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INFLUENCE OF FREE RADICALS (OXIDES OF NITROGEN) ON THE STABILITY OF LIPIDS IN EGG POWDERS

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INFLUENCE OF FREE RADICALS (OXIDES OF NITROGEN) ON THE STABILITY OF LIPIDS IN EGG POWDERS

By

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ABSTRACT

INFLUENCE OF FREE RADICALS (OXIDES OF NITROGEN) ON THE STABILITY OF LIPIDS IN EGG POWDERS

By

Shu-Mei Lai

Oxides of nitrogen (NOx), by-products of the combustion process in direct gas-fired spray drying operations, are free radical initiators of lipid oxidation. This study was conducted to investigate the influence of NOx and other free radicals on the stability of lipids in spray-dried egg powders, particularly with respect to cholesterol and carotenoids.

A method involving solid phase extraction and capillary gas chromatography was developed for the rapid quantification of cholesterol oxidation products (COPS) in egg powders. Homogeneous and consistently high recoveries of COPS, approximately 86%, were achieved by this analytical technique.

Concentrations of COPS in egg powders processed in the presence of free radicals (by using a gas burner or by the direct addition of NOx to air heated by an electric heating system) were approximately 2-5 times greater than those in powders produced by the electric heating system alone. The pathway of cholesterol oxidation initiated by NOx appeared

to be similar to the hydroperoxide-induced free radical chain reaction.

Results of a feeding trial established that supplementation of 4mg/kg oleoresin paprika carotenoids (OP) in hen diets based on white wheat provided egg yolk color equivalent to the color of eggs in supermarkets. Carotenoid stability in powders from the OP-supplemented eggs was less when processed with the direct gas-fired burner than with the indirect heating dryer. However, dietary supplementation of α-tocopherol acetate (200mg/kg) greatly enhanced the oxidative stability of cholesterol and carotenoids in eggs dried with the direct heating system.

The protective effect of dietary α-tocopherol supplementation on cholesterol and carotenoid stability in eggs exposed to NOx was also demonstrated in a lipid model system. The addition of carotenoids to egg lipids protected cholesterol from NOx-initiated oxidation. The rate constants of cholesterol oxidation and carotenoid degradation during storage increased with increasing concentrations of NOx. Cholesterol oxidation and carotenoid loss in the egg lipid model system were similar to those in egg powders processed by the direct gas-fired spray dryer.

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INTRODUCTION

Lipid oxidation is mainly responsible for the deterioration of food products during storage and adversely affects their color, flavor, nutritive value and even safety (Pearson et al., 1983). Dried egg products are susceptible to lipid oxidation not only because of their high lipid contents and low water activities, but also because of their exposure to oxides of nitrogen (NOx) produced during the combustion process in spray drying operations (Missler et al., 1985; Morgan and Armstrong, 1987, 1992; Kelly et al., 1989). NOx include nitric oxide (NO) and nitrous oxide (NO_2), and the latter has been demonstrated to be a free-radical initiator of oxidation of unsaturated lipids (Roehm et al., 1971; Pryor and Lightsey, 1981).

The presence of cholesterol oxidation products (COPS) in egg products is of considerable importance because of the high concentration of cholesterol in the yolk and the widespread use of dried egg products in foods such as scrambled eggs, pan cake mixes, Hollandaise sauce and cakemixes. Numerous COPS have been shown to possess biological activity and some are involved in atherogenesis, carcinogenesis, and cholesterol biosynthesis (Maerker, 1987; Kumar and Singhal, 1991). Although the presence of several COPS in dried egg products has been reported (Missler et al., 1985; Tsai and Hudson, 1985; Morgan and Armstrong,

1987, 1992), the effects of prooxidants produced during processing on the formation of COPS have not been conclusively investigated.

Another concern regarding the quality of dried egg products is the loss of pigments resulting from the oxidation process during dehydration and storage. The pigments responsible for egg yolk color are a group of compounds known as carotenoids, which are very susceptible to oxidation as a result of their high degree of unsaturation and the lipid environment in which they are located (Francis, 1985).

The red pigment extracted from paprika has been shown to modify and enhance the yellow color in yolk (Fletcher and Halloran, 1981, 1983). Most of the carotenoids in oleoresin paprika are esterified with fatty acids such as lauric, myristic and palmitic acids (Gregory et al., 1987; Biacs et al., 1989). The predominant carotenoid in saponified oleoresin paprika is capsanthin which accounts for 32 to 38% of the total carotenoids (Fisher and Kocis, 1987; Almela et al., 1991).

Although the saponified paprika carotenoids (free alcohol form) have been demonstrated to be more effectively absorbed by hens (Hamilton et al., 1990), the efficiency and mechanism of deposition of esterified and free (alcohol) carotenoids from oleoresin paprika in egg yolks have not been fully investigated. Furthermore, there are no reports in the literature on the stability of pigments in

carotenoid-supplemented egg products during processing and subsequent storage.

The effects of several natural antioxidants on the lipid stability of meats and meat products have been intensively investigated. The positive effects of dietary α-tocopherol supplementation on the oxidative stability of meats (Asghar et al., 1989, Monahan et al., 1992) and eggs (Faulkner, 1992) have been established. Oleoresin rosemary which contains a number of phenolic compounds, has been shown to be effective in various meat products (Barbut et al., 1985, Lai et al., 1991). However, none of these natural antioxidants have been used to stabilize the lipids or pigments of egg products during processing and storage. There is little information on the influences of free radicals (oxides of nitrogen) on lipid stability in egg powders, particularly with respect to cholesterol and carotenoids.

This research is therefore based on the hypothesis that lipid oxidation in spray dried eggs is initiated by the NOx formed during the combustion process. The addition of natural antioxidants such as oleoresin rosemary and α-tocopherol to whole liquid egg before drying and by supplementation of the laying hens diet in the case of α-tocopherol should therefore stabilize lipids as well as lipid-soluble pigments against oxidation during spray drying and storage.

Specific objectives of this study were to:

1. Develop a chromatographic method for the rapid quantitative determination of COPS in egg powders;

2. Investigate the influence of free radicals (NOx) which are formed during the combustion process in spray drying, on the formation of COPS in egg powder;

3. Evaluate the efficiency of deposition of carotenoids in egg yolk from groups of hens fed diets containing saponified or unsaponified oleoresin paprika, and to develop chromatographic methods for the characterization and quantification of carotenoids in oleoresin paprika and in egg yolk;

4. Evaluate the effects of spray drying method and dietary α-tocopherol on the stability of cholesterol and paprika carotenoids in egg during processing and storage;

5. Investigate the role of NOx in the formation of COPS and the degradation of paprika carotenoids using egg lipid model systems.

REVIEW OF LITERATURE

Lipid Oxidation in Spray-Dried Foods

Egg powders are usually produced by spray drying liquid whole eggs or yolks. Spray drying is a process where a liquid droplet is rapidly dried as it comes in contact with a stream of hot air. Figure 1 is a schematic diagram of a spray dryer where the atomized feed travels cocurrently with the drying air. According to the method of heating the air, spray dryers can be classified as: (1) direct heating spray dryers -- burning gas or fuel oil and the product of combustion heating the air directly; (2) indirect heating spray dryers -- using steam or electricity as heat sources and the heat being transferred across heat exchanger plates or coils to the air (Masters, 1976).

Jansen and Elgersma (1985) compared the economics of direct and indirect heating systems. They indicated that capital and maintenance costs associated with indirect systems are higher than with direct heating systems. In addition, the direct heating systems have been reported to be more efficient than the indirect systems due to less heat loss during heat transfer.

The method of spray drying has been reported to affect the stability of lipids in spray dried foods. Some investigators reported that the concentrations of

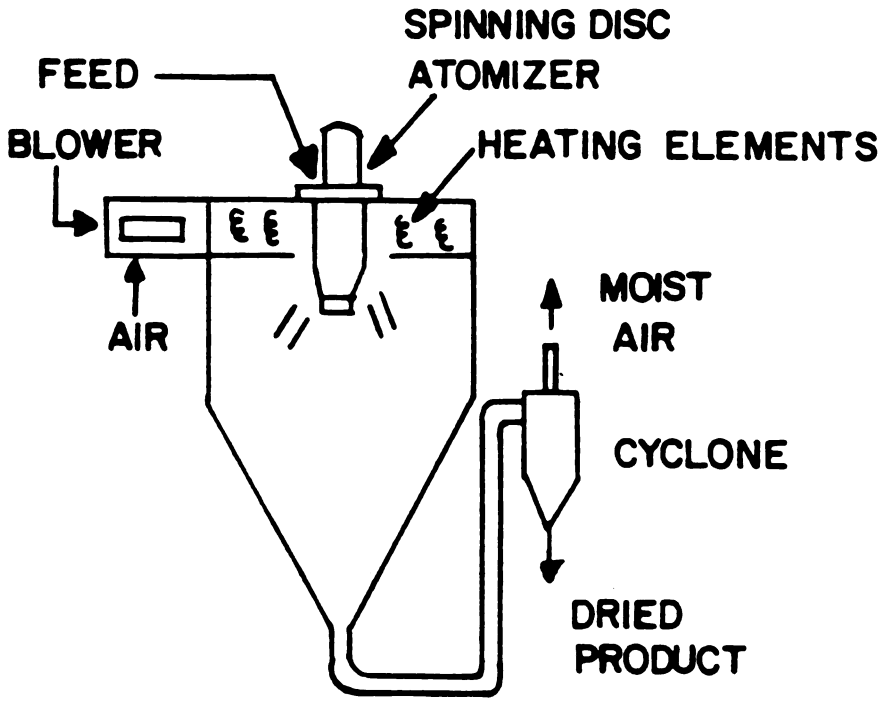


Figure 1. Schematic diagram for a spray dryer (Masters, 1976).

cholesterol oxidation products (COPS) are greater in foods dried with a direct gas-fired heating source than in foods dried with indirect heating (Missler et al., 1985; Tsai and Hudson, 1985; Faulkner et al., 1992; Chan et al., 1993). It was suggested that exposure of foods to oxides of nitrogen (NOx), by-products of combustion, was responsible for these greater quantities of COPS.

In this section, the mechanism of NOx formation during spray drying will be briefly reviewed. The role of NOx in the initiation of lipid oxidation will receive a major emphasis, as will be the formation of COPS in spray-dried egg products.

1. Formation of Oxides of Nitrogen during Spray Drying

Oxides of nitrogen (NOx), including nitrogen monoxide (also called nitric oxide, NO) and nitrogen dioxide (also called nitrous oxide, NO_2), are produced from air as a result of combustion processes. Wheeler (1980) summarized the mechanism of NO formation and reported that the formation of NO does not take place by the simple combination of nitrogen and oxygen, but through a set of interrelated, temperature-dependent reactions in which the predominantly active species are atomic oxygen (O), atomic nitrogen (N) and the hydroxyl radical ($OH^{\bullet}$):

$$N_2 + O \rightarrow NO + N$$

$$O_2 + N \rightarrow NO + O$$

$$OH^{\bullet} + N \rightarrow NO + H$$

The N and O atoms are produced by the thermal dissociation of N_2 and O_2 at elevated temperatures. The formation of NO is endothermic and quite rapid at flame temperatures above 1600°C (Wheeler, 1980).

On the other hand, the formation of NO_2 is a relatively low temperature reaction which takes place at approximately 600°C (Kelly et al., 1989) and likely occurs in the drying chamber. NO_2 is formed by the reaction of NO with diatomic oxygen:

$$NO + O_2 \rightarrow NO_2 + 1/2\ O_2$$

The rate of the reaction is dependent on the concentration of NO. Conditions which affect the rate of NO formation, e.g., temperature of combustion, are therefore the most critical factors involved in NOx ($NO + NO_2$) production (Kelly et al., 1989).

2. The Role of NOx in the Initiation of Lipid Oxidation

Lipid oxidation is mainly responsible for the deterioration of dried egg products and adversely affects the color, flavor, nutritive value and even safety of the products. The susceptibility and rate of oxidation vary according to the unsaturation of lipids and the presence of activating agents such as free radicals, metal catalysts, heat, light or enzymes (Nawar, 1985).

Classical studies have established the autoxidation of unsaturated lipids (RH) as a free-radical chain reaction

which can be divided into three separate processes (Chan et al., 1982):

Initiation: formation of lipid alkyl or allyl radicals (R·)

$$X\cdot + RH \rightarrow R\cdot + XH$$

(initiator)

Propagation: formation of lipid peroxyl radicals (ROO·) and hydroperoxides (ROOH)

$$R\cdot + O_2 \rightarrow ROO\cdot$$

$$ROO\cdot + RH \rightarrow ROOH + R\cdot$$

Termination: formation of non-reactive products

$$R\cdot + R\cdot \rightarrow RR$$

$$R\cdot + ROO\cdot \rightarrow ROOR$$

$$ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$$

The overall reaction between molecular oxygen and an unsaturated lipid (RH): $RH + O_2 \rightarrow ROOH$ is an addition reaction (Farmer, 1946). However, Heaton and Uri (1961) indicated that this reaction is endothermic (ΔH = 35kcal·mol^{-1}) and hence cannot proceed without the involvement of energy to abstract a proton from an unsaturated lipid molecule. This is due to the fact that the principle of spin conservation applies to an addition reaction between ground state molecules (Chan, 1987). There is a spin barrier which prevents the direct addition of triplet ground-state oxygen (i.e., contains two unpaired electrons) to singlet ground-state unsaturated lipid molecules (i.e., contains no unpaired electrons) because oxygen in the ground state is paramagnetic and unsaturated

lipids in the ground state are diamagnetic. This restriction can be overcome by abstracting a proton from an unsaturated lipid molecule with a free radical to form alkyl or allyl radicals which are paramagnetic and can react with oxygen in the ground state.

It is well established that a large number of different radicals can initiate lipid oxidation by abstracting hydrogen from RH to form R$^{\cdot}$ (Table 1)(Asghar et al., 1988). Because of the very small concentrations of radicals present and the likelihood of there being more than one process, the "initiation" of free radical-initiated lipid oxidation is a process that is very difficult to define. For example, initiators (X$^{\cdot}$) may be transition metal ions, radicals obtained by the decomposition of a hydroperoxide (e.g., RO$^{\cdot}$, HO$^{\cdot}$), radicals formed from an added initiator such as cumene hydroperoxide, or radicals existing in foods or which are produced during processing, e.g., NOx.

Among the many NOx, NO_2 and NO are free radicals. Although the biological effects of NO_2 and NO as strongly oxidizing toxicants have been investigated intensively (Kosaka et al., 1989), the mechanism of NO_2 and NO as initiators in lipid oxidation has not been fully established. Roehm et al. (1971) reported that trace quantities of NO_2 (0.5 to 5.4 parts per million, ppm) rapidly initiate the oxidation of methyl linoleate and methyl linolenate, both in thin films and in aqueous dispersions. They postulated that NO_2 adds directly to the

Table 1. Some possible initiators of free radical-initiated lipid oxidation[1].

Initiator	Formula
Hydroxyl radical	$HO^{\cdot}$
Hydroperoxyl radical	$HOO^{\cdot}$
Alkyl radical	$R^{\cdot}$
Alkoxyl radical	$RO^{\cdot}$
Peroxyl radical	$ROO^{\cdot}$
Superoxide radical	$O_2^{-\cdot}$
Nitrogen monoxide	$NO^{\cdot}$
Nitrogen dioxide	$NO_2^{\cdot}$
Ferrous ion	Fe^{2+}
Ferric ion	Fe^{3+}

[1] Adapted from Asghar et al., 1988; Simic and Taylor, 1987.

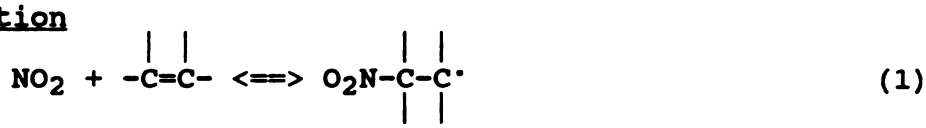
double bond of an unsaturated fatty acid to produce a nitroso radical, which in turn abstracts an allylic hydrogen from another unsaturated fatty acid molecule. This creates a second free radical which would then react with oxygen to form a peroxyl radical.

More recently, Pryor and Lightsey (1981) studied the reaction of NO_2 with cyclohexene as a model for the reactions that occur between NO_2 and unsaturated fatty acids. They proposed that the mechanism of NO_2-initiated oxidation is concentration-dependent.

At high concentrations (10,000 to 400,000 ppm in nitrogen), NO_2 initiates lipid oxidation by its direct addition to the double bonds of the unsaturated fatty acid, as shown in Figure 2 (Eq.1). At low concentrations (below 100 ppm in nitrogen), NO_2 reacts with unsaturated fatty

acids primarily by abstraction of the allylic hydrogen to form nitrous acid and an allylic radical (Figure 2, Eq.2). The allylic radical can combine with NO_2 to give an unsaturated nitro or nitrite compound, or with O_2 to give the allyic hydroperoxide.

<u>Addition</u>



(1)

<u>Abstraction</u>

(2)

Figure 2. Reactions of nitrogen dioxide (NO_2) with alkene (Pryor and Lightsey, 1981)

Once the reaction has been initiated, hydroperoxides are formed which subsequently undergo homolytic scission to form radicals capable of accelerating its oxidative process. The hydroperoxides may also undergo carbon-carbon cleavage to produce volatile compounds such as aldehydes, ketones and hydrocarbons. On the other hand, the lipid hydroperoxides can also react with oxygen to form secondary products such as expoxyhydroperoixdes, ketohydroperoxides and cyclic peroxides that can also breakdown and produce volatile compounds (Frankel, 1984).

Occurrence of Cholesterol Oxidation Products in Eggs

The proximate compositions of fresh eggs and egg powders are listed in Table 2. Lipids are one of the major constituents of eggs (11.15%) and whole egg powders (41.81%). The lipid content of yolk from fresh eggs of various strains of hens is between 32 and 36% (Marion et al., 1965). According to Privett et al. (1962), the composition of yolk lipid is 65.5% triacylglycerols, 28.3% phospholipids and 5.2% cholesterol. The cholesterol content in yolk lipids ranges from 2 to 6.2% (Tullett, 1987; Nourooz-Zaden, 1990) and is influenced by breeds or strains of hens (Edwards et al., 1960), dietary fat, and the method of determination (Weiss et al., 1964).

Although dietary cholesterol has long been considered a contributing factor in the atherosclerosis in humans (McGill, 1979; Kris-Etherton et al., 1988), recent studies have shown the possible role of COPS, rather than cholesterol, in the initiation of atherosclerotic plaque formation (Peng et al., 1982; Addis et al., 1989; Kubow, 1990; Kumar and Singhal, 1991). As shown in Table 3, the amount of cholesterol in eggs is relatively high among animal-derived foods. Therefore, the occurrence of COPS in eggs and egg products as well as the mechanism of their formation are of considerable importance and have been investigated intensely in recent years (Finocchiaro and

Table 2. Proximate composition of fresh eggs and egg powder (per 100g edible portion)[1].

Component		Fresh egg[2]			Egg powder	
		Whole	Yolk	White	Whole	Yolk
Weight per egg	(g)	50	17	33	--	--
Water	(g)	74.57	48.20	88.07	4.14	4.65
Protein	(g)	12.14	16.10	10.14	45.83	30.52
Lipid	(g)	11.15	34.10	trace	41.81	61.28
Carbohydrate	(g)	1.20	trace	1.23	4.77	0.39
Ash	(g)	0.94	1.69	0.56	3.45	3.16

[1] Adapted from USDA, 1976.
[2] Shell is 12% of weight of egg

Table 3. Cholesterol content of selected animal foods (per 100g)[1].

Food	Cholesterol content (mg)
HIGH	
Boiled calf brain	3100
Whole egg, poached	480
Butter	230
MEDIUM HIGH	
Lobster	150
Stilton cheese	120
Cooked pork, lean	110
MEDIUM LOW	
Cooked beef, lean	82
Boiled Chicken, white	80
Steamed cod	60
LOW	
Milk, whole	14
Cottage cheese	13
Milk, skimmed	2

[1] Adapted from Paul and Southgate, 1978.

Richardson, 1983; Maerker, 1987; Morgan and Armstrong, 1992).

In this section, the mechanism of cholesterol oxidation and the biological effects of COPS will be briefly reviewed. Other components of this review include summaries and discussion of the measurement of COPS in foods and factors that influence their formation in egg products.

1. General Mechanism of Cholesterol Oxidation

Cholest-5-en-3β-ol (cholesterol), with a 5,6-double bond in the B ring (Figure 3), is an unsaturated lipid which readily undergoes oxidation in the presence of oxygen (air) and light via a free radical reaction (Smith, 1981).

