whole body	% SFA	Y = 32.36 – 1.91·X	<0.001	0.87	4.07
		% MUFA	Y = 51.87 – 4.03·X	<0.001	0.85	6.67
		% PUFA	Y = 15.46 + 5.99·X	<0.001	0.89	6.27

[1]Coefficient of variation.

In both trials, the concentration of 18:2 ω6 and 18:3 ω3, the main precursors of the ω6 and ω3 series respectively, increase linearly with their respective intakes because they cannot be synthesized by the chicken, and are deposited directly from the diet. 18:3 ω3 increases much more markedly because the PUFA gradient of the diets has been achieved by using linseed oil as a vegetal source (see table 16 of the Materials and Methods chapter), and linseed is particularly rich in 18:3 ω3. Very long chain ω3 fatty acids increase with dietary PUFA for two reasons. First of all, fish oil, which is used in the high PUFA diets, is rich in these fatty acids. The second reason is that the concentration of 18:3 ω3 in the body is greatly increased in the high-PUFA treatments of both trials and, given that 18:2 ω6 and 18:3 ω3 compete for the same elongation and desaturation enzymes (Sprecher, 1989), 18:3 ω3 derivatives are more easily synthesized. Also, 18:3 ω3 is preferred to 18:2 ω6 as a substrate for Δ6-desaturase enzyme (Holman, 1998). For these reasons, very long chain ω 6 fatty acids are reduced with increasing dietary polyunsaturation in our experimental conditions.

7.2.3. Effect of dietary polyunsaturation on potential endogenous FA synthesis

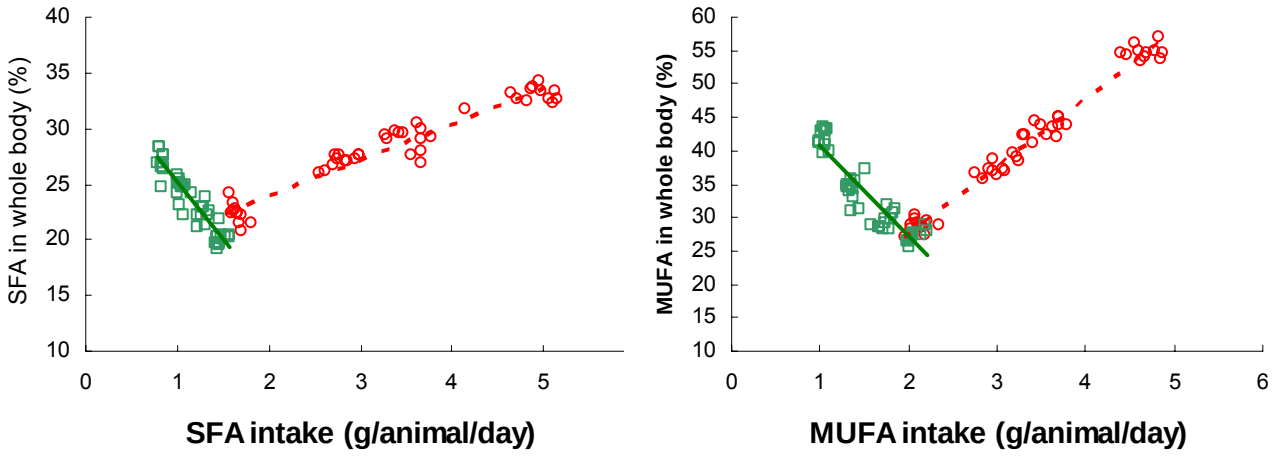
The body-to-dietary SFA, MUFA and PUFA ratios give us an idea of the role played by endogenous synthesis in the final FA profile of the whole body. We

calculated the ratio of body SFA, MUFA and PUFA to their respective dietary concentration as an indicator of net synthesis (higher than 1) or degradation (lower than 1). In the first trial, there is a net synthesis of SFA in the PU45 and PU61 treatments, and there is a net synthesis of MUFA in all treatments. The fact that there is a net synthesis of these FA when the level of dietary fat is high (10.2%, table 8), indicates that hepatic lipogenesis is not completely inhibited. This net synthesis of SFA and MUFA is higher in the PU61 treatment than in the rest ($p<0.05$), which could mean that high PUFA diets compared to SFA and MUFA rich diets exhibit a lower inhibition effect upon hepatic lipogenesis, as it was suggested by Crespo and Esteve-Garcia (2002*c*).