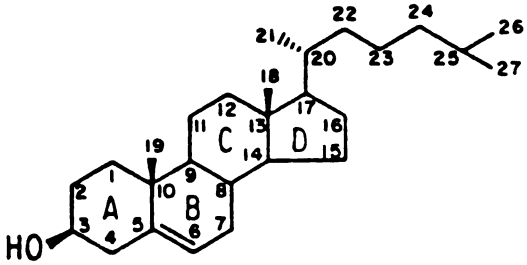
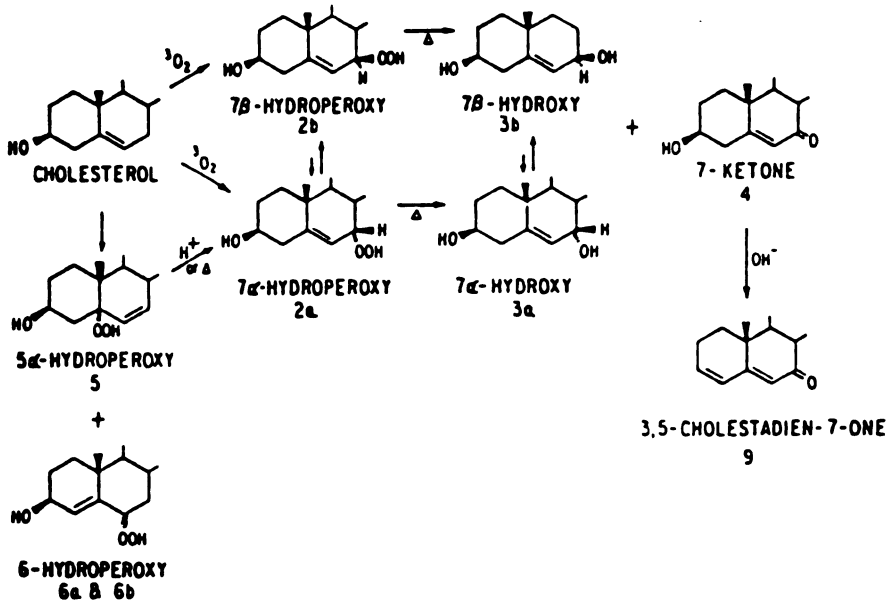
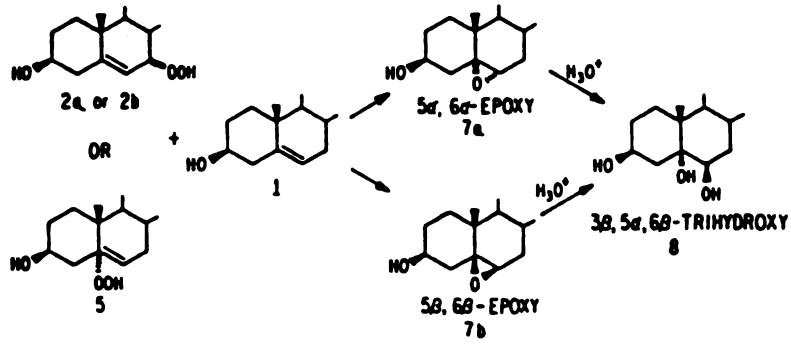


Figure 3. Structure of Cholesterol



SCHEME 1



SCHEME 2

Figure 4. Schemes for cholesterol oxidation (Maerker, 1987).

Cholesterol oxidation is initiated by abstraction of the allylic C-7 hydrogen and followed by reaction with triplet oxygen to form two epimeric hydroperoxides (**2a**, **2b**) as shown in Figure 4 (Smith and Hill, 1972). On the other hand, attack by singlet oxygen on cholesterol results in the formation (74-75%) of 5α-hydroperoxide (**5**) that is readily isomerized to 7α-hydroperoxide in presence of acid or heating (Kulig and Smith, 1973). Due to their thermal instability, the 7-hydroperoxides (**2a**, **2b**) are subsequently converted to more stable products such as the epimeric 7-hydroxycholesterol (**3a**, **3b**) and 7-ketocholesterol (**4**) (Van Lier and Smith, 1970).

The epoxides (**7a**, **7b**) are the products of attack by 7-hydroperoxides (**2a** or **2b**) or by 5α-hydroperoxide (**5**) on the 5,6-double bond of cholesterol and therefore are secondary oxidation products (Smith and Kulig, 1975). Hydration of epoxides (**7a** or **7b**) results in the formation of cholestan-3β, 5α, 6β-triol (Smith and Kulug, 1975). The α-epimers of COPS (**2a**, **3a** or **7a**) are thermodynamically less stable than the β-forms (**2b**, **3b** or **7b**), and interconversion occurs readily (Maerker, 1987).

Most studies of the pathway of cholesterol oxidation have been carried out in solutions or dispersions to provide a model for the state of cholesterol in most foods and in the aqueous environment of animal tissues. In solid phase or crystalline cholesterol, side chain oxidation occurs at the C-25 position producing initially the 25-hydroperoxy

derivative (Smith, 1981). This compound then degrades to the 25-hydroxycholesterol. Similarly, the 20α-hydroxy derivative is found when solid cholesterol oxidizes. By contrast, side-chain oxidation is not observed in autoxidation carried out in solution or in aqueous dispersions (Maerker, 1987).

2. Biological Effects of Cholesterol Oxidation Products

As a consequence of the documented relationship between elevated blood cholesterol and increased risk of coronary heart disease (CHD), researchers have attempted to elucidate the specific role of cholesterol in the disease process (McGill, 1979; Kris-Etherton et al., 1988). As a result of these investigations, it has been reported that COPS such as 25-hydoxycholesterol (Peng et al., 1982; Cox et al., 1988; Morin and Peng, 1989) and cholestan-3β, 5α, 6β-triol (Jacobson et al., 1985) possess greater atherogenicity than unaltered cholesterol.

Recent studies have indicated that low-density lipoprotein (LDL) must be modified (oxidized) before it can be recognized by scavenger receptors on monocytes in the vessel wall, a step which leads to foam cell formation (Jurgens et al., 1987; Heinecke, 1987). Although elevated plasma levels of COPS have been found in patients with CHD (Kumar and Singhal, 1991), it is still uncertain whether COPS in human lipoproteins are derived from in vivo

oxidation or from dietary sources or from both (Addis et al., 1989).

Emanuel et al. (1991) studied the absorption of COPS in humans following consumption of a powdered egg meal containing 30-90 ppm of each of four different COPS, and found increased COPS concentrations in both total plasma and plasma chylomicrons. By contrast, subjects consuming fresh eggs containing very low levels of the same four COPS (0-2 ppm) did not exhibit any significant rise in plasma COPS. These results demonstrated that the human subjects studied have the capacity to absorb COPS from the food source. Furthermore, the brief residence times of COPS observed in both chylomicrons and plasma suggest the rapid transfer of COPS among lipoprotein fractions from plasma.

The toxicity of dietary oxidized cholesterol has been studied using both in vivo and in vitro systems. Cholesterol α-epoxide has been reported to induce tumor formation in the skin of hairless mice (Bischoff, 1969; Black and Chan, 1976). Cox et al. (1988) demonstrated the cytotoxic effect of 25-hydroxycholesterol using sparse smooth muscle cells and human umbilical vein endothelial cells. Kandutsch et al. (1978) reported loss of DNA synthesis and cell division when 25-hydroxycholesterol or 7-ketocholesterol was added to L-cell cultures. In addition, Kandutsch and Chen (1973, 1974) found that 7-ketocholesterol and 25-hydroxycholesterol are potent inhibitors of cholesterol biosynthesis in cultured mouse cells by the

suppression of 3-hydroxy-3-methlyglutaryl coenzyme A (HMG-Co A) reductase activity.

Because most of the studies of cytotoxic effects of COPS have been carried out on cultured cells or experimental animals, it is difficult to speculate on the significance of COP-induced cytotoxicity as it relates to atherogenesis in humans. However, these findings create concerns about the presence of COPS in human foods and their possible effects on humans.

3. Measurement of COPS in Foods

In spite of the many established toxic effects of COPS, their occurrence in foods has only been successfully studied in recent years. There have been technical difficulties associated with the isolation of COPS from foods, a much more complicated matrix than air-aged or model system cholesterol (Addis, 1986). Other difficulties in the quantification of COPS are related to the limitation of analytical tools for detecting the trace amounts of COPS in foods. The development and limitations of the quantitative determinations of COPS in foods will be reviewed briefly.

(a) Isolation of COPS

The quantification of COPS from foods is difficult because their isolation is frequently impeded by large amounts of interfering cholesterol, triacylglycerols, phospholipids and other lipids. It is a common practice to

isolate cholesterol and COPS from lipid matrices by saponification and the subsequent extraction with a solvent such as diethyl ether (Finocchoaro, et al., 1984; Lee, et al., 1985; Higley, et al., 1986). However, hot alkaline treatment of cholesterol and COPS is a significant source of error in the quantification of COPS (Addis, 1986). Tsai et al. (1980) reported a loss of approximately 75% of the α-epoxide after saponification due to the hydrolysis of the epoxide ring. Substantial loss of 7-ketocholesterol also occurs as a result of saponification because of its high sensitivity to alkali and its subsequent degradation to 3,5 cholestadien-7-one and several other products (Maerker and Unrun, 1986). However, Park and Addis (1986) reported no loss of α-epoxide and 7-ketocholesterol after cold saponification with 1M KOH for 18 hours at room temperature.

Column chromatographic clean-up has been used intensively as an enrichment step in the quantification of COPS to avoid formation of artifacts and/or breakdown of COPS during saponification. Packed silicic acid columns and silica gel columns have been utilized to fractionate COPS from triacylglycerols and other lipids (Tsai and Hudson, 1985; Missler et al., 1985; Park and Addis, 1985a; Sugino et al., 1986). However, these columns can not remove cholesterol substantially to avoid its interference with the quantification of some COPS, e.g., 7α-hydroxycholesterol (Morgan and Armstrong, 1987; Sander et al., 1989; Monahan et al., 1992). Missler et al. (1985) used preparative high

performance liquid chromatography (HPLC) to further remove excess cholesterol from samples after an initial clean-up with silica gel column chromatography and prior to final gas chromatographic analysis. They reported that with this method, 20% of the 25-hydroxycholesterol present was also eluted out with the cholesterol fraction.

Recently, disposable columns or cartridges have been used effectively to enrich COPS from cholesterol and other lipids (Morgan and Armstrong, 1989, 1992; Nourooz-Zadeh, 1990). This method is rapid and suitable for the routine analysis of COPS in foods and eliminates cumbersome and tedious preparatory steps. However, the broad range of recoveries (63.9%-101.2%) of COPS reported with this method suggests that some of the COPS may be selectively washed out to various degrees with the cholesterol fraction during sample clean-up (Morgan and Armstrong, 1989). Thus, it was suggested that the loss of COPS due to the clean-up procedure should be corrected with recovery data (Morgan and Armstrong, 1989).

(b) Detection of COPS

Although thin-layer chromatography (TLC) is a rapid method for the analysis of COPS, the epimeric 5,6-epoxides and 7-ketocholesterol are poorly resolved by this technique (Maerker, 1987). HPLC has been used successfully for identification and quantification of some COPS (Tsai and Hudson, 1981; Park and Addis, 1985a; Sugino *et al.*, 1986).

However, α-and β-epoxides and their triol hydrolysis products are not detected by HPLC when using a UV detector (Csallany et al., 1989). The development of improved gas chromatographic (GC) techniques has facilitated the separation of the principal COPS and greatly enhanced cholesterol oxidation research in the past few years (Missler et al., 1985; Park and Addis, 1985b, 1986, 1987; Sander et al., 1989; Morgan and Armstrong, 1989, 1992).

The resolution of major COPS on different types of GC columns has been evaluated by Park and Addis (1985b). They reported that the resolution of epimeric 7-hydroxycholesterols improves when capillary columns are used instead of packed columns. However, the thermal decomposition of the diols, 7α- and 7β- hydroxycholesterols and 25-hydroxycholesterol was observed during the analysis. The instability of COPS during GC analyses can be overcome by converting the COPS to their trimethylsilyl ether (TMS) derivatives. Park and Addis (1985b) reported that the cholesterol diols are effectively resolved as their TMS derivatives with no evidence of thermal decomposition.

Missler et al. (1985) suggested the use of less harsh silylating conditions to prevent cleavage of the epoxide bonds of COPS as well as to improve peak shape and subsequent separation of COPS. Later, Park and Addis (1989) found that bis- and/or tris- TMS ethers may be formed when cholestan-3β, 5α, 6β-triol is derivatized for different lengths of time. The degree of silylation is also

temperature-dependent. Morgan and Armstrong (1989) evaluated the recovery data from several studies of COPS and reported that the extraction, concentration and derivatization procedures might account for most of the variation in the recovery data.

4. Factors Influencing the Formation of COPS in Egg Products

COPS have been detected in foods such as egg products, cheeses, butteroil, milk powders, french fries, tallow and meat products (Finocchiaro and Richardson, 1983; Sander et al., 1989). Among these, egg products are of major considerable interest because of their high contents of cholesterol.

Eight common COPS have been identified in cholesterol-rich foods: 25-hydroxycholesterol, α- and β-epoxides, 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, cholestan-3β, 5α, 6β-triol and cholestan-3,5-dien-7-one, which is actually an artifact derived from 7-ketocholesterol during isolation (Finocchiaro and Richardson, 1983; Tsai and Hudson, 1984). Many factors including elevated temperatures, prolonged storage, or processing in the presence of prooxidants can influence the rate of cholesterol oxidation as well as the quantities and distribution of COPS in foods (Sander et al., 1989). Several recent studies on formation of COPS in eggs or egg powders subjected to these oxidative conditions will be reviewed briefly.

(a) Heat or Light

Chicoye et al. (1968) identified 5,6-epoxides and 7-hydroxycholesterols in spray-dried egg yolks exposed to direct sunlight for 5 hours. The 7-hydroxycholesterols were also identified in dry egg nog mix which had been exposed to fluorescent light for 90 days (Herian and Lee, 1985). Naber and Biggert (1985) reported that fresh egg yolk and spray dried egg yolk powders do not contain 25-hydroxycholesterol, but this compound as well as the epimeric 7-hydroxycholesterols were present after egg yolk powders were heated at 100-110^{o}C for four days.

(b) Storage

Tsai and Hudson (1984) studied the effect of storage on the formation of COPS in egg powders and detected a 4.6 fold increase in cholesterol epoxides in samples stored in glass jars under lighting and ambient temperature conditions for 9 months. Nourooz-Zadeh and Appelqvist (1987) reported that the concentrations of COPS in dehydrated egg yolk stored in sealed plastic bags increased with the storage time as well as the temperature of storage. They found no COPS in fresh egg yolks and only traces of COPS in spray dried egg yolk powders which were fresh or stored for 2 months at 4^{o}C. However, samples stored at ambient temperature for 3 and 5 months contained α- and β-epoxides, 7α- and 7β-hydroxycholesterols and 7-ketocholesterol, and had greater concentrations of COPS than those stored at 4^{o}C. These

investigators also reported that after 8 years of storage, a dehydrated egg yolk sample contained the above COPS plus 25-hydroxycholesterol, 20α-hydroxycholesterol and cholestan-3β, 5α, 6- triol. These COPS (except 20α-hyroxycholesterol) also have been detected in dried egg mixes that had been stored in cans for 5 years (Missler et al., 1985).

(c) Processing

Several reports revealed that concentrations of COPS are higher in foods spray dried with a direct gas-fired heating source than in foods dried with indirect heating. Tsai and Hudson (1985) surveyed commercial dried egg products from 15 dehydration plants and reported a wide range of variation (3 to 74 ppm) in α-epoxide and β-epoxide concentrations in the samples. They found that egg samples dried by hot air heated directly with a gas burner contain greater amounts of cholesterol epoxides (up to 74 ppm) than those dried by air heated indirectly with steam (up to 5 ppm). Missler et al. (1985) investigated the COPS contents of a dried egg mix produced under different processing conditions and also found that samples dehydrated with a direct heat source generated considerably greater amounts of total COPS (168 ppm) than indirect heating (42 ppm). These investigators suggested that exposure to nitrogen oxides (by-products of combustion) may be responsible for the higher levels of COPS.

Recently, Morgan and Armstrong (1992) investigated the effects of processing conditions on formation of COPS concentrations in egg spray dried with direct heat source. They found that amounts of NOx in the combustion gases and outlet temperature of spray dryer are the only processing conditions responsible for the elevated COPS concentrations.

The presence of prooxidants (e.g. hydrogen peroxide) during spray-drying was also demonstrated to be necessary to promote formation of measurable quantities of cholesterol-5,6-epoxides in egg yolk powders dried with an indirect-heated system (Morgan and Armstrong, 1987). However, the concentrations of H_2O_2 in liquid yolk required to generate detectable epoxides in dried egg yolks at a outlet temperature of 90°C was 10%, a concentration not normally used in any food processing procedure.

Enhancement of Egg Yolk Color by Dietary Supplementation with Paprika Carotenoids

The color of egg yolks is of major concern to food processors who rely on egg yolks to impart color to various products such as noodles and pasta. It is also of concern to consumers who associate yolk color with quality and health. The pigments responsible for egg yolk color are a group of compounds known as oxycarotenoids or xanthophylls which originate in plants (Marusich and Bauernfeind, 1981).

At present, enhancing the pigmenting properties of poultry feeds by supplementation with xanthophylls costs from $5 to $15/ton (Williams, 1992).

During the past half century, many investigators have studied the pigmenting properties of various colored substances to identify one or a combination that can achieve the most consistent and economic pigmentation for egg yolk. As a result, hundreds of carotenoids have been found that can be absorbed from the diet and deposited in the yolk (Marusich and Bauernfeind, 1981; Fletcher, 1992). In this review, only recent studies of egg yolk pigmentation which involve the utilization of paprika carotenoids will receive major emphasis. In addition, the composition and the properties of the paprika carotenoids, as well as the stability of carotenoids in foods will be briefly reviewed.

1. Composition of Paprika Carotenoids

Paprika, *Capsicum annum*, is one of the oldest and most important source of natural carotenoid food colors. It is often referred as red pepper and is used in a variety of products such as chili, soups, stews and sausage products (Klaüs and Baurenfeind, 1981).

Most of the carotenoids in paprika extract or oleoresin paprika are esterified with fatty acids, thus making them oil-soluble (Philip *et al.*, 1971; Gregory *et al.*, 1987). Fisher and Kocis (1987) separated the carotenoids in

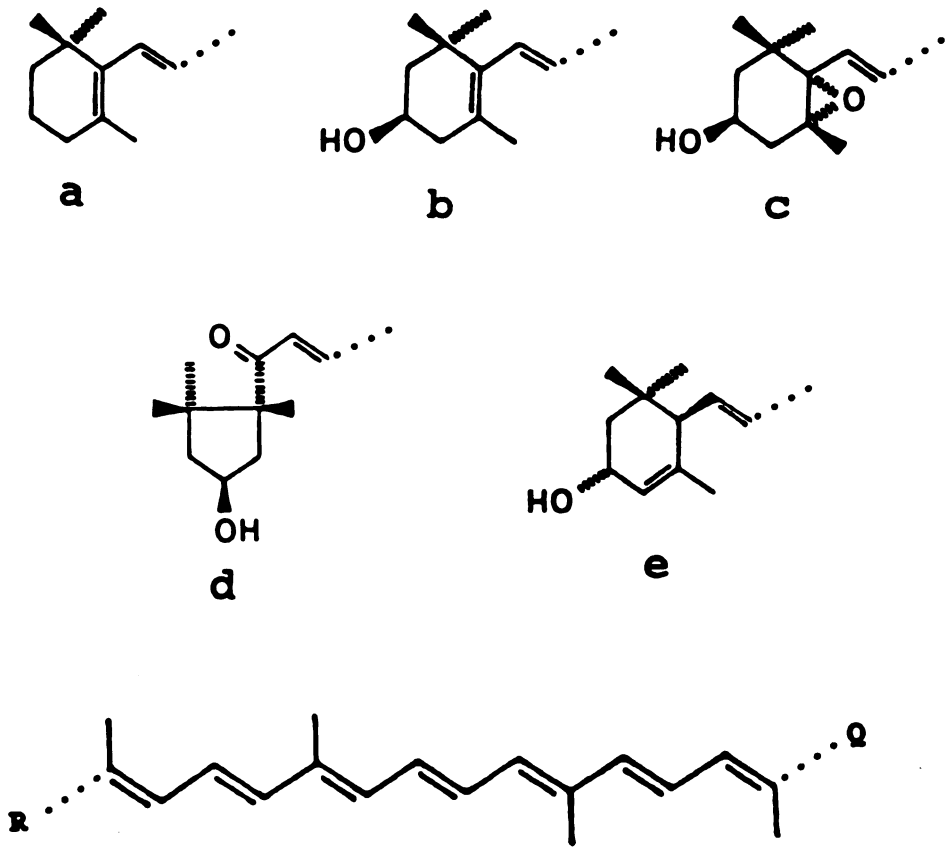
oleoresin paprika using reverse phase HPLC, and reported that hypophasic compounds such as capsorubin, capsanthin and zeaxanthin elute first, followed by the epiphasic pigments such as the monoesters of carotenoids, β-carotene and diesters of carotenoids. These investigators also indicated that the diesters of carotenoids are the principal pigments in oleoresin paprika, but did not identify the individual peaks for the esters of carotenoids. Biacs et al. (1989) further investigated the carotenoids in paprika and found that the majority of the carotenoid esters are mono- and diesters of capsanthin. The monoesters of capsanthin are generally esterified with unsaturated fatty acids, mainly linoleic acid, while diesters of both capsanthin and capsorubin are esterified with saturated fatty acids such as lauric, myristic and palmitic acids (Biacs et al., 1989).

Because most carotenoids in oleoresin paprika exist in the ester form, it is a common practice to hydrolyze the ester linkage by saponification for easier isolation and identification of the paprika pigments (Fisher and Kocis, 1987). Sixteen carotenoids have been identified in saponified oleoresin paprika (Almela et al., 1991), with capsanthin predominating to the extent of 32 to 38% of the total carotenoid content (Davies et al., 1970; Baranyai et al., 1982; Almela et al., 1991). The relative amounts of carotenoids in paprika cited in the literature are summarized in Table 4, while the structures of the major carotenoids are shown in Figure 5.

Table 4. Relative amounts (%) of carotenoid pigments in the saponified extract of paprika.

Carotenoid	Curl (1962)	Davies (1970)	Baranyai (1978)	Alemla (1991)
capsanthin	34.7	31.7	38.1	38.4
β-carotene	11.6	12.3	18.6	7.4
violaxanthin	9.9	9.8	7.9	3.9
crytoxanthin	6.7	7.8	4.2	2.8
capsorubin	6.4	7.5	9.5	5.0
cryptocapsin	4.3	5.0	1.8	0.7
zeaxanthin	2.3	6.5	4.0	2.9
antheraxanthin	1.6	9.2	2.6	1.6
capsanthin epoxide	0.9	4.2	2.6	4.7
lutein	---	---	---	1.2

Red paprika color is primarily caused by capsanthin and capsorubin, while β-carotene and violaxanthin contribute to the yellow-orange color (Reeves, 1987). Carotenoids with the end group "a" (Figure 5), e.g., β-carotene, have provitamin A activity and are more likely to be metabolized by poultry as a precursor of vitamin A than utilized for pigmentation (Marusich and Bauernfeind, 1981). On the other hand, dihydroxycarotenoids with hydroxyl groups at both ends of the carotenoid structure (e.g., capsanthin, zeaxanthin and lutein) can be effectively absorbed by hens and deposited in the egg yolks (Hamilton et al., 1990).



	R	Q		R	Q
capsanthin	b	d	cryptocapsin	a	d
β-carotene	a	a	zeaxanthin	b	b
violaxanthin	c	c	antheraxanthin	b	c
crytoxanthin	a	b	capsanthin epoxide	c	d
capsorubin	d	d	lutein	b	e

Figure 5. Structures of paprika carotenoids.

2. Absorption and Deposition of Carotenoids in Hens

A considerable amount of research has been conducted to evaluate carotenoids for their ability to be absorbed from the diet and deposited in the yolk (Fletcher, 1992; Hencken, 1992). Carotenoids can be divided into two main groups: (1) hydrocarbons containing only hydrogen and carbon and which are called carotenes; and (2) oxygen-containing derivatives which are called xanthophylls or oxycarotenoids (Goodwin, 1980). In hens, β-carotene is almost completely converted into vitamin A and does not contribute to the pigmentation of egg yolk (Marusich and Bauernfeind, 1981). Therefore, the pigmentation of chicken egg yolk is based on the absorption and deposition of oxycarotenoids or xanthophylls (Hencken, 1992).

The oxycarotenoids which are primarily responsible for the pigmentation of egg yolks from hens fed a typical corn diet include lutein, zeaxanthin and cryptoxanthin (Schaeffer et al., 1988). Therefore, color substances from other natural sources such as marigold concentrate and alfalfa, which are also rich in lutein, have often been utilized to enhance the yellow pigmentation of egg yolks (Middendor et al., 1980; Papa et al., 1985).

While most efforts have concentrated on yellow pigmenting substances, several investigators noted that some naturally occurring red substances can impart reddish tones to the yolk. Mackay et al. (1963) reported that paprika extract enhanced egg yolk pigmentation and that the improved

color would not adversely affect sponge cake color. Later, Nelson and Baptist (1968) found that incorporating small amounts of red pigments into typical poultry diets containing comparatively large amounts of yellow carotenoids had the effect of modifying and enhancing the yellow color in the yolks.

Fletcher and Halloran (1981) applied this concept in a low xanthophyll diet (white corn diet) to evaluate the egg yolk pigmentation properties of marigold extract and oleoresin paprika. They reported that the addition of small amounts of oleoresin paprika, e.g., 2.4mg/kg, to a diet containing marigold concentrate, e.g., 24mg/kg, produced egg yolk color equivalent to that produced using much higher levels of marigold concentrate, e.g., 60mg/kg. Furthermore, Fletcher and Halloran (1983) investigated the pigmenting properties of these two substances with a practical yellow corn diet. They found that the addition of small quantities (6mg/kg diet) of the oleoresin paprika to the yellow corn diet resulted in a greater color response than that provided by larger quantities (60mg/kg diet) of the marigold concentrate. These investigators suggested that small amounts of oleoresin paprika can be used to greatly enhance yolk color as opposed to much larger quantities of marigold concentrate.

Recent developments in HPLC for the separation and quantification of component oxycarotenoids from feedstuffs and egg yolks have permitted investigators to study the

metabolic changes of feed carotenoids during their absorption, and the deposition efficiency of individual carotenoids in poultry (Tyczkowski and Hamilton, 1986; Schaeffer et al., 1988; Hamilton et al., 1990). Tyczkowski and Hamilton (1986) used the free (alcohol) form of lutein as a model dihydroxycarotenoid to study the pigmentation in chickens. They found that part of the free lutein in the diet is esterified during its passage down the intestinal tract and regardless of its status when absorbed, it is transported in the body as the free alcohol. Later, Schaeffer et al. (1988) reported that carotenoid esters are hydrolyzed in vivo prior to absorption and that more than 90% of the carotenoids are deposited as free alcohols in egg yolks from hens fed a yellow corn-alfalfa diet.

More recently, Hamilton et al. (1990) investigated the influence of saponification on the deposition in egg yolk of carotenoids from oleoresin paprika. They demonstrated that saponified paprika carotenoids are deposited in egg yolks twice as efficiently as unsaponified paprika carotenoids. They suggested that saponification improves the digestibility of the originally esterified carotenoids (i.e., capsanthin), thus enhancing the efficiency of absorption and deposition of carotenoids.

3. Stability of Carotenoids in Foods

Carotenoids are very susceptible to oxidation because of the conjugated polyene system. The oxidation of

carotenoids is an autocatalytic, free-radical chain reaction and is accelerated by prooxidant factors such as free radicals, metals, light and elevated temperature (Francis, 1985). The oxidative degradation products of carotenoids play an important role in the flavor and aroma development of tea (Yamanishi et al., 1980) and cooked salmon (Josephson et al., 1991). However, in other foods such as dehydrated vegetables, egg powders and rainbow trout, oxidation of carotenoids can diminish the attractive color and flavor, leading to unacceptability and reduced storage life (Walter et al., 1970; Chen et al., 1984; Bergquist, 1986; Glória et al., 1993).

In processed foods, the mechanism of carotenoid oxidation is very complex because of the various carotenoids present and the complicated interactions of carotenoids with the surrounding media (Francis, 1985). Therefore, model systems have been frequently used to elucidate the mechanism of carotenoid oxidation. Philip and Francis (1971) investigated the oxidation of capsanthin, the predominant paprika carotenoid, by molecular oxygen at 40^{o}C in the solid state. They reported that the oxidation process involves primarily the conversion of hydroxyl groups to keto groups, followed by scission of the chain at the carbon-carbon bond 'a' to the in-chain carbonyl group. A number of keto-carotenoids such as capsanthone, 3-keto-kryptocapsone and 3-

keto-β-apo-8'-carotenal were identified among the oxidation products.

The mechanism of oxidation of carotenoids is influenced by the structure of the carotenoid in question and by the type of oxidation involved (Francis, 1985). For example, Terao (1989) reported that canthaxanthin and astaxanthin which possess oxo groups at the 4, and 4'-positions in the β-ionone ring, are more resistant to free radical-initiated oxidation than β-carotene and zeaxanthin which possess no oxo groups.

Recently, Glória et al. (1993) investigated the effect of different types of oxidation on β-carotene loss and volatile products formation in model systems. They found that the loss of β-carotene is faster during autoxidation at 80°C, followed by chemical reaction (initiated by metachloroperbenzoic acid), photosensitized oxidation and autoxidation at 20°C. Although similar degradation products were found for all types of oxidation, their relative concentrations varied. Autoxidation at 20°C and chemical reaction led to several volatile oxidation products originating from cleavage of bonds 7-8, 9-10, and 8-9 of β-carotene. However, autoxidation at 80°C and photosensitized oxidation resulted in more specific cleavage. The major volatile oxidation product from autoxidation at 80°C was dihydroactinidiolide, originating from cleavage of bond 8-9,

while photosensitized oxidation produced β-ionone, a product from cleavage of bond 9-10 of β-carotene.

Although the mechanisms of oxidation for some carotenoids in model systems have been established, there are few reported studies regarding the loss of carotenoids in foods as a consequence of processing and storage conditions. Carotenoids can also act as antioxidants or prooxidants depending on the system. For example, β-carotene has been reported to be an effective singlet oxygen quencher (Krinsky, 1989) and prevents light-catalyzed oxidation in soybean oil (Warner and Frankel, 1987; Lee and Min, 1990). However, in the absence of δ-tocopherol or in the dark, β-carotene is autoxidized rapidly and the oxidation products of β-carotene can induce autocatalytic oxidation of oils (Terao et al., 1980; Warner and Frankel, 1987). Therefore, β-carotene is an effective inhibitor of photooxidation of soybean oil only when its oxidation is prevented by tocopherols (Warner and Frankel, 1987).

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CHAPTER ONE

EVALUATION OF SOLID PHASE EXTRACTION AND GAS CHROMATOGRAPHY FOR DETERMINATION OF CHOLESTEROL OXIDATION PRODUCTS IN SPRAY-DRIED EGG POWDERS.

ABSTRACT

A method using solid phase extraction (SPE) and capillary gas chromatography (GC) was developed for the rapid quantification of cholesterol oxidation products (COPS) in egg powders. Total lipid extracts were fractionated on disposable silica SPE tubes to isolate COPS from triacylglycerols, phospholipids and cholesterol. The COPS (α- and β-epoxides, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol) were resolved as their trimethylsilyl ether derivatives on a non-polar capillary column. Combined GC-mass spectrometry was used to confirm the identities of the trimethylsilyl ethers of COPS. Homogeneous and consistently high recoveries of COPS standards as well as 6-ketocholesterol (internal standard), approximately 86%, were achieved with this analytical technique.

INTRODUCTION

Numerous cholesterol oxidation products (COPS) possess biological activity, with some involved in athergenesis, carcinogenesis, and cholesterol biosynthesis (Maerker, 1987; Kumar and Singhal, 1991). The adverse biological activities attributed to COPS have caused concern about their occurrence in foods (Finocchiaro and Richardson, 1983; Sander et al., 1989).

Over the past decade, various methods have been developed for the identification and quantification of COPS in food products exposed to heat and air during processing and/or storage. These include packed column gas chromatography (GC) (Morgan and Armstrong, 1987), capillary column GC (Park and Addis, 1985a; Missler et al., 1985; Nourooz-Zadeh, 1990; Morgan and Armstrong, 1992), and high performance liquid chromatography (HPLC) (Tsai and Hudson, 1981; Park and Addis, 1985b; Suginol et al., 1986). However, there is no standard method that isolates and quantifies all major COPS in foods accurately and rapidly. This is partly due to the difficulties associated with the isolation of small amounts of COPS in foods that contain large amounts of interfering cholesterol, triacylglycerols, phospholipids and other lipids (Missler et al., 1985; Park and Addis, 1985b; Nourooz-Zadeh, 1990).

Saponification has been commonly used as an enrichment step in the quantification of COPS in the presence of other

lipids (Finocchiaro et al., 1984; Higley et al., 1986). However, hot alkaline treatment of cholesterol and COPS is a significant source of error in the quantification of COPS (Addis, 1986). Tsai et al. (1980) reported a loss of approximately 75% of the α-epoxide after saponification due to the hydrolysis of the epoxide ring. Substantial loss of 7-ketocholesterol also occurs as a result of saponification because of its high sensitivity to alkali and subsequent degradation to 3, 5-cholestadien-7-one and several other products (Maerker and Unruh, 1986).

Recently, solid phase extraction (SPE) has been used intensively for COPS clean-up to avoid formation of artifacts and/or breakdown of COPS during saponification. Packed silicic acid columns and silica gel columns have been utilized to fractionate COPS from triacylglycerols and other lipids (Tsai and Hudson, 1984; Missler et al., 1985; Park and Addis, 1985b; Sugino et al., 1986). However, these columns can not remove cholesterol substantially to avoid its interference with the quantification of some COPS, e.g., 7α-hydroxycholesterol (Morgan and Armstrong, 1987; Sander et al., 1989; Monahan et al., 1992). Furthermore, this column chromatography procedure has cumbersome and tedious preparatory steps.

On the other hand, disposable columns or cartridges have become popular for sample clean-up because of their convenience and the improved reproducibility of data obtained. Although they have been used effectively to

enrich COPS from cholesterol and other lipids (Morgan and Armstrong, 1989, 1992; Nourooz-Zadeh, 1990), a broad range of recoveries (63.9%-101.2%) of COPS has been reported with this method (Morgan and Armstrong, 1989). It has been suggested that some of the COPS may be selectively washed out to varying degrees with the cholesterol fraction during sample clean-up. However, no information about the capacity of the disposable columns or cartridges or the flow rate of solvents used for SPE, is provided in these reports.

The objective of this study was to develop a method that can be used routinely for the quantification of all major COPS in egg powder. Some parameters that may affect the efficiency of SPE and the accuracy of quantification such as solvent flow rate and conditions for derivatization were also investigated.

MATERIAL AND METHODS

Reagents

Cholesterol (cholest-5-en-3β-ol) and 6-ketocholesterol (5-cholesten-3β-ol-6-one) standards were purchased from Sigma Chemical Co. (St. Louis, MO). Cholesterol oxide standards, α- and β-epoxides (cholestan-5α, 6α-epoxy-3β-ol and cholestan-5β, 6β-epoxy-3β-ol), 7α- and 7β-hydroxycholesterols (5-cholesten-3β, 7α-diol and 5-cholesten-3β, 7β-diol), 7-ketocholesterol (5-cholesten-3β-

ol-7-one), 20α-hydrocholesterol (5-cholesten-3β, 20α-diol), 25-hydroxycholesterol (5-cholesten-3β, 25-diol), and cholestan-3β, 5α, 6β-triol were purchased from Steraloids Inc. (Wilton, NH). Bis-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS) was obtained from Pierce Chemical Co. (Rockford, IL). Other reagents and solvents used in this study were analytical grade and/or HPLC grade.

Food Samples

Whole liquid egg was spray dried with a direct gas-heating spray dryer (Kelly et al., 1989) at the National Dairy Products Research Centre (Moorepark, Cork, Ireland). The egg powders were vacuum packaged in polyethylene-laminated nylon pouches (Koch, Kansas City, MO) and immediately air freighted to Michigan. These pouches (90μm thickness) have a water-vapor transmission rate of 0.041 $ml/m^2 \cdot day \cdot mmHg$ and an oxygen transmission rate of 0.124 $ml/m^2 \cdot day \cdot mmHg$ at 22.7°C, 50% RH. Once received (within two weeks of processing), the egg samples were repacked in low density polyethylene bags (8" X 10", 75μm thickness, Whirl-Pak, Fisher Scientific, Fair Lawn, NJ) without heat sealing and stored at ambient temperature (22 ± 2°C) in the dark for 6 months. Sampling for analysis of the egg samples was performed randomly after thoroughly mixing the egg powder in the bags with a spatula (approximately 100g per bag).

Extraction of Lipids

Fifteen μg of the internal standard, 6-ketocholesterol, were added to 1.000±0.005 g egg powder prior to extraction. The sample was homogenized for 2 min with 30ml chloroform in a 100ml beaker using an Ultra-Turrax type of homogenizer (Tekmar Co., Cinn., OH). The extract was then filtered through 5g anhydrous sodium sulfate and Whatman No.4 filter paper to remove moisture and egg powder particles. Another 30ml aliquot of chloroform was used to rinse the probe and to reextract the residue. The combined extracts were evaporated to dryness with a vacuum rotary evaporator (Büchi Rotavapor, Postfach, Switzerland) and redissolved in 5ml hexane.

Solid Phase Extraction

The COPS in the lipid extract were isolated using a 3ml Superclean^{TM} LC-Si SPE tube (Supelco, Bellefonte, PA) filled with 300mg silica packing (40 μm particles, 60 Å pores). The SPE tube was wetted with 3ml hexane to activate the packing before the sample was added. After adding the sample, the following solvent combinations were applied to the SPE tube when approximately 1mm of the previous solvent remained above the top of the tube frit: 10ml hexane/ethyl ether (95:5, v/v), 25ml hexane/ethyl ether (90:10, v/v), and 15ml hexane/ethyl ether (80:20, v/v). Finally, the COPS were eluted out of the column with 5ml acetone. Chromatography was accelerated with a vacuum of 20kPa using

a vacuum manifold (Supelco, Bellefonte, PA) which was attached to a water-suction arrangement. The vacuum manifold also controlled the flow rate of the solvents (0.6 ± 0.1ml/min). This system, through the use of independent screw-type valves, permitted the simultaneous extraction of up to 12 samples.

Derivatization of COPS to TMS Ethers

After the solvent was evaporated under a stream of nitrogen, the COPS were redissolved in 100μl BSTFA + 1% TMCS in a 1/2 dram glass vial, capped and mixed with a vortex mixer for 30 sec. This mixture was placed in the dark at room temperature for 50 min to form the trimethylsilyl (TMS) ether derivatives of the COPS. Subsequently, the TMS reagent was removed under nitrogen and the residue was dissolved in 100μl hexane.

GC Analysis

TMS ethers of COPS were analyzed using a Hewlett Packard (HP) 5890A GC (Avondale, PA) equipped with a flame ionization detector. A 15m x 0.25mm i.d. DB-1 (0.1μm film thickness) capillary column (J&W Scientific Inc. Folsom, CA) operated with a helium carrier gas (column flow rate of 1ml/min) was used for the separation of the TMS ethers. The oven temperature was programmed from 170^{o}C to 220^{o}C at a rate of 10^{o}C/min, then increased to 236^{o}C at a rate of 0.4^{o}C/min. After the peaks of interest (COPS) were eluted,

the oven temperature was increased at a rate of 10°C/min to a final temperature of 300°C and held for 25 min or until all lipid residues were eluted out of the column. The temperatures of the injection port and detector were held at 275°C and 320°C, respectively. Two microliters of the TMS derivatives of the COPS were injected onto the column with a split ratio of 11:1. Peak areas were integrated with a HP 3392A integrator and converted to quantity of COPS using the internal standard method.

GC-Mass Spectrometry (GC-MS)

A HP 5890A GC equipped with a 5970A Mass Selective Detector was employed. GC-MS analyses were performed using the same capillary column and temperature programming as for the quantification of COPS. The operation conditions were: ion source temperature, 200°C; electron voltage, 70eV; and electron multiplier, 2000V. The mass spectra of the TMS ether derivatives of COPS, scanned within the mass range m/e 50-650, were recorded.

Recovery of COPS

Recovery studies on COPS through the SPE procedures were performed with various concentrations of the COPS standards. Each recovery experiment was repeated three times and the extracts analyzed in duplicate.

RESULTS AND DISCUSSION

Isolation and Identification of COPS

Incomplete separation by capillary GC of some COPS or overlapping pairs, such as cholesterol and 7α-hydroxycholesterol (Park and Addis 1986, Sander et al 1989) and the isomeric forms of 5,6-epoxides (Fisher et al., 1985) has been a major difficulty in the quantification of COPS. In this study, the complete separation of all relevant COPS was achieved by non-polar capillary column (DB-1) gas chromatography after extensive evaluation of the gas chromatographic parameters. Separation of cholesterol, 7α- and 7β-hydroxycholesterols, α-epoxide, β-epoxide, 7-ketocholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol standards chromatographed as their TMS ethers is shown in Figure 1. Retention times and retention times relative to that of the internal standard, 6-ketocholesterol are presented in Table 1.

Although 5α-cholestane has been frequently used as the internal standard for the quantification of COPS (Park and Addis 1986, Sander et al 1989), under current GC conditions it was eluted out of the column after 10 min and interfered with other BSTFA by-products. For this reason, 6-ketocholesterol was selected as the internal standard because it was found to elute between 7β-hydroxycholesterol and 7-ketocholesterol and did not interfere in the

Table 1. Retention times and relative retention times[1] for cholesterol and cholesterol oxidation products.