In trial 2, there is a net synthesis of SFA and MUFA and a net oxidation of PUFA in all cases. But the SFA and MUFA body-to-dietary ratios are lower, and PUFA ratio is higher, when added fat inclusion level is high compared to the other treatments. This suggests that, in the low fat treatments, PUFA are oxidized in order to obtain energy, substrate or both to synthesize SFA and MUFA. This idea is supported by the fact that the slope of the regression equations (table 40) between PUFA intake and PUFA deposition is higher in trial 1 than in trial 2 ($p<0.001$), suggesting that when added fat level is high, increasing dietary PUFA are deposited more readily from the diet, whereas in trial 2 the efficiency of deposition is lower, possibly due to their oxidation in the low-fat treatments in order to synthesize SFA and MUFA.

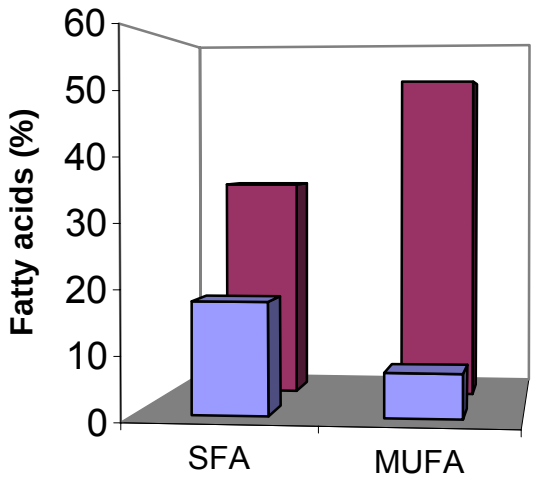
The regression equations between SFA and MUFA intake and their concentration in the whole body, which are shown in table 26, are plotted in figure 14. The intervals marked by the intercept values from trial 1 (lower values, when no SFA/MUFA is consumed, 10% dietary fat) and trial 2 (higher values, when no fat is consumed) show the variation of endogenous synthesis when dietary fat is increased from 0% to 10%. That is, increasing from 0 to 10% of dietary fat, SFA from endogenous synthesis found in the whole body decrease from 35% to a 17%, and MUFA decrease from 53% to 7% of total fatty acids (figure 15).

Figure 14. Regression equations between saturated (SFA) and monounsaturated (MUFA) fatty acids intake (g/animal/day) and its concentration (g/100g of total fatty acids) in the whole body. Data represented by ○ are from trial 1 (gradient of polyunsaturation achieved keeping added fat constant) and data represented by □ are from trial 2 (gradient of polyunsaturation achieved increasing added fat inclusion level).



It can be observed that the variation range of SFA proportion is lower than the range of variation of MUFA. This further supports the hypothesis already mentioned that there is a homeostatic mechanism in the cellular membranes to keep the SFA: unsaturated fatty acids ratio inside a relatively narrow range to maintain membrane fluidity. Also, comparing these equations with the ones obtained from breast muscle of animals from the same experiment (PhD thesis of L. Cortinas, 2004), we can see that breast SFA and MUFA have a lower range of variation than in the whole body. This shows that fat composition of intramuscular fat (main fat depot present in breast muscle) is less modifiable by the diet than storage fat (main fat present in the whole body), which seems logical given that intramuscular fat is comprised mainly of membrane phospholipids, and phospholipid composition affects the execution of different metabolic activities (Merrill and Schroeder, 1993). Changes in this composition could seriously affect cell metabolism, hence phospholipid fatty acids are less affected by diet composition than triglyceride fatty acids, whose main role is to store energy.

Figure 15. Percentage of saturated (SFA) and monounsaturated (MUFA) fatty acids when administering a diet with no dietary fat, or with 10% dietary fat but without SFA and MUFA respectively.



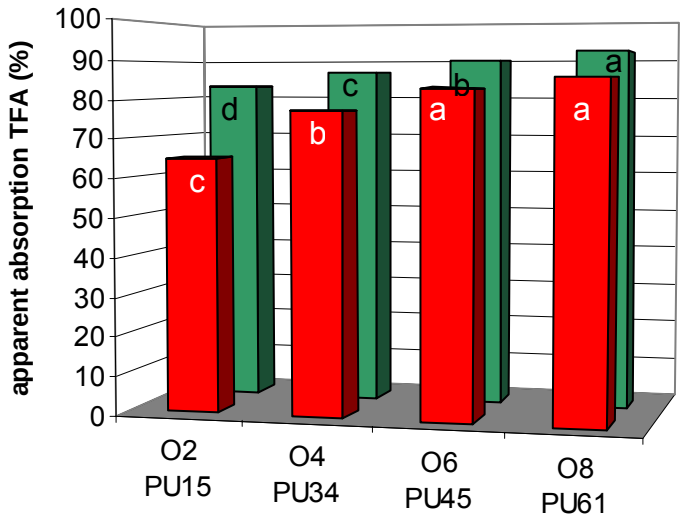
7.3. α-tocopherol apparent absorption and deposition

The type and amount of dietary fat influence the digestion and absorption of fatty acids. Given that vitamin E is a fat-soluble vitamin, it is believed that dietary fat also influences the intestinal uptake of this vitamin. Unsaturated FA have better apparent absorption values because of their polar nature, which allows them to incorporate readily into mixed micelles. Moreover, unsaturated FA facilitate the incorporation of SFA into the mixed micelles, improving the absorption of the latter (Young and Garret, 1963).

In our experiments, the use of saturated fats resulted in low FA apparent absorption coefficients (figure 16). The animals fed the more saturated diet (PU15) had an apparent absorption coefficient of crude fat of 60.7% and total fatty acids of 65.0%, whereas the more unsaturated diet (PU61) had a coefficient of 79.1 and 85.7% respectively. This was reflected in a lower organic matter apparent absorption coefficient in the PU15 treatment, which, in turn, reduced AME of the PU15 diet and resulted in an increase of average daily intake of the animals fed this diet. Despite the increase in the intake, it was not enough to compensate for the lower EMA.

However, in trial 2, TFA apparent absorption values were over 80% in all cases (figure 16), and reaching values around 90% in the more unsaturated diets (O6 and O8). Despite the better TFA apparent absorption with increasing added fat, organic matter digestibility was lower in the high-fat treatments. The reason for this is that almond husk was included as an energy dilutor and the high percentage of fibre in the high fat diets has impaired organic matter apparent absorption, both at 20 and at 39 days of age. However, as to TFA apparent absorption, the unsaturation level of the diets seems to have counteracted the negative fibre effect.

Figure 16. Apparent absorption values of total fatty acids (TFA) in response to a dietary polyunsaturation gradient, achieved by varying fat source (red) or by increasing added fat inclusion level (green).



Apparent absorption values of α-tocopherol in broilers varied between 11 and around 50%, with an average value of 42.1% under our experimental conditions.

The decreased intestinal uptake of the saturated fat mentioned before has affected α-tocopherol apparent absorption. Unsaturated fatty acids form micelles in the intestine more readily than saturated ones, thus enhancing vitamin E inclusion in these micelles. These results are in contradiction with the supposed existence of an interference of PUFA with vitamin E intestinal

absorption based on the work of Gallo-Torres et al. (1971), cited by several authors since then (Weber, 1981; Bjorneboe et al., 1990; Cohn, 1997, among others). Tijburg et al. (1997), in a study with rats using diets with increasing levels of linoleic acid (18:2 ω6), questions the methodology of the cited authors because they used free linolenic acid instead of using linolenic acid-containing TAG , which could affect the formation of mixed micelles. In the same study, they found a lower faecal excretion of α-tocopherol as the dietary linoleic level increased. This decrease in faecal excretion is reflected in a higher apparent absorption of α-tocopherol. In a recent work with broilers, Knarreborg et al. (2004) found lower ileal α-tocopherol absorption coefficients in 35-day old birds fed animal fat compared to those fed soybean oil. In the first trial of this dissertation we only have data corresponding to 20-day old birds. It is known that SFA absorption improves markedly with age, it would be interesting to study whether older birds (40 days) would present the same impairment in α-TE apparent absorption when fed 9% of tallow.