Peak No.		Retention Time (Min)	Relative Retention Time (Min)
1	cholesterol	18.4	0.59
2	7α-hydroxycholesterol (7α-OH)	19.3	0.62
3	cholesterol-5β,6β,epoxide (β-epoxide)	23.7	0.76
4	cholesterol-5α,6α,epoxide (α-epoxide)	24.2	0.78
5	7β-hydroxycholesterol (7β-OH)	24.9	0.80
6	20α-hydroxycholesterol (20α-OH)	27.2	0.87
7	25-hydroxycholesterol (25-OH)	28.0	0.90
8	6-ketocholesterol (6-keto)	31.1	1.00
9	7-ketocholesterol (7-keto)	32.0	1.03
10	cholestan-3β,5α,6β-triol (triol)	33.5	1.08

[1] Retention time relative to the internal standard (IS), 6-ketocholesterol (6-keto).

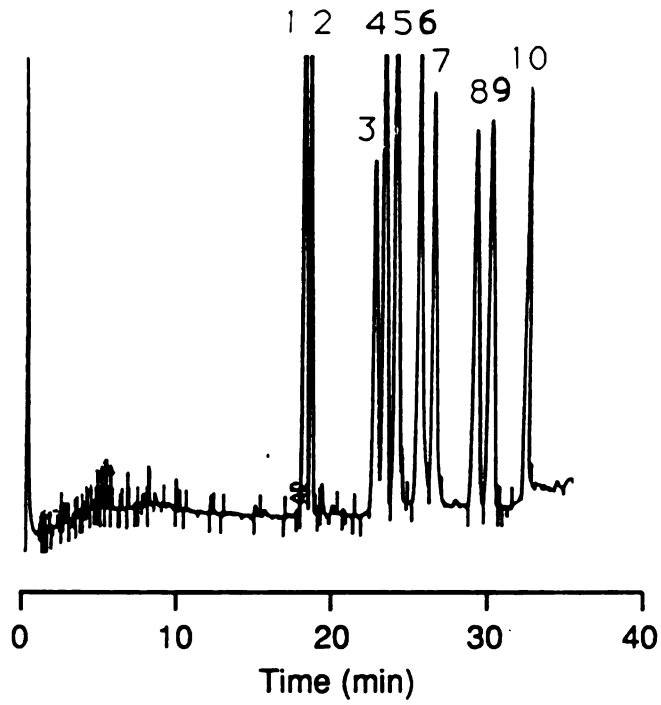

Figure 1. Gas chromatogram of TMS ethers of cholesterol oxide standards; peak numbers correspond to those listed in Table 1.

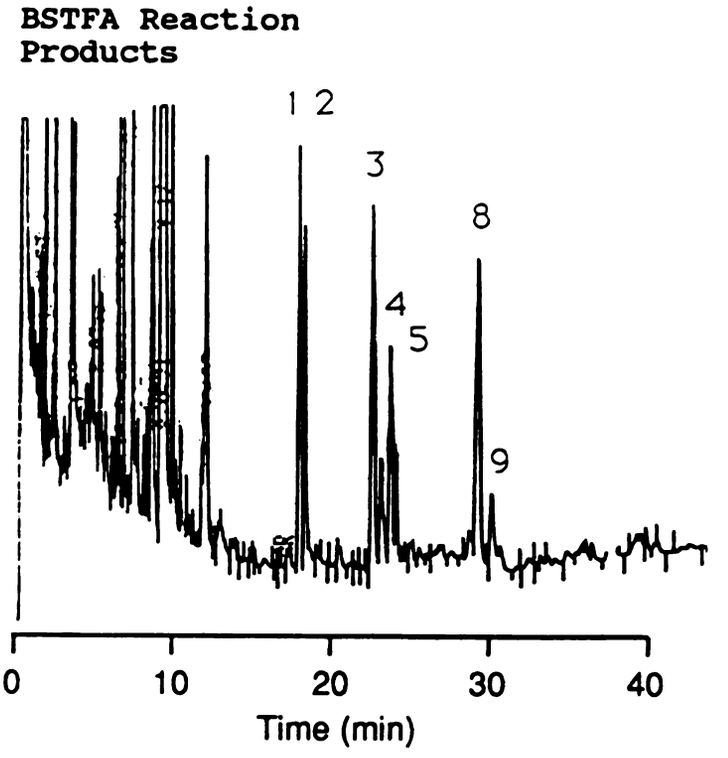

Figure 2. Gas chromatogram of TMS ethers of cholesterol oxidation products from egg powder; peak numbers correspond to those listed in Table 1.

quantification of other oxides during GC and GC-MS analyses. In addition, it is structurally similar to 7-ketocholesterol, yet has not been reported as a product of cholesterol oxidation in foods (Smith, 1981).

The identification of COPS in egg samples was based on the relative retention times and mass spectra of sample TMS ethers compared to those of the relevant COP standards. The gas chromatogram of a spray-dried egg sample (Figure 2) showed separations of COPS as well as cholesterol. This indicated that the clean-up procedure removed sufficient amounts of cholesterol to prevent interference with the quantification of COPS.

The mass spectra of TMS ethers of 7α- and 7β-hydroxycholesterols, α- and β-epoxides and 7-ketocholesterol, 20α-hydroxycholesterol, 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol standards were in agreement with those published in the literature (Brooks et al. 1973; Park and Addis 1985a; Park and Addis 1986; Park and Addis 1987). Based on the molecular ion peaks of TMS sterols (Table 2), COPS with one or two hydroxyl groups were identified as the corresponding mono- and bis- TMS ethers, respectively. The presence of characteristics peaks corresponding to M-90 or M-180 in TMS sterols supported the identity of sterols as either the mono- or bis- TMS ethers, in accordance with the number of the hydroxyl groups. For example, in the mass spectrum of the cholesterol TMS ether (Figure 3), the molecular ion (M)

Table 2. Mass spectrometric data for TMS ethers of cholesterol and cholesterol oxidation products.

TMS ether	Characteristic ion						
	B[1]	M[2]	M-15	M-90	M-105	M-180	M-195
cholesterol	129	458 (42)[3]	443 (12)	368 (77)	353 (36)	--	--
7α-OH	456	546 (2)	531 (1)	456 (100)	441 (2)	366 (1)	351 (1)
7β-OH	456	546 (1)	531 (1)	456 (100)	441 (1)	366 (2)	351 (1)
α-epoxide	73	474 (15)	459 (8)	384 (16)	369 (6)	--	--
β-epoxide	73	474 (35)	459 (8)	384 (35)	369 (12)	--	--
7-keto	472	472 (100)	457 (17)	382 (20)	367 (34)	--	--
20α-OH	69	546 (3)	531 (1)	456 (7)	441 (2)	366 (3)	351 (5)
25-OH (mono-)	129	474 (25)	459 (8)	384 (50)	369 (13)	--	--
25-OH (bis-)	131	546 (2)	531 (1)	456 (6)	441 (2)	366 (2)	351 (2)
triol (bis-)	75	564 (2)	--	474 (12)	459 (5)	384 (9)	369 (7)
triol (tris-)	75	636 (2)	--	546 (6)	531 (2)	456 (18)	441 (4)

[1] Base peak.
[2] Molecular ion.
[3] Percentage of base peak.

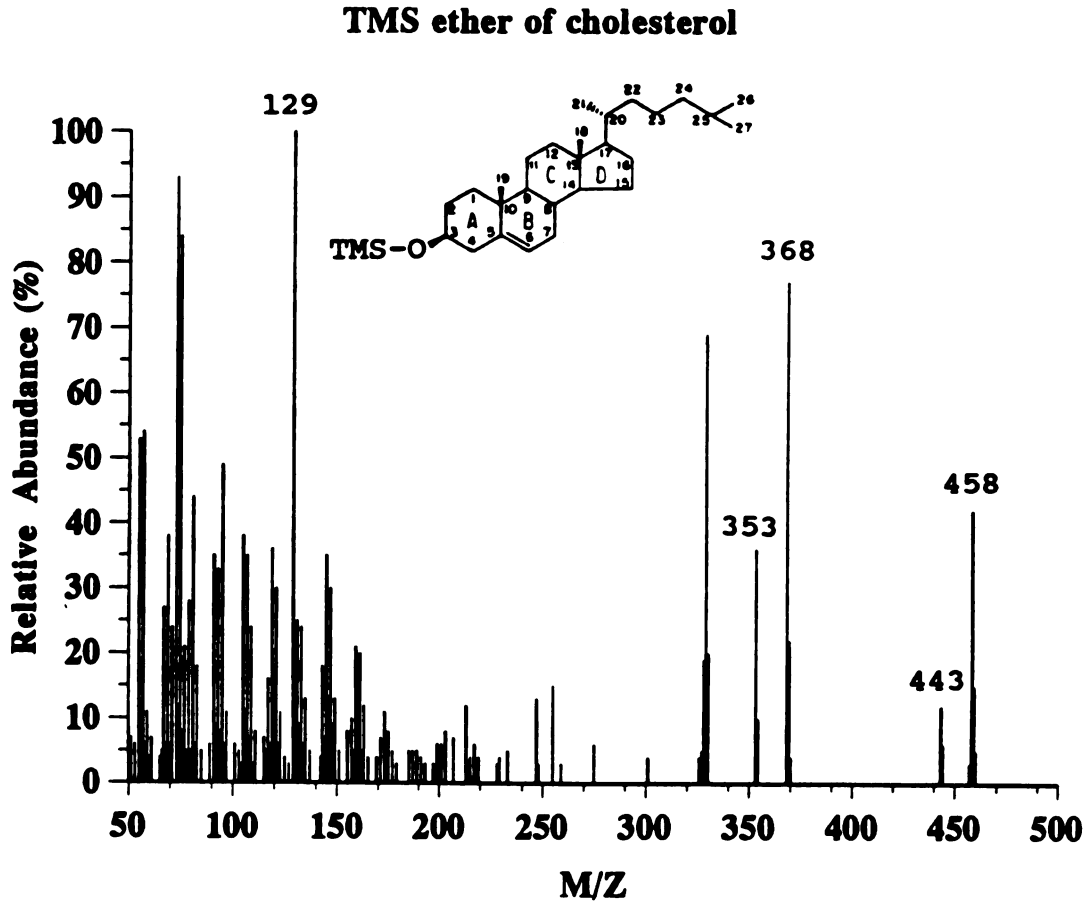

Figure 3. Mass spectrum of the TMS ether of the cholesterol standard.

appeared at m/z (relative intensity) 485. Some principal ions were present at m/z 443 [M-15, loss of CH_3], 368 [M-90, loss of trimethylsilanol, TMS-O°], 353 [M-(15+90)]. The base peak at m/z 129 of cholesterol is well confirmed as originating from the fragmentation of the A ring as TMS-O-CH-CH=CH_2 (Brooks et al., 1973).

By comparing the relative GC retention times and fragmentation patterns of mass spectra of COPS peaks from the egg samples with those of COPS standards, five COPS were identified. They were α- and β-epoxides, 7α- and 7β-hydroxycholesterols and 7-ketocholesterol. These compounds have been reported in egg samples previously. For example, Nourooz-Zadeh and Appelqvist (1987) reported that after 8 years of storage, a dehydrated egg yolk sample contained these COPS plus 20α-hydroxycholesterol, 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol. Missler et al (1985) detected 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol in dried egg mixes that had been stored in cans for 5 years. However, 20α-hydroxycholesterol and 25-hydroxycholesterol, the products of side chain oxidation of cholesterol, as well as cholestan-3β, 5α, 6β-triol, the hydration products of cholesterol epoxides, were not detected in the spray-dried whole egg powders that were stored at room temperature for 6 months in this study.

Derivatization and Response Factors of COPS

The thermal decomposition of cholesterol diols such as 7α- and 7β-hydroxycholesterols and 25-hydroxycholesterol has been observed during GC analysis (Park and Addis, 1985a). The instability of COPS during GC analysis can be overcome by converting the COPS to their TMS derivatives (Park and Addis, 1985a). To set the optimum silylating conditions for COPS, BSTFA + 1% TMCS was used under different time-temperature conditions (room temperature and 60°C for 0.5, 1, 2, 6, 12 and 24 hr) to convert COPS to their TMS ethers.

The maximum peaks areas for 7α- and 7β-hydroxycholesterols, α- and β-epoxides, 6-ketocholesterol and 7-ketocholesterol were reached when they were derivatized with BSTFA + 1% TMCS alone for 1 hour. However, GC-MS analysis of the TMS ethers of COPS standards revealed incomplete derivatization of some COPS with this derivatization procedure. The 25-hydroxycholesterol was converted to the mono-TMS derivatives, with the TMS ether formed at the 3 position. The cholestan-3β, 5α, 6β-triol was found to form the bis- TMS ether with the hydroxyl groups at the 3 and 6 positions being derivatized.

With increase of derivatization time and/or temperature, the peak areas of the mono- TMS derivative of 25-hydroxycholesterol and the bis-TMS ether of cholestan-3β, 5α, 6β-triol decreased, while the peaks corresponding to the bis TMS ether of 25-hydroxycholesterol and the tris-TMS ether of cholestan-3β, 5α, 6β-triol appeared at retention

times of 33 min and 31.5 min, respectively. The presence of two TMS derivatives for 25-hydroxycholesterol or cholestan-3β, 5α, 6β-triol could be a source of error in quantification, and thus is not desirable.

Complete derivatization of 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol to their bis- and tris- TMS ethers was achieved by silylating these COPS with BSTFA + 1% TMCS at 60°C for 1 hour or at room temperature for 24 hours. However, decreases in the peak areas of other COPS were observed when complete derivatization of 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol was performed. Missler et al (1985) indicated that the harsher silylating conditions used to completely derivatize 25-hydroxycholesterol and cholestan-3β, 5α, 6β-triol resulted in cleavage of the epoxide bonds in cholesterol epoxide isomers, and formation of enol-TMS derivatives from the COPS with ketone groups. Decomposition of COPS derivatives when using a stronger silylating agent such as Sylon BTZ (N-trimethylsilylimidazole + N,O-bis-[trimethylsilyl] acetamide + TMCS, 3:3:2, V/V/V) has also been reported by Nawar et al.(1991).

On the other hand, BSTFA with 1% TMCS as an acid catalyst not only gave maximum peak areas for COPS with free hydroxyl groups such as 7α- and 7β-hydroxycholesterols, α- and β-epoxides, 6-ketocholesterol and 7-ketocholesterol, but also provided the best combination of good peak shape and separation during the GC analysis of COPS. Therefore, in

this study, isolated COPS were derivatized using BSTFA with 1% TMCS at room temperature for 50 min and analyzed by capillary GC. The average response factors of COPS for this study are listed in Table 3. The response factors of COPS were the ratios of absolute response factor (weight divided by peak area) of each COP to that of the internal standard (6-ketocholesterol). Some investigators have assumed that the response factor for all COPS is 1 (Park and Addis, 1985a; Nourooz-Zadeh, 1990) when quantifying the amounts of COPS in foods. Investigators should be aware that the response factor for each COP may vary with the different derivatization conditions, type of GC column and GC operating conditions. Therefore, the response factors of COPS should be calculated for each individual study instead of using the values cited in the literature.

Evaluation of SPE Clean-up

The average lipid content of whole egg powder is approximately 40%, triacylglycerol and phospholipids being the major components. The cholesterol content in egg lipids ranges from 2 to 6.2% (Tullett, 1987; Nourooz-Zadeh, 1990), with approximately 90% being present in the free form (Nourooz-Zadeh, 1990). On the other hand, the concentration of each COP in egg powder only ranges from 0 to 100 μg/g (Tsai and Hudson, 1985). Therefore, it is necessary to isolate COPS from other major lipids and increase their relative concentration prior to GC analysis.

Table 3. Response factor[1] and percent recovery of cholesterol oxidation products (COPS).

Cholesterol oxidation products	Response factor		Percent recovery	
	mean[2]	CV (%)	mean[3]	CV (%)
7α-OH	1.9356	5.7	86.7	2.9
β-epoxide	1.6582	5.1	84.9	3.5
α-epoxide	1.0261	3.0	84.3	2.5
7β-OH	1.0471	2.1	85.0	2.2
20α-OH	1.0842	7.2	86.3	3.6
25-OH	1.4800	8.0	86.8	5.2
6-keto	1.0000	0.0	86.8	3.5
7-keto	1.0788	1.8	84.2	2.7
triol	1.5000	6.2	86.3	4.6

[1] Response factor = $(W_i/A_i)/(W_{is}/A_{is})$; W_i and W_{is} are weight of COPS and internal standard (IS, 6-ketocholesterol); A_i and A_{is} are peak areas of COPS and IS.

[2] Mean of six replicated experiments (i.e., derivatization and GC analysis).

[3] Mean of three replicated experiments (i.e., solid phase extraction, derivatization and GC analysis).

In this study, SPE was used to separate COPS from other lipids and cholesterol according to their differences in polarity. Lipid components in order of increasing polarity are esterified cholesterol, triacylglycerols, free fatty acids, cholesterol, COPS and phospholipids (Park and Addis, 1985b). Egg lipids extracted from egg powder (1g) were dissolved in hexane, a non-polar solvent, and then applied to a disposable SPE tube containing a highly polar silica packing. To ascertain the capacity of SPE tubes for retaining cholesterol and COPS, egg lipid extracts were applied to SPE tubes filled with various amounts of silica packing: 100mg, 300mg and 500mg. Results indicated that 300mg of packing effectively held up to 500mg lipids without elution of any COP with hexane or solvent combinations of hexane and ethyl ether.

After the less polar lipids such as triacylglycerols and cholesterol were eluted out of the SPE tube using hexane/ethyl ether combinations of slightly increasing polarity, acetone was used to recover all COPS, while leaving most of the phospholipids on the column. A solvent flow rate of approximately 0.6 ml/min was maintained by a vacuum manifold throughout the chromatographic process.

Five ml fractions of solvent were collected to investigate the distribution of cholesterol and COPS during SPE. GC analyses showed that more than 99% of the cholesterol was eluted using the 90:10 and 80:20 hexane/ethyl ether solvents. The acetone fraction contained

all recoverable COPS and only small amounts of cholesterol (approximately 20 μg/g) which could be completely separated from 7α-hydroxycholesterol during GC analysis.

Because cholesterol and COPS have similar polarities, it is difficult to remove enough cholesterol to prevent its interference in the quantification of COPS without losing any of the latter compounds at the same time. Missler et al. (1985) used preparative HPLC to further remove excess cholesterol from samples after an initial sample clean-up with silica gel column chromatography and prior to final GC analysis. They reported that with this method, 20% of the 25-hydroxycholesterol present was also eluted out with the cholesterol fraction. Nourooz-Zadeh (1990) used three enrichment steps to isolate COPS from egg samples. First, NH_2 cartridges were used to separate cholesterol and COPS from triacylglycerols and phospholipids. The eluate from the NH_2 cartridge containing cholesterol and COPS was applied to a Sep-Pak C_{18} cartridge. After the second enrichment step, most of the COPS (>90%) and 40% of the cholesterol were recovered. Prior to GC analysis, preparative HPLC on a cyanopropyl column was used for the removal of the remaining cholesterol from COPS. In the present study, a single step clean-up using a silica-packed SPE tube and a well controlled solvent flow rate not only removed cholesterol and other lipids sufficiently to avoid the interference with the quantification of COPS in egg powder but also gave reproducible and satisfactory

recoveries (86% ± 3%) for the internal standard (6-ketocholesterol).

To study the recovery of each COP in the SPE procedure, standard mixes of nine COPS (1:1, w/w) at three different concentrations (10, 50, 100μg/g) were applied to the SPE tubes containing 300mg silica packing, and the acetone fractions were collected, derivatized, and analyzed by capillary GC. No significant difference ($P < 0.05$) in percent recoveries of COPS at different concentrations were found. Therefore, data at all levels were combined and averaged (Table 3). The percent recoveries of COPS ranged from 84.2% to 86.8% with coefficients of variation (CV) from 2.2% to 5.2%.

The percent recoveries of COPS reported in the literature range from 23.6% (Higley et al., 1986) to nearly 100% (Park and Addis, 1985b), although only two or three COPS were quantified in each report. Morgan and Armstrong (1989) developed a wide-bore capillary GC method to quantify the five most common COPS in egg powder after COPS were cleaned up using a Sep-Pak silica cartridge. They reported average recoveries of 78.2% and 95.1% for α-epoxide and 7-ketocholesterol, respectively. In addition, large variations within each concentration of COPS as well as among different COPS were observed with this method.

Homogeneous ($P<0.05$) and sufficiently high recoveries of COPS as well as 6-ketocholesterol were achieved in this study with SPE tubes and solvent systems similar to those

used by Morgan and Armstrong (1989). The solvent flow rate was controlled by a vacuum manifold with a vacuum of 20kPa to provide a slow and constant flow rate (0.6 ± 0.1ml/mim). No information about the flow rate of solvents has been reported in the other studies in which SPE was used to isolate COPS from other lipids and/or cholesterol. Therefore, the large variations among the recoveries of COPS reported in the literature may be attributed to inconsistent solvent flow rates. In addition, this system where up to twelve samples can be cleaned up simultaneously in 1.5 hours is relatively faster than the conventional chromatographic clean-up procedure (Monahan et al., 1992) which takes approximately three hours to prepare columns and extract four samples.

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CHAPTER TWO

INFLUENCE OF FREE RADICALS AND OTHER FACTORS ON FORMATION OF CHOLESTEROL OXIDATION PRODUCTS IN SPRAY-DRIED EGG POWDERS

ABSTRACT

Factors influencing formation of cholesterol oxidation products (COPS) in spray-dried egg powders, particularly free radicals generated during the combustion process, were investigated. Total COPS formed in products dried in the presence of free radicals (by using a gas burner or addition of prooxidants into a electric heating system) were approximately 2-5 times greater than those in powders processed by an electric heating system. Addition of antioxidants did not significantly affect ($p < 0.05$) the formation of COPS in eggs spray dried with direct, gas-fired heating during processing and/or storage. Room temperature storage significantly promoted ($p < 0.05$) further formation of COPS regardless of the initial content of COPS in the egg powders. The pathway of cholesterol oxidation initiated by oxides of nitrogen appeared to be similar to the hydroperoxide-induced free radical reaction.

INTRODUCTION

Although dietary cholesterol has long been considered a contributing factor to athersclerosis in humans (McGill, 1979; Kris-Ztherton et al., 1988), recent studies have indicated a possible role of cholesterol oxidation products (COPS) in the initiation of atherosclerotic plaque formation (Peng et al., 1982; Addis et al., 1989; Addis and Park, 1989; Kubow, 1990; Kumar and Singhal, 1991). Furthermore, plasma COPS concentrations in humans have been found to increase with consumption of oxidized egg powders containing approximately 230μg/g COPS (Emanuel et al., 1991). Thus, the presence and quantity of COPS in foods resulting from processing and subsequent storage have received considerable attention in recent years (Finocchiaro and Richardson, 1983; Morgan and Armstrong, 1987; Sander et al., 1989).

The method of spray drying affects the stability of lipids, including cholesterol, in dehydrated foods. For example, it has been reported that the concentrations of COPS are greater in egg powders processed by a direct gas-fired heating source than in powders produced with indirect heating (Missler et al., 1985; Tasi and Hudson, 1985; Faulkner et al., 1992). It was suggested that the exposure of foods to oxides of nitrogen (NOx) in the direct gas-fired spray dryer may be responsible for the elevated COPS concentrations.

NOx, including nitric oxide (NO) and nitrous oxide (NO_2) are produced from air as a result of combustion processes (Wheeler, 1980). NO_2 has been demonstrated to be a free radical initiator of oxidation of unsaturated lipids in model systems (Roehm et al., 1971; Pryor and Lightsey, 1981). Morgan and Armstrong (1992) investigated the effect of NOx on the formation of COPS in egg yolk powders produced with direct gas-fired heating. They manipulated the levels of NOx in the combustion gas by delivery of NO_2 to the gas burner where NO_2 dissociates into a mixture of oxidizing nitrogen oxide gases. They demonstrated that the quantities of COPS in spray-dried egg yolk powders increased proportionally to the NOx concentrations in the combustion gas. However, there is still no direct evidence that NOx are mainly responsible for the elevated COPS concentrations in egg powders processed with direct gas-fired heating.

The present study was carried out to investigate factors influencing the formation of COPS in spray-dried egg powders, particularly the role of free radicals generated during the combustion process. The effects of adding prooxidants and antioxidants to the liquid egg before drying on egg product stability were also determined.

MATERIALS AND METHODS

Preparation of Liquid Egg

Freshly processed liquid whole egg was purchased from a commercial supplier in Cork, Ireland, and stored under refrigeration (4^{o}C) until dried (within eight hours).

Cumene hydroperoxide (80%, Sigma Chemical Co., St. Louis, MO) was added directly to the liquid egg to achieve a concentration of 0.05% (w/w). Oleoresin rosemary (OR)(Herbalox^{TM} Seasoning, Type WM, 100% activity, Kalsec Inc., Kalamazoo, MI) was diluted with distilled water to prepare a 15% (w/v) stock solution. A stock solution (0.6%, w/v) of tertiary butylhydroquinone (TBHQ)(Eastman Chemical Products, Inc., Kingsport, TN), was prepared in 50% (v/v) ethanol solution. The stock solutions of OR and TBHQ were then added to the liquid egg in appropriate amounts to produce the desired final concentrations based on the lipid content (12%) of the liquid egg (Table 1).

Preparation of Spray-dried Egg Powders

Two spray-drying systems, indirect (electric) heating and direct gas-fired heating, were used to dehydrate liquid whole egg (Table 1). Prooxidants were added during processing by introducing NOx gas to the drying air and by adding cumene hydroperoxide into the liquid egg immediately before drying. Antioxidants were also added to the liquid egg before drying.

Table 1. Liquid egg treatments and drying conditions used to prepare egg powders.

Heating source	NOx in drying air	Liquid egg
Indirect, electric	0ppm	control
Indirect, electric	0ppm	cumene-OOH 0.5%
Indirect, electric	15ppm[1]	control
Indirect, electric	15ppm	OR 0.05%[3]
Direct, gas-fired	8ppm[2]	control
Direct, gas-fired	8ppm	OR 0.05%
Direct, gas-fired	8ppm	OR 0.1%
Direct, gas-fired	8ppm	TBHQ 0.02%

[1] Amount of NOx added to the drying air which originally contained no detectable NOx.
[2] Amount of NOx generated by gas combustion.
[3] Concentrations of antioxidants (OR and TBHQ) are based on lipid content of liquid egg.

All egg samples were spray-dried using a pilot scale Anhydro Lab3 spray dryer with inlet and outlet temperatures of 200 and 95°C, respectively, at the National Dairy Products Research Centre (Moorepark, Fermoy, Cork, Ireland). The Anhydro Lab3 model is a single stage, conical dryer equipped with a pneumatic nozzle and electric heating elements for indirect air heating. Conversion to direct gas-fired heating was achieved by the attachment of a gas burner to the extended air inlet duct (Kelly et al., 1989).

NOx gases consisting of 50% NO and 50% NO_2 (B.O.C. Ltd., London, England) were introduced into the drying air in the indirect heating system through the duct between the electric heater and the inlet of the drying chamber. The

concentrations of NOx in the drying air were measured by Dräeger tubes (Dräegerwerk AG, Lübeck, Germany) at the inlet of the drying chamber. A similar location was used to quantify the levels of NOx in the direct gas-fired heating system. A sample of the air was drawn through the Dräeger tube and any NOx present reacted with N,N'-diphenylbenzidine in the tube to form bluish-grey compounds (Leichnitz, 1985). NOx ($NO + NO_2$) concentration was determined from the scale on the tube at the point where the color reaction terminated. The range of measurement for NOx with this method was 0.5 to 50 parts per million (ppm).

The egg powders were vacuum packaged in polyethylene-laminated nylon pouches (Koch, Kansas City, MO) and immediately air freighted to Michigan. These pouches (90μm thickness) have a water-vapor transmission rate of 0.041 $ml/m^2 \cdot day \cdot mmHg$ and an oxygen transmission rate of 0.124 $ml/m^2 \cdot day \cdot mmHg$ at 22.7^oC, 50% RH. Once received (within two weeks of processing), the egg samples were repacked in low density polyethylene bags (8" X 10", 75μm thickness, Whirl-Pak, Fisher Scientific, Fair Lawn, NJ) without heat sealing and stored at ambient temperature (22 $\pm$ 2^oC) in the dark for 6 months. Sampling for analysis of the egg samples was performed randomly after thoroughly mixing the egg powder in the bags with a spatula (approximately 100g per bag). Total solids were determined using the AOAC (1984) vacuum oven method. COPS in the samples were determined at time 0

(corresponding to two weeks post processing) and after storage for three and six months.

Quantification of Cholesterol Oxidation Products

Five COPS (cholesterol α- and β-epoxides, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol) in the egg powders were quantified by the method described in Chapter 1. Lipids were extracted from the egg powders with chloroform and the extracts applied to silica-packing tubes. COPS in the lipid extracts were isolated using solid phase extraction, derivatized to their trimethylsilyl ethers, and subsequently determined by capillary gas chromatography using a 15m x 0.25mm i.d. DB-1 (0.1μm film thickness) column.

Statistical Analysis

The experiment was designed as a three factor (treatment x time x replication) complete randomized model with balanced data. The whole experiment was repeated twice. Analysis of variance (ANOVA) for data was calculated using the MSTATC microcomputer statistical program (Michigan State University, 1991). Bonferroni t statistics were performed to analyze specific contrasts among treatments (Gill, 1978).

RESULTS AND DISCUSSION

Cholesterol is an unsaturated lipid and susceptible to oxidation by a free radical mechanism (Smith, 1981). Many factors including elevated temperatures, prolonged storage, and processing in the presence of prooxidants can influence the rate of cholesterol oxidation as well as the quantities and distribution of COPS in foods (Sander et al., 1989). Although the presence of several COPS in egg powders have been reported (Missler et al., 1985; Tasi and Hudson, 1985; Morgan and Armstrong, 1987; Nourooz-Zadeh and Appelqvist, 1987), the effects of prooxidants produced during processing on the formation of COPS have not conclusively investigated.

In this study, whole liquid egg was spray-dried with two spray drying systems, electrical heating and gas-fired heating, with or without the addition of prooxidants and/or antioxidants. The average total solid contents of egg powders was 96.47% ± 0.56% (standard errors for means). The mean values of the total solids did not differ significantly ($p < 0.01$) among samples from different treatments. Results of ANOVAs for total COPS as well as individual COPS in egg samples showed significant effects ($p < 0.05$) of treatments and storage times. On the other hand, replication, as well as interactions of each combination from these factors, did not significantly ($p < 0.05$) affect the formation of COPS.

Effect of drying method

No measurable quantities of NOx in the drying air were detected by the Dräeger tubes at the inlet of the drying chamber (minimum detection: 0.5ppm) when the dryer was used in the indirect heating mode. However, when this system was converted to direct gas-fired heating, 8ppm of NOx were found in the drying air.

The concentrations of total COPS in the egg samples spray-dried with direct gas-fired heating were significantly ($p < 0.01$) greater than concentrations in samples dried with indirect heating. This trend in concentrations in the samples was observed initially (two weeks after processing) and after three and six months of storage (Table 2). Similar findings have been reported in the literature. For example, Missler et al. (1985) found that scrambled egg mixes dried using a direct, gas-fired heating system contained greater amounts of COPS than those dried by the indirect (electric) heating process. Tsai and Hudson (1985) surveyed commercial egg powders from 15 plants and reported that egg samples dried by air heated directly with a gas burner contained greater amounts of cholesterol epoxides (up to 74μg/g) than those dried by air heated indirectly with steam (up to 5μg/g). These investigators suggested that exposure to NOx may be responsible for the greater quantities of COPS in the egg samples processed by the direct heating process.

Table 2. Effects of drying method on the concentrations of cholesterol oxidation products (μg/g) in egg powders stored at 22°C for six months[1].

Cholesterol oxidation products	Month 0[2]		Month 3		Month 6	
	Direct	Indirect	Direct	Indirect	Direct	Indirect
Cholesterol α-epoxide	1.76	---[3]	4.35	3.21	6.10	4.21
Cholesterol β-epoxide	5.46	2.96	21.29	14.05	26.69	20.43
7α-hydroxy-cholesterol	2.57	1.81	7.25	4.92	6.89	6.00
7β-hydroxy-cholesterol	2.13	2.32	7.79	4.08	10.70	6.90
7-keto-cholesterol	2.44	1.48	3.54	2.71	5.48	5.00
Total	14.36	8.57	44.22	28.97	55.86	42.54

[1] All values represent the average of two replicated experiments analyzed in duplicate.
[2] Month 0: two weeks after processing due to shipping.
[3] Not detectable (minimum detection limit: 0.1μg/g).

Five COPS, cholesterol α- and β-epoxides, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, were found in samples spray-dried with direct, gas-fired heating. However, α-epoxide was not detected in samples dried with indirect, electric heating before storage but appeared after three months of storage. The effects of storage times and the mechanism of formation of cholesterol epoxides (α- and β-epoxides) will be discussed later.

Effects of prooxidants

Cumene hydroperoxide and NOx gases (NO and NO_2) were used to investigate the influence of free radical on the formation of COPS in spray-dried eggs. Cumene hydroperoxide, added to liquid egg (0.5%) prior to spray drying, will undergo decomposition by heat to generate alkoxyl or peroxyl radicals (Hiatt, 1975). These radicals will accelerate the chain reaction of lipid oxidation, including cholesterol oxidation (Smith, 1981). NOx gases, as free radical initiators (Pryor and Lightsey, 1981), were introduced into the drying air heated with the indirect, electric heating source. The amount of NOx in drying air measured at the inlet of the drying chamber was increased from the original non-detectable amount (< 0.5ppm) to 15ppm.

Results presented in Table 3 suggest that the presence of prooxidants play an important role in the oxidation of cholesterol in spray-dried egg powders. The amounts of COPS in egg powders produced by indirect heating were increased

Table 3. Effects of adding prooxidants and antioxidants to liquid egg on the concentrations of cholesterol oxidation products (μg/g) in egg powders produced with an indirect heating system[1,2].

Cholesterol oxidation product	Control	Cumene-OOH (0.5%)	NOx (15ppm)	NOx (15ppm) + OR (0.05%)[3]
7α-hydroxy-cholesterol	1.81	3.49	5.07	3.59
7β-hydroxy-cholesterol	2.32	2.77	6.15	3.77
7-keto-cholesterol	1.48	3.03	2.45	2.70
Cholesterol α-epoxide	---[4]	2.08	3.83	2.19
Cholesterol β-epoxide	2.96	8.76	21.32	12.65
Total	8.57	20.13	38.82	24.90

[1] All values represent the average of two replicated experiments analyzed in duplicate.
[2] Two weeks after processing due to shipping.
[3] Based on lipid content of liquid egg.
[4] No detectable cholesterol α-epoxide (minimum detection limit: 0.1μg/g).

2.3- fold and 4.5-fold by the addition of cumene hydroperoxide and NOx gases, respectively. Morgan and Armstrong (1987) also demonstrated that the addition of prooxidants such as hydrogen peroxide to liquid egg before spray drying promoted formation of measurable quantities of cholesterol epoxides in egg yolk powder produced by indirect heating. Hydroxyl radical, a product from thermolytic cleavage of hydrogen peroxide (Simic and Taylor, 1987), like NOx is highly reactive with unsaturated lipids such as cholesterol.

Similarly, results of the indirect heating studies, with and without cumene hydroperoxide and NOx suggest that the presence of prooxidants is necessary to explain the greater quantities of COPS in egg powders produced by indirect heating system. These results support the hypothesis that cholesterol oxidation in spray-dried egg powders is initiated by NOx formed during the combustion process.

Effects of antioxidants

Antioxidants, OR and TBHQ, which function as free radical scavengers, were added to liquid egg to ascertain their effectiveness in preventing cholesterol oxidation in powders produced by direct, gas-fired spray drying. Although there appeared to be a trend toward reduced COPS in samples dried in the presence of antioxidants, the observed differences in COPS concentrations were not statistically

significant ($p < 0.05$). The ineffectiveness of TBHQ and OR may be due to their volatilization and subsequent loss during spray drying.

On the other hand, OR reduced the formation of COPS by 36% in the egg powders produced with the indirect spray dryer with the addition of 15ppm NOx in the drying air. It is possible that the lower temperatures attained during the drying operation using indirect heating may reduce the loss of antioxidant principles from OR during egg processing. However, further studies on the effect of temperature of the drying operation on the effectiveness of antioxidants are needed to evaluate this suggestion.

Effects of storage times

The length of storage time significantly ($p < 0.05$) affected the formation of COPS in all egg samples. This result is predictable because the samples were stored in a oxygen-permeable package (polyethylene bags without sealing) at ambient temperature to facilitate cholesterol oxidation. As shown in Figure 1, COPS concentrations increased more rapidly in the first three months than in the subsequent three months.

The presence of cholesterol α- and β-epoxides, 7α- and 7β-hydroxycholesterols and 7-ketocholesterol in the egg powders is consistent with similar reports in the literature. However, 20α-hydroxycholesterol and 25-hydroxycholesterol, the products of side chain oxidation of

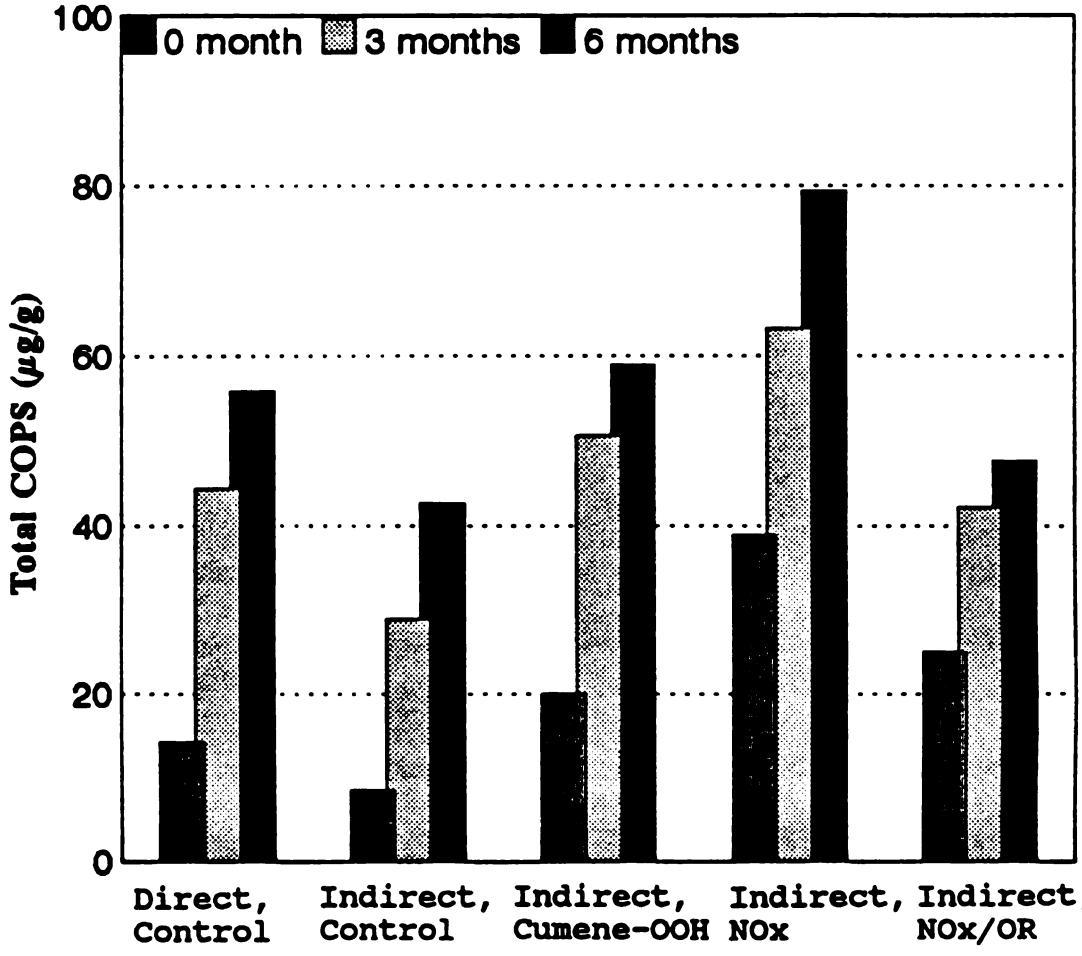

Figure 1. Formation of cholesterol oxidation products (COPS) in egg powders stored at 22 ± 2°C for six months.

Table 4. Effects of storage time on the concentrations of cholesterol oxidation products (μg/g) in egg powders produced with an indirect heating system[1].

Storage time	Control	Cumene-OOH (0.5%)	NOx(15ppm)	NOx(15ppm)[2]+ OR (0.05%)
Month 0[3]				
Total COPS	8.57	20.13	38.82	24.90
% C-7	65.5	46.2	35.2	40.4
% epoxides	34.5	53.9	64.8	59.6
Ratio β/α[4]	---[5]	4.2	5.6	5.8
Month 3				
Total COPS	28.97	50.56	63.20	42.18
% C-7	40.4	42.1	39.0	41.5
% epoxides	59.6	57.9	61.0	58.5
Ratio β/α	4.4	4.3	4.1	4.5
Month 6				
Total COPS	42.54	58.93	79.38	47.52
% C-7	42.1	48.0	39.7	48.4
% epoxides	57.9	52.0	60.3	51.6
Ratio β/α	4.9	4.0	4.9	4.6

[1] All values represent the average of two replicated experiments analyzed in duplicate.
[2] Based on lipid content of liquid egg.
[3] Two weeks after processing due to shipping.
[4] Ratio of cholesterol β-epoxide to α-epoxide.
[5] No detectable cholesterol β-epoxide (minimum detection limit: 0.1μg/g).

cholesterol, as well as cholestan-3β, 5α, 6β-triol, the hydration products of cholesterol epoxides, were not detected. These compounds have been reported in egg powders stored for more than five years (Missler et al., 1985; Nourooz-Zadeh and Appelqvist, 1987).

The most abundant COP in the egg powders was β-epoxide. The ratios of this compound to its α-isomer in the samples ranged from 4:1 to 4.9:1 after three and six months of storage (Table 4). The relative abundance of β-epoxide comparded to its α-isomer is due to the relative thermal instability of the latter (Maerker, 1987). Tsai and Hudson (1985) reported a ratio of 4:1 in commercial dried egg powders. Nourooz-Zadeh and Appelqvist (1987) also reported a ratio of 5:1 for egg powders and 10:1 for scrambled egg mixes.