It seems that tallow inclusion at 9% impairs α-tocopherol intestinal uptake. Other saturated fats may have the same effect. In an experiment carried out by our team in order to study the effect of different fats upon α-TE apparent absorption in female broiler chickens between 30 and 50 days (data not published), the animals fed palm oil (10 %) had in lower apparent absorption values than the ones fed soybean or linseed oils (22.30 % vs 43.90 %, $p<0.001$). Interestingly, in the same experiment, coconut oil (rich in medium chain FA) included at 10% had apparent absorption values comparable to soybean and linseed oils (47.8%). There is no data in chickens regarding to the effect of medium chain FA upon α-tocopherol absorption.

In trial 2, where dietary fat used is a mixture of highly unsaturated oils (linseed and fish oils), we have not found differences between treatments. Although it is accepted that dietary fat is necessary to a certain extent for the correct absorption of vitamin E, the minimum amount required for optimal absorption has not been determined in chickens. Regarding other species, the minimum amount of dietary fat in humans is being studied but has not been still

established and results are controversial. Some authors found no differences in vitamin E availability between high-fat or low-fat diets (Roodenburg et al., 2000) and other authors found that the amount of fat in a meal clearly influenced α-tocopherol absorption (Jeanes et al., 2004). In rats, Brink et al. (1996) found no differences in α-TE apparent absorption between a diet with 0.7% and 5.2% of added fat, and Losowsky (1972) found no differences between diets containing 5 and 23% added fat. Our results suggest that 2% highly unsaturated added fat (4.4% total dietary fat) is not limiting for a proper α-tocopherol absorption in chickens. These results, however, may be different with the use of a more saturated dietary fat.

As to the effect of α-TA supplementation dosage, our results are not conclusive. In trial 1, 400 mg/kg of dietary α-TA resulted in lower α-TE apparent absorption coefficients, although the difference is only significant for the more saturated treatment (PU15). In trial 2, α-TA dosage does not seem to have an effect upon α-TE apparent absorption.

We can conclude that there is not a clear effect of α-TA supplementation level upon α-TE apparent absorption, which could be expected by the fact that α-tocopherol is absorbed passively. Other authors (Traber et al., 1998) have found constant absorption values with increasing vitamin E supplementation (from 15 to 150 mg/day).

The previous step for α-TE absorption is the hydrolysis of α-TA into free α-tocopherol. When feeding the animals with a 9% of added fat, we have found that the lower hydrolysis values correspond to the lower α-TE apparent absorption values. This finding suggests that the inclusion of 9% tallow impairs α-TE apparent absorption partly due to a reduction in α-TA hydrolysis in the small intestine. Our results suggest that this step is a limiting factor in α-tocopherol intestinal uptake, as has been suggested by Combs (1978).

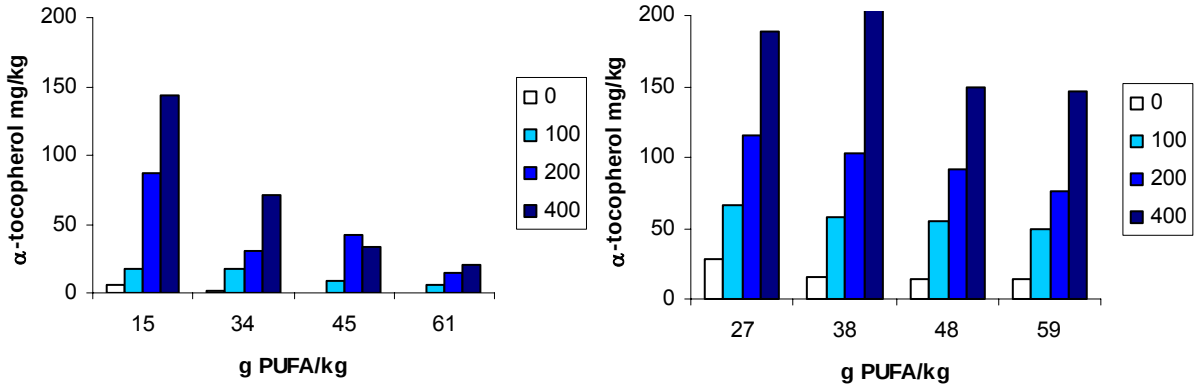
When feeding the animals with increasing levels of a PUFA-rich added fat, there were no clear differences among treatments in α-TA hydrolysis percentage.