Cholesterol oxidation is initiated by the abstraction of the allylic C-7 hydrogen, with the subsequent formation of C-7 COPS such as 7α- and 7β-hydroxycholesterols and 7-ketocholesterol (Smith, 1981). On the other hand, cholesterol α- and β-epoxides are the products of attack by cholesterol 7-hydroperoxide on the 5, 6-double bond of cholesterol, and therefore are the secondary oxidation products (Maerker, 1987). Other lipid hydroperoxides and some organic hydroperoxides such as cumene hydroperoxide and hydrogen peroxide likewise favor epoxidation of cholesterol and form cholesterol epoxides (Smith, 1981).

Many investigators have claimed C-7 COPS as the predominant cholesterol oxides in foods such as beef, tallow and milk powders (Park and Addis, 1986; 1987; Chan et al., 1993). However, data presented in Table 4 indicated that under conditions of NOx- or cumene hydroperoxide-induced oxidation of cholesterol, epoxides were the predominant COPS. These findings support the results of other studies in which epoxides were identified as the major COPS formed in egg powders spray-dried with a direct heating source (Missler et al., 1985; Morgan and Armstrong, 1992).

In conclusion, this study has demonstrated that the presence of NOx, free radicals generated during the combustion process, is responsible for the rapid formation of COPS in egg powders produced by the direct gas-fired spray dryer. The pathways of cholesterol oxidation initiated by NOx appear to be similar to the hydroperoxide-induced free radical chain reaction. More specific studies on the mechanism of NOx-initiated cholesterol oxidation in a lipid model system were conducted. The results are reported in Chapter 5.

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CHAPTER THREE

DEPOSITION OF CAROTENOIDS IN EGGS FROM HENS FED DIETS CONTAINING SAPONIFIED AND UNSAPONIFIED OLEORESIN PAPRIKA

ABSTRACT

Deposition of carotenoids in saponified paprika (SP) and unsaponified oleoresin paprika (OP) in egg yolks as well as the dietary level for desired pigmentation were evaluated. Sixty-four hens on a carotenoid-depletiion diet were divided into two replicated groups of each of 8 dietary treatments containing from 0 to 16 mg paprika carotenoids/kg feed. Color and the carotenoid content of egg yolk increased linearly ($p < 0.01$) with the amounts of paprika carotenoids in the diets. The color of egg yolks from hens fed similar concentrations of OP or SP were not significantly different ($p < 0.01$). A low dose (4mg/kg) of OP or SP provided yolk color equivalent to the color of eggs in supermarkets. High-performance liquid chromatographic analyses showed that carotenoids deposited in the yolk are in the free alcohol form, regardless of the form of carotenoids in the diet. Capsanthin, the predominant carotenoid in paprika, was deposited in egg yolk less efficiently than zeaxanthin and lutein.

INTRODUCTION

The pigments responsible for egg yolk color are a group of compounds of plant origin known as oxycarotenoids or xanthophylls (Marusich and Bauernfeind, 1981). Many investigators have studied the pigmenting properties of various colored substances that, when used singly or combination, can achieve the most consistent and economic pigmentation for egg yolk (William, 1992).

While most efforts have concentrated on yellow pigmenting substances, several investigators noted that some naturally occurring red substances such as paprika extracts had the effect of modifying and enhancing the yellow color in the yolks (Mackay et al., 1963; Nelson and Baptist, 1968). Fletcher and Halloran (1981) applied this concept in a low xanthophyll diet (white corn diet) to evaluate the egg yolk pigmentation properties of marigold extract and oleoresin paprika. They reported that the addition of small amounts of oleoresin paprika to a diet containing marigold concentrate produced egg yolk color equivalent to that produced with much higher levels of marigold concentrate.

The red paprika color is primarily due to capsanthin and capsorubin, while β-carotene and violaxanthin contribute to the yellow-orange color (Reeves, 1987). Most of the paprika carotenoids are esterified with fatty acids such as lauric, myristic and linoleic acids (Biacs et al.,1989). Hamilton et al. (1990) investigated the influence of

saponification on the deposition in egg yolk of carotenoids from oleoresin paprika. They found that saponified paprika carotenoids were deposited in egg yolks twice as efficiently as unsaponified paprika carotenoids. Schaeffer et al. (1988) reported that carotenoid esters are hydrolyzed in vivo prior to absorption and that more than 90% of the carotenoids are deposited as free alcohols in yolks of eggs from hens fed a yellow corn-alfalfa diet. Therefore, it was suggested that saponification improves the digestibility of esterified carotenoids, thus enhancing the efficiency of absorption and deposition of carotenoids.

This study was conducted to ascertain the optimal pigmentation of yolks by feeding various concentrations of saponified and unsaponified oleoresin paprika added to a depletion white wheat diet, and to characterize the carotenoids deposited. Another objective was to investigate the relative deposition efficiency of carotenoids.

MATERIALS AND METHODS

Experimental Description

The deposition of saponified and unsaponified oleoresin paprika carotenoids in egg yolk as well as the dietary level for desired pigmentation were evaluated by a feeding trial. Eight diets with various concentrations of paprika carotenoids from saponified paprika (SP) and unsaponified

oleoresin paprika (OP) (Kalsec Inc., Kalamazoo, MI) were prepared by diluting the paprika premixes with the control diet immediately prior to the feeding trial (Table 1). The diets were stored at -20°C throughout the study to minimize oxidation of the carotenoids. The concentrations of carotenoids in the paprika premixes, in which OP or SP was mixed with maltodextrin at approximately 8% (w/w), were determined as described later in the text.

Table 1. Concentrations of paprika carotenoids in the diets of laying hens.

Diet[1]	Paprika carotenoids (mg/kg feed)
control	0
OP low	4
OP med.	8
OP high	16
SP 1/2 low	2
SP low	4
SP med.	8
SP high	16

[1] OP: oleoresin paprika; SP, saponified paprika.

Sixty-four White Leghorn hens were randomized into two replicated groups of each of eight dietary treatments. The hens were caged individually with an independent feeder at the Michigan State University Poultry Science Research and Teaching Center. All groups of hens were initially fed for 21 days a basal diet (Table 2) containing white wheat meal

Table 2. Composition of white wheat diet.

Ingredient	% Ration
White wheat	65.55
Soybean meal (44%)	21.00
Meat and bone meal (50%)	2.50
Limestone (ground)	8.30
Dicalcium phosphate	1.50
Layer special premix[a]	0.25
Methionine (dl)	0.10
Soybean oil	0.50
salt	0.30
Calculated analysis	
Crude protein (%)	17.50
Crude fiber (%)	3.24
Fat (%)	2.00
Calcium (%)	3.82
Metabolizable energy (kcal/kg)	2586

[1] Layer special premix contains: Vitamin A, 3,530,000USPU/kg; vitamin D_3, 1,320,000ICU/kg; vitamin E, 4,400IU/kg; riboflavin, 1760mg/kg; d-pantothenoic acid, 4,400mg/kg; niacin, 13,200mg/kg; vitamin B_{12}, 6.6mg/kg; choline, 115,000mg/kg; folic acid, 88mg/kg; Mn, 25.6g/kg; Zn, 20.4g/kg; Fe, 14.4g/kg; Cu, 2g/kg; I, 0.2g/kg; Se, 0.12g/kg.

in place of yellow corn in order to deplete the concentrations of carotenoids deposited in the yolk. Each group of hens was then fed one of experimental diets for an additional 20 days, during which time eggs were collected on even numbered days and stored at 4°C until analyzed.

The eggs from each group (an average of three eggs was obtained daily from a group of four hens) were combined as indicated below and served as an experimental unit. Yolks of eggs from each group were separated from the whites, with the adhering albumen being removed by rolling the yolks over moistened paper towels. The membranes surrounding the yolks were punctured, and the free flowing yolks were combined.

Determination of Egg Yolk Color

Yolk (12 ± 0.5g) from each experimental unit was transferred to a disposable petri dish (60 x 15mm). The intensity of yolk color was determined visually using a Roche color fan (RCF) which consists of 15 shades of color ranging from light cream-yellow to deep orange in the form of a folding fan (Vuilleumier, 1969). The lightness (L), redness (a) and yellowness (b) of the yolk were measured using a Hunterlab ColorQUEST 45°/0° colorimeter (Hunter Assoc. Lab. Inc., Reston, VA).

Determination of Total Carotenoids in Paprika Premix, Chicken Feed and Egg Yolk

Paprika premix (1g) or chicken feed (5g) was homogenized for 2 min with 75ml acetone in a 150ml beaker using an Ultra-Turrax type of homogenizer (Tekmar Co., Cinn., OH). The extract was filtered through Whatman NO.4 paper to remove solid particles. Another 20ml acetone aliquot was used to rinse the probe and reextract the residue. The combined extracts were diluted to 100ml with acetone and used as the stock solution. Due to the large concentration of paprika carotenoids in the paprika premix, a 5ml aliquot of the stock solution was taken and diluted to 50ml with acetone for absorbance readings.

Extraction of carotenoids from egg yolk was performed using a slightly modified version of the Bligh and Dyer procedure (1959). Egg yolk (2g) was mixed in a 150ml beaker with 7.5ml 2M NaCl solution prior to the addition of 20ml methanol and 10 ml chloroform. The mixture was homogenized for 1 min. Chloroform (10ml) was added to the homogenate and the mixture homogenized for another 15 seconds. Deionized water (10ml) was added to the mixture and after homogenizing for 15 seconds, the mixture was transferred to a 100ml centrifuge tube and centrifuged at 700xg for 20 min using an IEC centrifuge (International Equipment Co., Needham Hts., MA). The upper aqueous layer was removed by aspiration. The lower chloroform phase containing all extractable carotenoids was then filtered through Whatman

NO.4 paper and diluted to 50ml with acetone for absorbance readings.

The absorbance of the carotenoid extract was measured at 460nm with a double beam Bausch and Lomb Spectronic 2000 spectrophotometer (Rochester, NY). Total carotenoid concentrations were obtained from the absorbance at 460 nm (maximum wavelength of capsanthin) using the experimentally determined absorptivity at 1% in acetone of 1922 (Fisher, 1993, personal communication).

Analysis of Carotenoids by High Performance Liquid Chromatography

Carotenoids in samples were extracted as previously described. The carotenoid extract was evaporated to dryness with a rotary evaporator (Büchi Rotavapor, Postfach, Switzerland) and redissolved in 10ml hexane. Triacylglycerols in the extract were removed by passing the carotenoid extract through a prewetted Superclean™ LC-Si tube (Supelco, Inc., Bellefonte, PA) filled with 500mg of silica packing. The carotenoids were eluted out of the tube with 10ml acetone. The acetone extract was then concentrated to 2ml under a stream of nitrogen.

The carotenoids (20µl injection) were separated on a 250 x 4.6mm (i.d.) Supelcosil LC 18™ column (Supelco, Inc.) with gradient elution at a flow rate of 1ml/min. Eluants were: A, 75:25 (v/v) acetone-methanol; B, 75:25 (v/v)

acetone-water and the gradient was programmed as 0% A to 65% A in 10 min, to 80% A in 30 min, and to 100% A in 60 min.

A Hitachi Model 655A-11 liquid chromatograph equipped with a Hitachi Model L-5000LC controller (EM Science, Gibbstown, NJ) and a Waters 712 WISP pump (Waters Assoc., Milford, MA) was used. A Waters Model 990 photodiode array detector was used to record the absorbance (440 to 550nm) and the retention time of each peak as it was eluted. After all peaks were eluted, the absorbance of each peak as relative area at any given wavelength (i.e., 460nm) was calculated. The identification of major carotenoids in egg (i.e., capsanthin, zeaxanthin and lutein) was based on the spectra and the elution order of these compounds (Fisher and Kocis, 1987).

Statistical Analysis

The analyses of variance (ANOVAs) for data from all experiments were based on a three-stage nested (hierarchical) model (dietary treatments, experimental units and batches of eggs from different days) with balanced data (Gill, 1986). Bonferroni t statistics were performed to analyze specific contrasts among treatments (Gill, 1978). Linear and quadratic relationships of dose-response for OP and SP treatments were teated with orthogonal polynomial contrasts (Gill, 1978).

RESULTS AND DISCUSSION

Effects of Dietary Treatments on Pigmentation of Egg Yolks

Color development in egg yolks during the duration of the feeding trial is shown in Figure 1 and in Appendices B and C. Color of egg yolks reached its maximum value after feeding for approximately 10 to 12 days. This is probably due to the fact that the development of an ova requires a similar period of time (North, 1984). During this time, the carotenoids used for yolk pigmentation are supplied by the feed. Stored carotenoids such as those found in the skin of the hen are not utilized for this purpose (Nelson *et al.*, 1990).

As the color of egg yolk was stabilized in all treatments after ten days, results of the carotenoid analyses of eggs collected on days 12, 16 and 20 were used to analyze the variances. The ANOVAs of egg yolk color measurements (RCF scores, Hunter color values and total carotenoids) all indicated that dietary treatment is the only factor influencing the pigmentation of egg yolks. Therefore, results of analyses from each treatment were averaged and are presented in Table 3.

The color of egg yolks (RCF scores, L values, a values and total carotenoids) increased linearly ($p < 0.01$) with the amounts of OP or SP in the diets. Addition of 2mg/kg SP increased the color of egg yolks, but the difference between the control and the treatment provided less than 90%

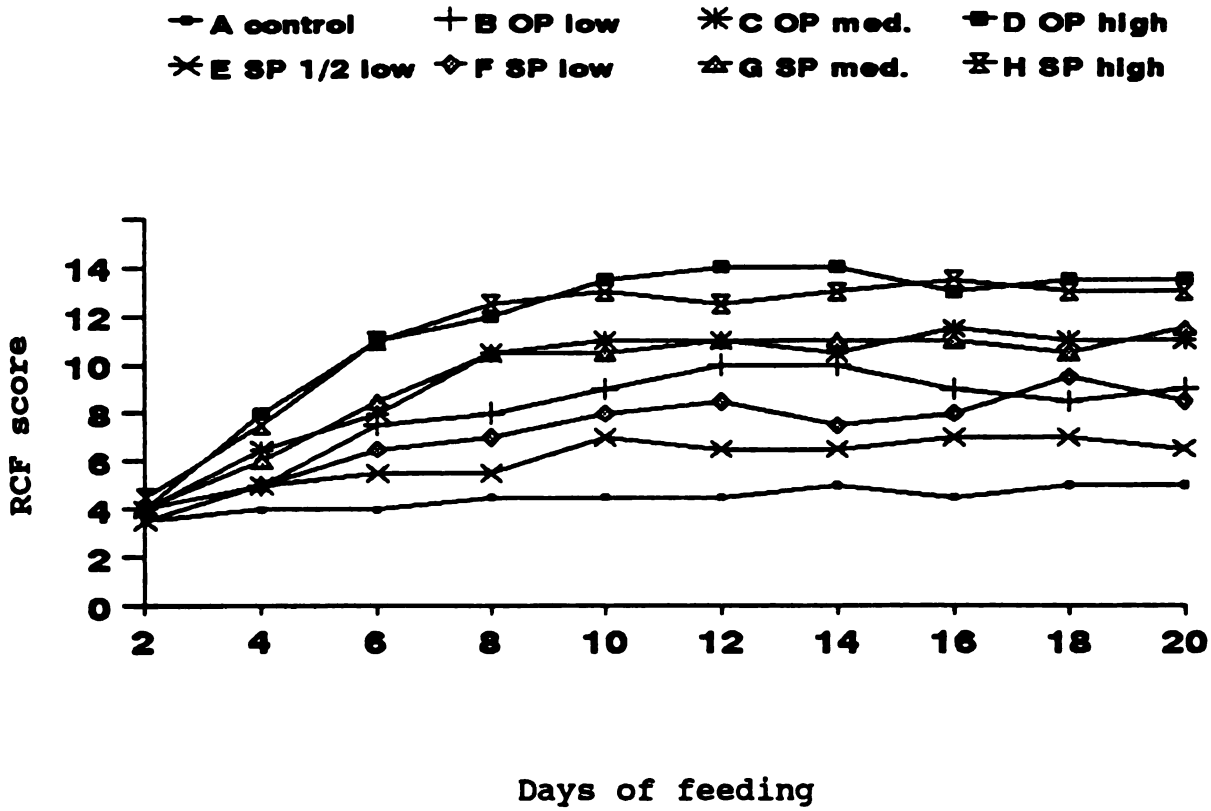


Figure 1. Roche color fan (RCF) scores of egg yolks obtained during the feeding trial (OP, oleoresin paprika; SP, saponified paprika; 1/2 low, 2mg/kg feed; low, 4mg/kg feed; med, 8mg/kg feed; high, 16mg/kg feed).

confidence. However, supplementation of the diet with 4mg/kg paprika carotenoids (OP or SP) significantly enhanced ($p < 0.01$) the color of egg yolk. Fletch and Halloran (1983) also reported that a small amount (6mg/kg) of OP greatly enhances yolk color as opposed to much higher quantities (60mg/kg) of yellow carotenoids such as marigold concentrates.

Table 3. Effects of dietary treatments on Roche color fan (RCF) scores, total carotenoid concentrations and Hunter color values of egg yolks.

Dietary treatment[1]	RCF score	Total carotenoids[2]	Hunter color[3]		
			L	a	b
Control	4.6	5.4	54.6	-4.5	29.5
OP, low	9.3	7.9	51.7	2.2	28.7
OP, med	11.2	10.5	49.2	5.9	27.7
OP, high	13.5	13.5	47.0	12.3	27.5
SP, 1/2 low	6.7	6.1	53.3	-1.4	28.4
SP, low	8.3	7.6	52.4	1.6	28.2
SP, med	11.2	9.3	49.8	5.6	28.3
SP, high	13.0	12.4	47.1	11.2	27.5

[1] OP, oleoresin paprika; SP saponified paprika; 1/2 low, 2mg/kg feed; low, 4mg/kg feed; med, 8mg/kg feed; high, 16mg/kg feed.
[2] µg carotenoids/g egg yolk.
[3] L, lightness; a, redness; b, yellowness.

Low levels (4mg/kg) of OP and SP provided egg yolk color with RCF scores of 9.3 and 8.3, respectively. RCF scores of eggs purchased from local supermarkets range from 7 to 9 depending on individual store and season of the year. A RCF score of 9 is regarded as a very satisfactory color for fresh eggs (Anderson et al., 1991).

Higher levels of dietary paprika carotenoids (i.e., 8mg/kg and 16mg/kg) produced a reddish color in the egg yolks (Appendix C). In addition, the b values (yellowness) of yolk color were also not influenced by dietary supplementation of paprika carotenoids. This is due to that the predominant carotenoid in paprika is capsanthin which contributed to the reddish color of egg yolks, unlike lutein which provided the yellow color of egg yolks from hens fed typical corn diets (Schaeffer et al., 1988).

The reddish yolks may be not acceptable to consumers universally , except for a few limited areas around the world (Hamilton et al., 1990). However, the dark orange color of egg yolks resulting from higher levels of dietary paprika carotenoids (i.e., 8mg/kg and 16mg/kg) may be desired by food processors who rely on egg yolks to impart color to various products such as noodles and pasta.

Influence of Saponification on Deposition of Paprika Carotenoids in Egg Yolk

Results of the color measurements of egg yolks from hens fed the same levels of OP or SP were not significantly

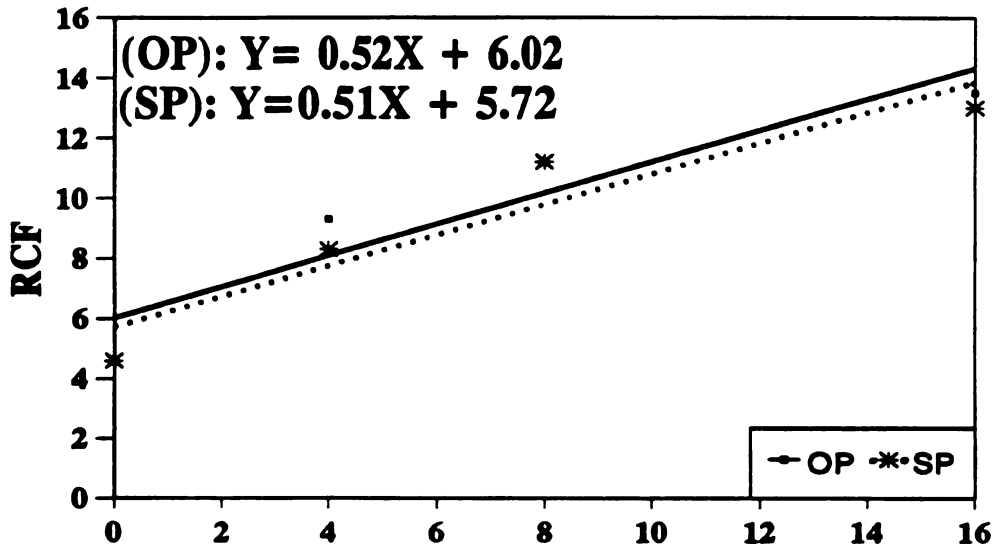


Figure 2. Linear relationship between concentrations of paprika carotenoids in diet and Roche color fan (RCF) scores of egg yolk color (OP, oleoresin paprika; SP, saponified paprika).

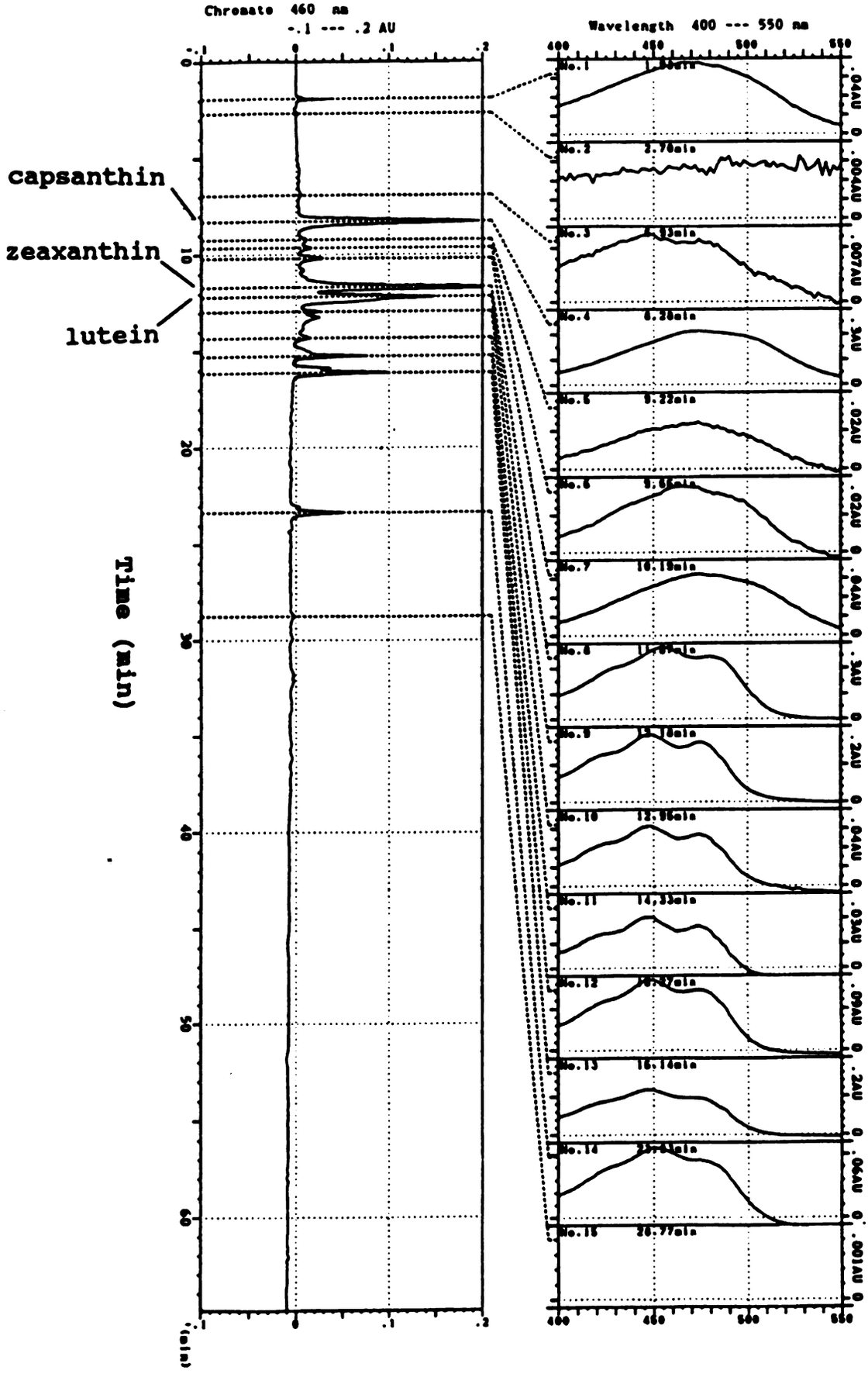
different ($p < 0.01$). Therefore, saponification did not affect the deposition of paprika carotenoids in the egg yolks. The slopes for the concentrations of OP and SP in the diets to the RCF scores of egg yolks were 0.52 and 0.51, respectively (Figure 2). The slightly lower color responses in eggs with the SP treatments may be due to the lower stability of the carotenoids in SP (Biacs et al., 1989), although all diets were stored at -20°C during the feeding trial.

These findings are contradictory to the results reported by Hamilton et al. (1990). They demonstrated that the carotenoids in SP were deposited in egg yolks twice as efficiently as those in OP because the digestibility of the carotenoids (i.e., capsanthin) was improved after saponification. However, Hencken (1992) reported that the improved digestibility of carotenoids by in vitro saponification was found only in young broilers (but not in laying hens) because of the poor fat absorption ability of young chickens.

Deposition Efficiency of Carotenoids

The carotenoids in egg yolks were analyzed by reversed phase high performance liquid chromatography (HPLC). A HPLC chromatogram of the carotenoid extract of an egg sample from hens fed OP is shown in Figure 3. Although fifteen peaks were observed in the extracts of egg yolks from hens fed OP or SP, only three major carotenoids, i.e.,

Figure 3. HPLC chromatogram of the carotenoid extract of egg yolks from hens fed oleoresin paprika-supplemented diet.


Chromato 460 nm
-.1 --- .2 AU
Wavelength 400 --- 550 nm
capsanthin
zeaxanthin
lutein
Time (min)

capsanthin, zeaxanthin and lutein were identified due to limited literature information. No peaks were found in the region where monoester and diester carotenoids are eluted (Fisher and Kocis, 1987). This implies that all carotenoids deposited in the egg yolk are in the free alcohol form, regardless of the form of carotenoids in the diets.

The amounts of zeaxanthin and capsanthin relative to lutein in diets and egg yolks are listed in Table 4. No capsanthin was found in the control diet. The presence of lutein and zeaxanthin in the control diet was attributed to the soybean meal in the basal hen diet. The relative amounts of zeaxanthin and capsanthin increased proportionally with the amounts of paprika carotenoids in the diets. Similarly, no capsanthin was found in eggs from hens fed the control diet and the relative amounts of zeaxanthin and capsanthin in egg yolks also increased proportionally with the amounts of paprika carotenoids in the diets.

The relative deposition efficiency of each carotenoid was obtained from the ratio of the relative amounts of the carotenoid in the egg yolk to that in the diet. In the present study, zeaxanthin and capsanthin were deposited approximately 43% and 13% as efficiently as lutein, respectively. According to Marusich and Bauernfeind (1981), zeaxanthin is deposited in yolks with a efficiency of 56%. Because capsanthin was deposited in this study at approximately 30% (0.13 + 0.43 = 0.30) as efficiently as

zeaxanthin, 17% (0.30 x 56%) of the capsanthin in the diet was deposited in the yolk. The results agree with those of Hamilton et al. (1990) who reported a deposition efficiency of 16% in egg yolks for capsanthin.

Table 4. Ratios of lutein, zeaxanthin and capsanthin (L:Z:C) in diets, egg yolks and deposition efficiencies.

Dietary treatment[1]	L:Z:C		
	Diet	Egg yolk	Deposition efficiency[2]
Control	100:18:0	100: 7: 0	1:0.39:0
SP, low	100:32:120	100:15:16	1:0.47:0.13
SP, med	100:52:240	100:22:31	1:0.42:0.13
SP, high	100:72:360	100:31:44	1:0.43:0.12

[1] SP saponified paprika; low, 4mg/kg feed; med, 8mg/kg feed; high, 16mg/kg feed.
[2] L:Z:C in egg yolk divided by L:Z:C in diet.

However, the deposition efficiency of lutein calculated in the same way was 130% (1 + 0.43 x 56%). This result implies that transformation of carotenoids (i.e., lutein) in vivo during the metabolism of carotenoids in hens or after deposition in egg yolks may occur. Research should be conducted to investigate the distribution of carotenoids by feeding the hens with individual radioactive-labeled carotenoids.

In conclusion, this study has established that supplementation of 4mg/kg OP or SP in a low carotenoid diet (white wheat diet) for hens provided an egg yolk color equivalent to that produced using commercial corn diets. Therefore, 4mg/kg OP was selected for the next feeding trial to produce the eggs for the study of the stability of carotenoids in the paprika-supplemented eggs during processing and subsequent storage. The results are reported in Chapter 4.

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CHAPTER FOUR

STABILITY OF CHOLESTEROL AND PAPRIKA CAROTENOIDS IN EGG POWDERS AS INFLUENCED BY DIETARY AND PROCESSING TREATMENTS

ABSTRACT

The effects of dietary α-tocopherol and/or oleoresin paprika (OP) on cholesterol and carotenoid stability in egg powders during spray drying and subsequent storage were investigated. Cholesterol oxidation and loss of carotenoids in eggs dried with direct gas-fired spray dryer were greater ($p < 0.05$) than in eggs dried using a indirect heating dryer. Dietary supplementation of α-tocopherol acetate (200mg/kg feed) significantly increased ($p < 0.01$) the oxidative stability of cholesterol and carotenoids in eggs dried with the direct heating system. Supplementation of OP (7.5μg/g egg lipids) through diet or by direct addition to liquid eggs did not affect the formation of cholesterol oxidation products (COPS) during storage. However, increased concentrations of OP in liquid eggs (15 and 30 μg/g lipids) suppressed the formation of COPS during processing and subsequent storage.

INTRODUCTION

Lipid oxidation is mainly responsible for the deterioration of food products and adversely affects their color, flavor, nutritive value and even safety (Pearson et al., 1983). Dried egg products are susceptible to lipid oxidation not only because of their high lipid contents and low water activities, but also because of their exposure to oxides of nitrogen (NOx) produced during the combustion process in direct gas-fired spray drying operations (Missler et al., 1985; Morgan and Armstrong, 1987, 1992; Kelly et al. 1989).

The presence of cholesterol oxidation products (COPS) in dried egg products is of considerable importance because of the widespread use of dehydrated eggs in foods such as scrambled eggs, pan cake mixes, Hollandaise sauce and cakemixes (Nourooz-Zadeh and Appelqvist 1987). Numerous COPS have been shown to possess biological activity and some are involved in atherogenesis, carcinogenesis, and cholesterol biosynthesis (Maerker,1987; Kumar and Singhal, 1991). Another concern regarding the quality of dried egg products is the loss of pigments resulting from the oxidation process during dehydration and storage. A considerable amount of research has been conducted to evaluate a variety of carotenoids for their ability to be absorbed from the diet and be deposited in the yolk (Fletcher, 1992; Hencken, 1992). However, there are few

reports in the literature on the stability of pigments in carotenoid-supplemented egg products during processing and subsequent storage.

Cholesterol and carotenoids are unsaturated lipids and as such undergo oxidation in the presence of oxygen (air) via a free radical reaction (Smith, 1981; Francis, 1985). Several free radical scavenger-type antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tertiary butylhydroquinone (TBHQ) and oleoresin rosemary have been used to investigate their effectiveness in controlling and/or preventing cholesterol in aqueous model systems (Rankin and Pike, 1993) and in spray-dried eggs (Morgan and Armstrong, 1987, Lai et al., 1992). However, the results are variable and generally inconclusive.

Initial studies have indicated that α-tocopherol is ineffective in retarding cholesterol oxidation in spray-dried egg during processing and subsequent storage when added to liquid egg prior to spray drying. However, dietary α-tocopherol supplementation has been demonstrated to be effective in improving cholesterol stability in meats (Monahan et al., 1992) and eggs (Faulkner et al., 1992). The effect of dietary α-tocopherol on the stability of carotenoids in eggs during spray drying and subsequent storage has not yet been investigated.

The objective of the present study was to evaluate the effects of dietary α-tocopherol supplementation on

cholesterol and carotenoid stability in dried egg powders during processing and subsequent storage. The stability of paprika carotenoids added to liquid egg prior to spray drying, and the relationship between carotenoid and cholesterol oxidation in eggs during processing and storage were also investigated.

MATERIALS AND METHODS

Dietary Supplementation of Laying Hens

Six hundred White Leghorn hens were randomized into three groups and caged individually with an independent feeder at the Michigan State University Poultry Science Research and Teaching Center. All groups of hens were initially fed a diet containing white wheat meal (Table 1) for 21 days in order to deplete the carotenoids deposited in the yolks. Each group of hens was then fed one of the following experimental diets for another five weeks: (A) control (depletion white wheat) diet; (B) oleoresin paprika (OP) diet (4mg/kg feed); (C) OP (4mg/kg feed) + α-tocopherol acetate (200mg/kg feed). Initial studies indicated that optimal color of egg yolk was reached after 10 to 12 days of feeding supplemented diets (Chapter 3). Therefore, eggs produced after two weeks of dietary treatments were collected and stored at 4^{o}C until processed or analyzed.

Table 1. Composition of white wheat diet.

Ingredient	% Ration
White wheat	65.55
Soybean meal (44%)	21.00
Meat and bone meal (50%)	2.50
Limestone (ground)	8.30
Dicalcium phosphate	1.50
Layer special premix[a]	0.25
Methionine (dl)	0.10
Soybean oil	0.50
salt	0.30
Calculated analysis	
Crude protein (%)	17.50
Crude fiber (%)	3.24
Fat (%)	2.00
Calcium (%)	3.82
Metabolizable energy (kcal/kg)	2586

[1] Layer special premix contains: Vitamin A, 3,530,000USPU/kg; vitamin D_3, 1,320,000ICU/kg; vitamin E, 4,400IU/kg; riboflavin, 1760mg/kg; d-pantothenoic acid, 4,400mg/kg; niacin, 13,200mg/kg; vitamin B_{12}, 6.6mg/kg; choline, 115,000mg/kg; folic acid, 88mg/kg; Mn, 25.6g/kg; Zn, 20.4g/kg; Fe, 14.4g/kg; Cu, 2g/kg; I, 0.2g/kg; Se, 0.12g/kg.

OP diets (B and C) were prepared weekly by diluting an OP premix with the control diet to produce the desired final concentration of paprika carotenoids, and stored at ambient temperature. The concentration of paprika carotenoids in the paprika premix, in which OP was mixed with maltodextrin at approximately 8% (w/w), was determined by the method described in Chapter 3. In diet C, α-tocopherol acetate (BASF Co., Wyandotte, MI) was added to the OP premix in appropriate amounts prior to blending with the control diet.

Preparation of Liquid Egg

Liquid whole egg was freshly processed in the Department of Food Science and Human Nutrition at Michigan State University. After manual separation from the shell, eggs from each dietary group were blended in a mixer (Model A-200, Kitchenaid Division, Hobart Co., Troy, OH) set at low speed for one minute in order to mix yolk and white. The blended whole egg was stored under refrigeration (4^{o}C) until spray-drying (within eight hours).

In addition to the eggs from hens receiving the supplemented diets, three concentrations (7.5, 15, 30 μg/g egg lipids) of OP were added directly to the eggs from hens fed the control diet immediately before spray drying. A stock solution of OP (approximately 1%, w/w) was prepared in propylene glycol (Fisher Scientific, Fair Lawn, NJ). The concentration of carotenoids in the stock solution of OP was calculated from the absorbance at 460nm (maximum. wavelength

of capsanthin) using the experimentally determined absorptivity at 1% in acetone of 1922 (Fisher, 1993, personal communication). The stock solution of OP was then added to the control liquid egg in appropriate amounts to produce the desired final concentrations of carotenoids.

Preparation of Spray-Dried Egg Powders

All egg samples were spray-dried using a single stage spray dryer with a conventional conical-based tower design (Marriott Walker Co., Birmingham, MI) at the Michigan State University Dairy Plant. The pilot scale spray dryer was equipped with a pneumatic nozzle atomizer, electric heating elements for indirect air heating and a gas burner for direct heating. Both spray drying systems, indirect (electric) heating and direct (gas-fired) heating, were used to dehydrate liquid whole egg (Table 2). The egg samples were packed in low density polyethylene bags (8" X 10", 75μm thickness, Whirl-Pak, Fisher Scientific, Fair Lawn, NJ) without heat sealing and stored at ambient temperature (22 ± 2^{o}C) in the dark for 4 months. Sampling for analysis of the egg samples was performed randomly after thoroughly mixing the egg powders in the bags with a spatula (approximately 100g per bag). Total solids of the egg powders were determined using the AOAC (1984) vacuum oven method. The formation of COPS and the loss of total carotenoids in the egg powders were monitored immediately after processing and

after storage for two and four months. The processing and storage studies were repeated twice.

Table 2. Liquid egg treatments and drying conditions used to prepare egg powders.

Heating source	Egg treatment[1]	
	Dietary treatment	Direct addition
Indirect, electric	A. control	---
Indirect, electric	B. OP (4mg/kg feed)	---
Direct, gas-fired	A. control	---
Direct, gas-fired	B. OP (4mg/kg feed)	---
Direct, gas-fired	C. OP (4mg/kg feed) + α-tocopherol (200mg/kg feed)	---
Direct, gas-fired	A. control	OP (7.5μg/g lipids)
Direct, gas-fired	A. control	OP (15μg/g lipids)
Direct, gas-fired	A. control	OP (30μg/g lipids)

[1] OP, quantities of oleoresin paprika carotenoids.

Lipid Extraction and Quantification of Cholesterol Oxidation Products in Egg Powders

Five COPS (cholesterol α- and β-epoxides, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol) in the egg powders were quantified by the method described in Chapter 1. Lipids were extracted from the egg powders with chloroform and the extracts applied to silica-packing tubes. COPS in the lipid extracts were isolated using solid phase extraction, derivatized to their trimethylsilyl ethers, and

subsequently determined by capillary gas chromatography using a 15m x 0.25mm i.d. DB-1 (0.1μm film thickness) column.

Determination of Total Carotenoids in Egg Powders

Egg powder (2g) was homogenized with 75ml acetone for 2 min in a 150ml beaker using an Ultra-Turrax type of homogenizer (Tekmar Co., Cinn., OH). The extract was filtered through Whatman NO.4 paper to remove solid particles. Another 20ml aliquot of acetone was used to rinse the probe and reextract the residue. The combined extracts were diluted to 100ml with acetone. The absorbance of the carotenoid extract was measured at 460nm with a double beam Bausch and Lomb Spectronic 2000 spectrophotometer (Rochester, NY). Quantification of the total carotenoids was calculated from the absorbance at 460nm using the experimentally determined absorptivity at 1% in acetone of 1922 (Fisher, 1993, personal communication).

Statistical Analysis

This experiment was designed as a two factor (treatment and storage time) split-plot model with the storage time as the factor for repeat-measurement of the formation of COPS and the loss of total carotenoids during storage. Two batches of eggs from all treatments were processed individually so that the batch of the processed egg powders is a within-treatment factor. The analyses of variance

(ANOVAs) for data were then based on the split-plot, repeat-measurement structure (Gill, 1986). Bonferroni t statistics were performed to analyze specific contrasts among treatments (Gill, 1978).

RESULTS AND DISCUSSION

The average total solid contents of the egg powders was 96.03% ± 0.46% (standard errors for means). The mean values of total solids did not differ significantly ($p < 0.01$) among samples from different treatments. Results of ANOVAs for the data revealed that the formation of COPS and the loss of carotenoids in the egg powders were significantly ($p < 0.05$) influenced by treatment (dietary and processing) as well as storage time. The variance between two processing batches of egg powders did not significantly affect the results ($p < 0.05$). However, a strong interaction of treatment and storage time was found in the formation of COPS in egg powders. On the other hand, the effect of storage time on the stability of carotenoids was not interacted with the effect of treatment. Therefore, the treatment effect was the major emphasis in the discussion of carotenoid stability, while the tests of the treatment effect and the time effect on the formation of COPS were performed conditionally, i.e., the contrasts for the

treatments were calculated separately at each stage of storage (0, 2 and 4 months).

Effects of Drying Method and Dietary α-Tocopherol on the Stability of Carotenoids in Egg Powders

Results showed that carotenoids in egg powders from hens fed the OP-supplemented diet and subsequently processed with indirect heating were significantly ($p < 0.05$) more stable than those in equivalent egg powders produced with the direct gas-fired heating system (Table 3). Although the carotenoids in the control eggs spray-dried with indirect heating also tended to be more stable than those processed by direct heating, the difference was not significant ($p < 0.05$). This statistical indifference may be due to the low initial content of carotenoids in the sample.

The method of spray drying has been reported to affect the stability of lipids in dehydrated foods (Kelly et al., 1989; Morgan and Armstrong, 1992; Chan et al., 1993). Oxides of nitrogen (NOx), by-products of the combustion process in the direct gas-fired spray dryer, are established free radical initiators of oxidation of unsaturated lipids (Roehm et al., 1971; Pryor and Lightsey, 1981). In this study, carotenoids, with their conjugated polyene structure, were also shown to be susceptible to oxidation when exposed to NOx or NOx-generated lipid radicals in the direct spray drying system.