It has to be considered, though, that α-tocopherol can be degraded before its absorption while protecting PUFA from oxidation in the gastrointestinal tract, as suggested by Tijburg et al. (1997). With the methodology used in these experiments, we cannot rule out this possibility, making it difficult to conclude that dietary PUFA inclusion enhances vitamin E absorption. However, the concentration of free α-tocopherol in the excreta can be an indicator of gastrointestinal degradation.

As mentioned previously in the Materials and Methods chapter, we carried out an experiment with broiler chickens studying the effect of the use of 6% oxidized sunflower oil (rich in primary oxidation products) on α-TE apparent absorption, using 100 ppm of α-TA supplementation. There were no differences among treatments regarding α-TE apparent absorption (average value 49.96 ± 3.27%), but the concentration of free α-tocopherol in the excreta was higher in the animals fed the fresh oil than in the ones fed oxidized oil (100.88 vs. 70.23 mg/kg of dry matter, $p<0.001$). This suggested that there was α-tocopherol degradation in the gastrointestinal tract due to an increase in the peroxidation processes in the gut caused by dietary fat.

Similarly, a high intake of PUFA can favour the appearance of peroxidation processes in the gut, as well as in the body tissues. The concentration of α-tocopherol in the excreta of birds in both experiments follows the same pattern (figure 17). Free α-tocopherol concentration in the excreta of both experiments is lower in the high-fat high-PUFA treatments, and our results suggest a protective effect of α-tocopherol in the gastrointestinal tract prior to its absorption. Sklan et al (1982) suggested that at least 50% of the ingested tocopherol (administered as non esterified α-tocopherol) is degraded in the intestinal tract before absorption takes place.

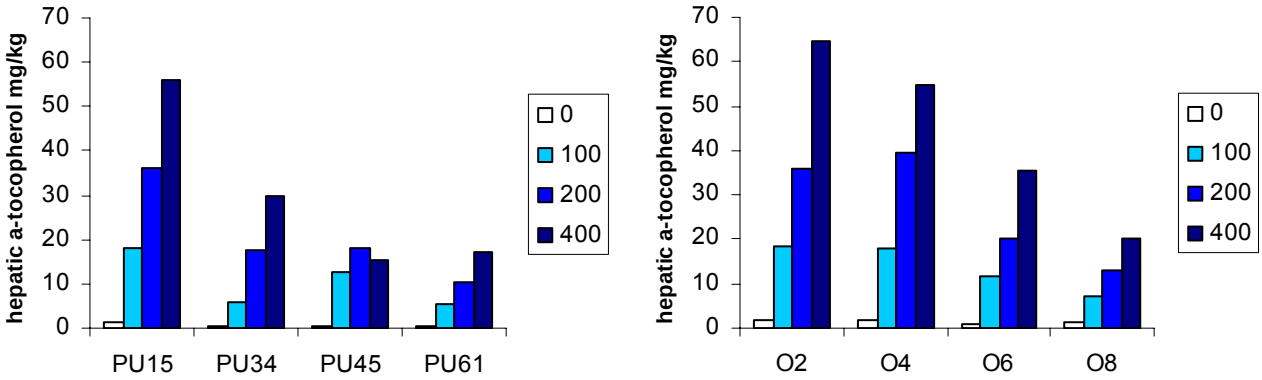
Figure 17. Concentration of free α-tocopherol in the excreta of chickens (mg/kg dry matter, 20 days of age approximately) in response to a dietary polyunsaturation gradient, achieved by varying fat source (left side) or by increasing added fat inclusion level (right side).



Interestingly, non-esterified α–tocopherol concentration in excreta of the E0 and E100 treatments is not affected by dietary polyunsaturation. This could suggest that there is a recycling of α-tocopherol in the gut when vitamin E concentration is relatively low. When supplemental vitamin E is high, a recycling mechanism may not be necessary and therefore, a high presence of PUFA results in lower α-tocopherol presence in excreta. The difference between the two trials can be attributed partly to the use of an antioxidant solution in excreta samples from trial 2, which prevented α-tocopherol in these samples to be degraded during storage.

Regarding vitamin E tissue concentration, it is well described the linear increase of α-tocopherol concentration in chicken tissues with the increase in its dietary supplementation (Jensen et al., 1999; Flachowsky et al., 2002). The α-tocopherol concentration of liver in both trials (figure 18) increased with α-TA supplementation, but the increase was lower in the high-PUFA treatments in both trials, despite the similar α-TE apparent absorption coefficients among the different added fat treatments and the markedly lower value in the animals fed the saturated diet (PU15).

Figure 18. Concentration of α-tocopherol (mg/kg of fresh matter) in the liver of chickens (40 and 44 days of age) in response to a dietary polyunsaturation gradient, achieved by varying fat source (left side) or by increasing added fat inclusion level (right side).



This interaction is similar to that found in the non-esterified α-tocopherol concentration in the excreta. It has been suggested that there is α-tocopherol recycling by other redox molecules such as ascorbate and glutathione (Surai, 1999). It is possible that the recycling is decreased when dietary α-tocopherol supplementation is high.

To confirm this, we carried out linear regressions between dietary and hepatic α-tocopherol concentration for each added fat treatment (O2, O4, O6 and O8, which have 28, 38, 48 and 59 g PUFA/kg diet). They are presented in table 41, and it can be observed that the slope of the equations is lower for the high-fat high-PUFA treatments. Given that the slope was not statistically different between O2 and O4 treatments, only an equation is presented.

Table 41. Regression equations between dietary α-tocopherol (mg/kg, x) and its hepatic concentration (mg/kg, y) depending on the inclusion level of added fat.

Added oil	Equation	p-value	R^2	CV[1] (%)
O2-O4	y = 0.16·x	<0.001	0.89	23.82
O6	y = 0.10·x	<0.001	0.95	14.85
O8	y = 0.07·x	<0.001	0.95	15.32

[1]CV, coefficient of variation.

These equations shows that to achieve, for instance, 15 mg α-tocopherol/kg fresh matter in liver it is necessary to provide 94, 150 and 214 mg/kg of α-tocopherol in the diet if dietary PUFA are 28-38 g/kg, 48 g/kg; and 59 g/kg respectively. That is, the relationship between dietary α-tocopherol and PUFA to achieve the same tissue α-tocopherol concentration ranges between 2.5 and 3.7 mg/g, which are values comparable to the ones proposed by companies such as BASF (3-4 mg/g). However, if one wants to achieve 30 mg α-tocopherol/kg fresh matter in liver, the relationship between dietary α-tocopherol and PUFA ranges between 4.9 and 7.3. Thus, the relationship between the two nutrients is not constant.

The high-fat treatments are also richer in PUFA, and PUFA enrichment of tissues predisposes them to lipid peroxidation processes, where vitamin E plays a protective role. Several authors have found that feeding diets rich in PUFA results in lower α-tocopherol concentration in tissues, in rats (Tijburg et al., 1997) and in poultry (Ruiz et al., 1999; Surai and Sparks, 2000; Sijben et al., 2002). These results suggest that there is some α-tocopherol destruction between absorption and deposition, or during deposition, due to the high PUFA content of the high-fat diets, which would result in a high PUFA deposition in the body. These results imply an important role of vitamin E in the increase of poultry meat shelf-life when highly unsaturated diets are fed to these animals.

In trial 2 we have measured α-tocopherol not only in liver but also in the whole body and in plasma. We have found that whole body, liver and plasma respond in a similar way to the experimental factors. This is expected, because liver is considered a main α-tocopherol depot tissue, and plasma is the means to transport α-tocopherol to all body tissues. The good correlation between total and hepatic α-tocopherol (r=0.93) shows that α-tocopherol concentration in liver is a useful indicator of total α-tocopherol status in chickens. Correlation of both body and hepatic vitamin E with its concentration in plasma are good but somewhat lower (r=0.91 and 0.87 respectively), probably due to the fact that α-tocopherol in plasma is not exactly a deposit, whereas liver and whole body are.

It is also true that the number of samples per treatment is lower for the plasma (n=3) than for liver and whole body (n=6). However, plasma α-tocopherol showed the same differences among treatments and can also be considered a useful indicator of α-tocopherol status in birds. However, the coefficients of variation of these equations are too high to consider plasma and liver α-tocopherol estimators of total body α-tocopherol under our conditions. It is possible that the use of more samples can improve this coefficient of variation.

The use of plasma α-tocopherol is the most used biomarker in mammals (especially humans) to assess α-tocopherol status (Morrissey and Sheehy, 1999). Its advantages are that is non-invasive and is technically simple, but there are a high number of confounding factors (for example age, sex, plasma lipids, smoking). The possibility of analyzing α-tocopherol of the whole body of chickens allows us to confirm the usefulness of both hepatic and plasma α-tocopherol in assessing the vitamin E status in chickens.

Chapter 8

Conclusions

All's well that ends.

Always keep your words soft and sweet, just in case you have to eat them.

An expert is one who knows more and more about less and less until he knows absolutely everything about nothing.

Schumpeter's Observation of Scientific and Nonscientific Theories:
Any theory can be made to fit any facts by means of appropriate additional assumptions.

The more knowledge you gained the less certain you are of it.

If you think you understand science (or computers or women), you're clearly not an expert

From the results presented in this dissertation, the following conclusions can be drawn:

1. PUFA-rich chicken diets (61 g/kg of PUFA, 15 g/kg of SFA) compared to saturated-rich ones (15 g/kg of PUFA, 44 g/kg of SFA), at the same fat inclusion level, cause a decrease in body fat deposition, observed in abdominal fat weight (35%) and in the total fatty acid and gross energy content (10 and18% respectively) of the whole chicken.

2. Increasing dietary added PUFA-rich fat levels from 2 to 8% does not result in any changes neither in abdominal fat pad weight nor in total fatty acids and gross energy content of the whole chicken.

3. Increasing dietary PUFA inclusion results in a linear increase in PUFA deposition in the whole body of chickens. However, the efficiency of deposition is higher when dietary fat is kept constant at 9% (7.54) compared to increasing dietary polyunsaturation with varying added fat inclusion levels (5.99).

4. Increasing dietary PUFA results in a linear decrease of SFA and MUFA concentration in the body. For each gram of PUFA intake, there is a decrease of 2 percentage units of SFA and of 5.44-4.03 percentage units of MUFA.

5. The inclusion of α-tocopheryl acetate up to levels of 400 mg/kg did not affect significantly fatty acid profile in chicken, not even in dietary PUFA levels of around 60g/kg, except for very long chain ω6 FA, that were in slightly higher percentages in the animals supplemented than in the non-supplemented.

6. Regarding endogenous synthesis potential, the estimated deposition of fatty acids in the whole body coming from endogenous synthesis is reduced when dietary fat increases from 0 to 10%, varying between 35.34 % (33.94 - 36.74) and 17.66 % (16.86 - 18.45) for SFA; and

between 52.70 % (49.38 - 56.03) and 7.01 % (3.98 - 10.03) for MUFA respectively.

7. Apparent absorption values of α-tocopherol in broilers during the growth phase vary between 11 and 50%, with an average value of 42.1% under our experimental conditions. There were no differences in this parameter between 20 and 39 days of age.

8. Tallow inclusion at 9% impairs α-tocopherol intestinal uptake. A 2% of added fat rich in PUFA (4.4% total dietary fat) is not limiting for a proper α-tocopherol apparent absorption in chickens.

9. The concentration of α-tocopherol in whole body, liver and plasma is directly proportional to its amount in the diet. At high vitamin E dietary supplementation, there is an inverse relationship between α-tocopherol concentration in the studied tissues and dietary polyunsaturation level.

10. The concentration of α-tocopherol in liver and plasma in poultry are useful parameters to study the response to different dietary factors that can affect vitamin E bioavailability.

Chapter 9

Literature cited

“Que haces leyendo, pero si los de ciencias no sabéis leer”.

The file you are looking for is always at the bottom of the largest pile.

Finagle's Laws of Information:

1. The information you have is not what you want.
2. The information you want is not what you need.
3. The information you need is not what you can obtain.
4. The information you can obtain costs more than you want to pay.

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