Table 3. The effects of drying method and dietary α-tocopherol on the concentrations of carotenoids (μg/g lipids) in egg powders stored for 4 months at 22°C[1,2].

Dietary treatments	Drying method	Storage time Month 0	Month 2	Month 4
Control	Indirect	15.27[c]	13.18[c]	11.86[b]
	Direct	13.73[c]	11.21[c]	9.15[bc]
OP (4mg/kg)	Indirect	22.91[a]	18.98[a]	16.09[a]
	Direct	19.84[b]	15.18[b]	12.05[b]
OP (4mg/kg) + α-tocopherol (200mg/kg)	Direct	21.27[ab]	18.11[a]	15.95[a]

[1] All values represent the average of two replicated experiments analyzed in triplicate.
[2] All values in the same column bearing the same superscript do not differ significantly at $p < 0.05$.

Results also revealed that the susceptibility of carotenoids to oxidation in eggs spray dried with direct gas-fired heating was positively influenced by dietary supplementation of α-tocopherol. The stability of carotenoids in these egg powders was comparable to that in egg powders processed with the indirect heating procedure. Alpha-tocopherol is a naturally occurring lipid-soluble compound which functions as an antioxidant by donating a hydrogen from the chromanol ring to a lipid free radical, thus terminating the free-radical chain reaction (Mahoney and Graf, 1986). The protective effect of α-tocopherol on

carotenoid stability has been also demonstrated in vegetable oils by Terao et al. (1980) and Warner and Frankel (1987).

Effects of Drying Method and Dietary α-Tocopherol on the Stability of Cholesterol in Egg Powders

The concentrations of COPS in egg powders after processing and storage are presented in Tables 4, 5 and 6. Total COPS concentrations in egg powders (with and without dietary OP supplementation) processed by direct gas-fired spray drying were approximately two times greater than those in similar eggs spray-dried by the indirect heating process. Similar trends were observed at all stages of the storage period. These data agree with those reported in the literature (Missler et al., 1985; Tasi and Hudson, 1985; Lai, 1992). It has been demonstrated that NOx or other free radicals produced during gas-fired spray drying are responsible for the acceleration of cholesterol oxidation in egg powders processed with the direct heating system (Chapter 2).

Results also showed that the concentrations of COPS in powders from eggs of hens receiving dietary OP were greater than the concentrations in egg powders from the control diet ($p < 0.05$) immediately after processing. However, the difference in the concentrations of COPS in these egg powders did not change over storage time, which indicated that dietary OP supplementation did not promote COPS formation during ambient storage. On the other hand, in

Table 4. Effects of drying method and dietary α-tocopherol on the concentrations of cholesterol oxidation products (μg/g) in egg powders immediately after processing[1,2,3].

Cholesterol oxidation products	Indirect		Direct		
	Control	OP	Control	OP	OP/α-tocopherol
7α-hydroxy-Cholesterol	3.35	3.50	4.72	6.72	3.38
7β-hydroxy-Cholesterol	0.13	0.15	1.48	2.52	---[4]
7-keto-cholesterol	0.84	1.51	2.18	2.70	1.41
Cholesterol α-epoxide	1.09	0.87	1.05	1.89	---[4]
Cholesterol β-epoxide	2.09	2.77	4.61	6.62	1.64
Total	7.50[c]	8.80[c]	14.04[b]	20.45[a]	6.43[c]
% C-7	58	59	60	58	74
% epoxides	42	41	40	42	26

[1] All values represent the average of the two replicated experiments analyzed in duplicate.
[2] All values in the same row bearing the same superscript do not differ significantly at $p < 0.05$.
[3] OP, 4mg paprika carotenoids per kg feed; α-tocopherol, 200mg α-tocopherol acetate per kg feed.
[4] Not detectable (minimum detection limit: 0.1μg/g)

Table 5. Effects of drying method and dietary α-tocopherol on the concentrations of cholesterol oxidation products (μg/g) in egg powders stored for two months at 22°C[1,2,3].

Cholesterol oxidation products	Indirect		Direct		
	Control	OP	Control	OP	OP/α-tocopherol
7α-hydroxy-Cholesterol	4.36	5.13	6.61	7.41	4.85
7β-hydroxy-Cholesterol	2.87	2.83	4.94	5.82	2.71
7-keto-cholesterol	1.29	1.97	3.51	3.11	2.40
Cholesterol α-epoxide	1.43	1.79	3.15	3.88	1.53
Cholesterol β-epoxide	5.71	6.43	14.62	18.01	5.97
Total	15.66[b]	18.15[b]	32.83[a]	38.23[a]	17.46[b]
% C-7	54	55	46	43	57
% epoxides	46	45	54	57	43

[1] All values represent the average of the two replicated experiments analyzed in duplicate.
[2] All values in the same row bearing the same superscript do not differ significantly at $p < 0.05$.
[3] OP, 4mg paprika carotenoids per kg feed; α-tocopherol, 200mg α-tocopherol acetate per kg feed.

Table 6. Effects of drying method and dietary α-tocopherol on the concentrations of cholesterol oxidation products (μg/g) in egg powders stored for four months at 22°C[1,2,3].

Cholesterol oxidation products	Indirect		Direct		
	Control	OP	Control	OP	OP/α-tocopherol
7α-hydroxy-Cholesterol	5.34	6.71	10.00	10.86	6.23
7β-hydroxy-Cholesterol	5.03	6.11	8.89	8.70	6.51
7-keto-cholesterol	3.12	2.29	2.37	3.17	2.37
Cholesterol α-epoxide	2.69	2.72	4.45	4.76	2.45
Cholesterol β-epoxide	7.73	8.61	19.59	23.47	8.45
Total	23.91[b]	26.44[b]	45.30[a]	50.96[a]	26.01[b]
% C-7	56	57	47	45	58
% epoxides	44	43	53	55	42

[1] All values represent the average of the two replicated experiments analyzed in duplicate.
[2] All values in the same row bearing the same superscript do not differ significantly at $p < 0.05$.
[3] OP, 4mg paprika carotenoids per kg feed; α-tocopherol, 200mg α-tocopherol acetate per kg feed.

samples processed by indirect heating, the difference in the concentrations of COPS between the two dietary treatments was not statistically significant ($p < 0.05$) at all test periods.

Dietary supplementation of OP in a low-carotenoid (depletion) diet provided yolk color equivalent to that produced using a commercial corn diet. However, the carotenoids in eggs from hens fed a diet supplemented with OP oxidized rapidly when exposed to NOx in a direct gas-fired spray dryer, and it is possible that their oxidation products might accelerate the oxidation of cholesterol. No specific information can be found in the literature to support this speculation, although the rates of cholesterol oxidation have been reported to be influenced by other lipids such as triacylglycerols and fatty acids in model systems (Zulak and Maerker, 1989; Nawar et al., 1991; Kim and Nawar, 1991).

Dietary supplementation of 200mg/kg α-tocopherol acetate significantly ($p < 0.01$) increased the stability of cholesterol in egg powders processed by the direct gas-fired heating system. The concentrations of COPS in these samples were similar to those in powders processed with indirect heating after two and four months of storage. The protective effect of α-tocopherol for carotenoids was also demonstrated in this study. Therefore, dietary α-tocopherol reduced the formation of COPS in egg powders during processing with a direct gas-fired spray dryer and

subsequent storage, by stabilizing cholesterol and/or other lipids such as carotenoids.

Cholesterol oxidation is a free radical reaction and is initiated by the abstraction of the allylic C-7 hydrogen, with the subsequent formation of primary C-7 COPS such as 7α- and 7β- hydroxycholesterols and 7-ketocholesterol (Smith, 1981). Cholesterol α- and β-epoxides are products of attack by cholesterol-7-hydroperoxide on the 5, 6-double bond of cholesterol, and therefore are classified as secondary oxidation products (Maerker, 1987).

At the beginning of the storage period, C-7 COPS were formed at a faster rate than epoxides, 7α-hydroxycholesterol being the predominant COPS in all samples. After four months, the ratio of 7α-hydroxycholesterol to its β-isomer changed from approximately 3:1 to 1:1. It appears that 7α-hydroxycholesterol was formed initially and then was converted to its β-isomer during subsequent storage as a result of the thermodynamic stability of β-isomer.

Data also showed that epoxides were the major COPS after two months of storage in samples spray dried with the direct gas-fired system except for those samples prepared from eggs supplemented with dietary α-tocopherol. It has been reported that epoxides are the predominant COPS in eggs spray dried with a direct heating source (Missler et al., 1985; Morgan and Armstrong, 1992). Free radicals such as NOx in the drying air not only react with cholesterol but also with other unsaturated lipids leading to the formation

of lipid hydroperoxides. These hydroperoxides can accelerate the epoxidation of cholesterol and form cholesterol epoxides (Smith, 1981). On the other hand, α-tocopherol can terminate free radical reactions and reduce the formation of lipid hydroperoxides. Therefore, the distribution of COPS in the α-tocopherol-supplemented powders should be similar to that of COPS in eggs spray dried using the indirect heating source where formation of epoxides is less favored than the formation of C-7 COPS. The data obtained showed this to be true.

Stability of Paprika Carotenoids in Egg Powders

Total carotenoid concentrations in eggs from hens fed control and OP diets were 16.10 ± 1.06 and 23.53 ± 1.23 μg/g egg lipids, respectively. Thus, the difference in the carotenoid concentrations in eggs from the two dietary treatments was approximately 7.5μg/g egg lipids. In this experiment, 7.5μg/g (low) and two other concentrations of OP, 15μg/g (Med) and 30μg/g (high) (based on an egg lipid concentration of 12%), were added directly to the eggs from hens on the control diet prior to drying in order to investigate the stability of paprika carotenoids and cholesterol in eggs spray dried with the direct gas-fired heating system.

Comparisons of total carotenoid and COPS concentrations in these samples are presented in Figures 1 and 2, respectively. Strong linear effects ($p < 0.05$) of OP

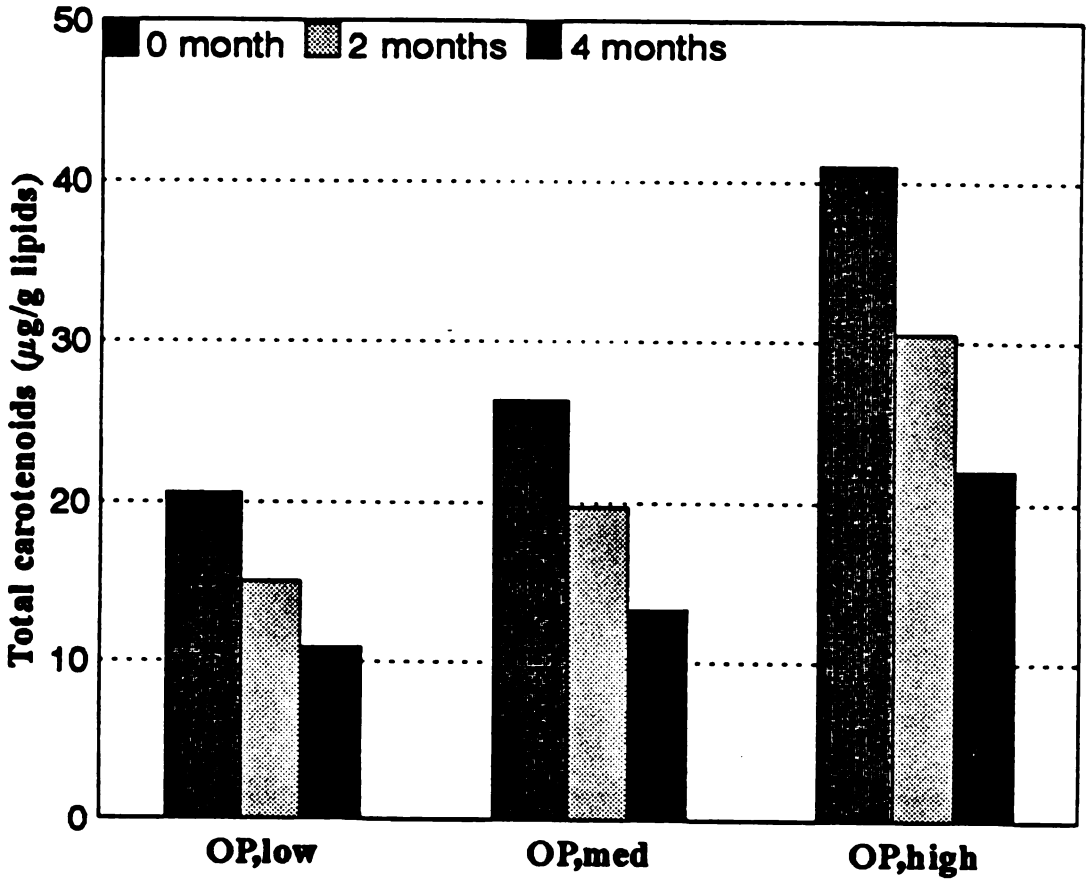

Figure 1. Stability of oleoresin paprika carotenoids in eggs dried with a direct gas-fired spray dryer and stored at 22°C for four months (OP,low: 7.5µg paprika carotenoids per g egg lipids; OP, med: 15µg paprika carotenoids per g egg lipids; OP, high: 30µg paprika carotenoids per g egg lipids).

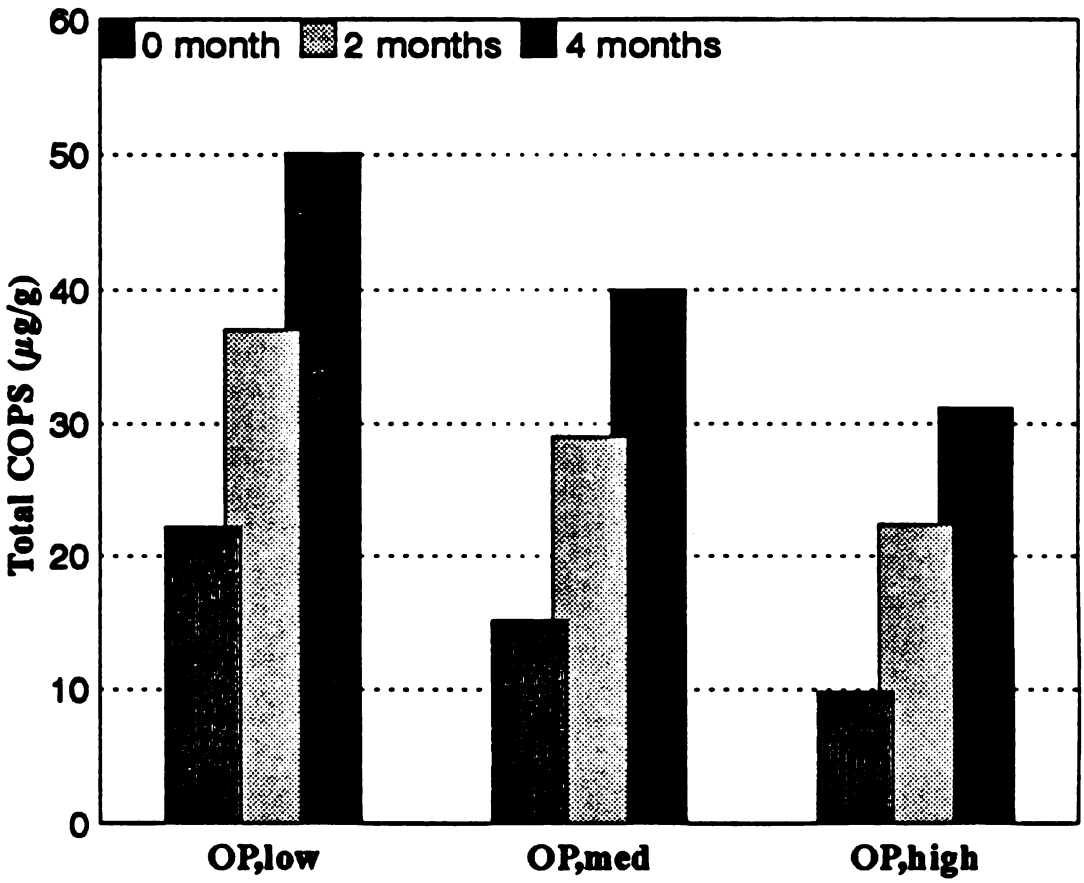

Figure 2. Formation of cholesterol oxidation products (COPS, µg/g) in eggs dried with a direct gas-fired spray dryer and stored at 22°C for four months (OP,low: 7.5µg paprika carotenoids per g egg lipids; OP, med: 15µg paprika carotenoids per g egg lipids; OP, high: 30µg paprika carotenoids per g egg lipids).

treatments on the stability of carotenoids in eggs were found at each test period, which indicated that the carotenoid concentrations in egg samples after processing and during storage were proportional to the amounts of OP added to these samples.

The formation of COPS in eggs during spray drying was influenced ($p < 0.01$) by the concentrations of OP added to the eggs before drying. Although the low concentration of OP produced a comparable quantity of COPS to those in eggs with dietary OP supplementation, the highest concentration of OP (30μg/g lipids) appeared to increase the oxidative stability of cholesterol in eggs during processing and storage.

It has been reported that carotenoids can act as antioxidants or prooxidants depending on the system (Warner and Frankel, 1987; Krinsky, 1989). For example, Burton and Ingold (1984) reported that β-carotene functioned as a radical quenching antioxidant at low oxygen tensions but acted as a prooxidant at 760 torr (100% oxygen), based on the appearance of an increased initial rate of oxidation of free radical-initiated oxidation of methyl linoleate. However, there are no reports in the literature relative to the effects of different concentrations of carotenoids on their roles in lipid oxidation. More research on the effect of different concentrations of carotenoids on cholesterol oxidation in model systems is needed to explain the

interactions between paprika carotenoids and cholesterol in oxidizing food systems.

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CHAPTER FIVE

NITROGEN OXIDE-INITIATED CHOLESTEROL OXIDATION AND CAROTENOID DEGRADATION IN AN EGG LIPID MODEL SYSTEM

ABSTRACT

The influence of nitrogen oxide (NOx) concentrations on the oxidation of cholesterol and carotenoids in an egg lipid model system was investigated. The effects of dietary α-tocopherol supplementation on cholesterol and carotenoid stability in egg lipids were also evaluated. The rate constants for cholesterol oxidation and carotenoid degradation in egg lipids during storage at 40°C were affected by both the egg treatments and the level of exposure to NOx before storage. The presence of NOx greatly promoted the oxidation of cholesterol and carotenoids. Dietary supplementation with α-tocopherol greatly improved the stability of carotenoids and cholesterol in egg lipids exposed to NOx. The addition of carotenoids to egg lipids also protected cholesterol from NOx-initiated oxidation. The patterns of cholesterol oxidation and carotenoid loss in this lipid model system were similar to those in egg powders processed by the direct gas-fired spray dryer.

INTRODUCTION

Lipid oxidation is mainly responsible for the deterioration of food products and adversely affects their color, flavor, nutritive value and even safety (Pearson et al., 1983). For example, the formation of cholesterol oxidation products (COPS) and the loss of pigments are results of oxidation of cholesterol and carotenoids during dehydration and storage (Bergquist, 1986; Morgan and Armstrong, 1987, Kumar and Singhal, 1991). Many investigators have studied the influence of processing conditions on lipid stability in spray-dried foods and reported that lipids, including cholesterol, in foods processed by a direct gas-fired heating source oxidized to a greater extent than in foods produced with indirect heating (Missler et al., 1985; Morgan and Armstrong, 1987; Kelly et al., 1989; Faulkner et al., 1992; Chan et al., 1993). They suggested that the exposure of foods to oxides of nitrogen (NOx) in the direct gas-fired spray dryer may be responsible for the accelerated lipid oxidation.

NOx, including nitric oxide (NO) and nitrous oxide (NO_2), are produced from air as a result of combustion processes (Wheeler, 1980). NO_2 has been demonstrated to be a free radical initiator of oxidation of unsaturated lipids in model systems (Roehm et al., 1971; Pryor and Lightsey, 1981). In a previous study (Chapter 2), the influence of NOx on the cholesterol oxidation in spray-dried egg powders

was investigated by introducing NOx gas to drying air heated by an indirect, electric heating source. The concentrations of COPS in egg powders produced by indirect heating increased by approximately 230 percent when the amount of NOx in drying air increased from the original non-detectable amount (< 0.5 parts per million, ppm) to 15ppm. The pathway of cholesterol oxidation initiated by NOx also appeared to be similar to the cumene hydroperoxide-induced free radical chain reaction. Thus, the role of NOx in the rapid formation of COPS in egg powders produced by the direct gas-fired spray dryer was indirectly confirmed.

Another study (Chapter 4) was conducted to evaluate the effect of dietary α-tocopherol supplementation on the stability of lipids in egg powders. It was demonstrated that dietary α-tocopherol acetate (200mg/kg feed) was very effective in improving stability of cholesterol and carotenoids in eggs spray-dried by the direct gas-fired system. The present study was designed to investigate the influence of NOx concentrations on the rate of cholesterol oxidation and carotenoid degradation in an egg lipid model system. The effects of dietary α-tocopherol supplementation on stability of cholesterol and carotenoids in egg lipids were also evaluated

MATERIALS AND METHODS

Source of Eggs

Eggs used in this study were obtained from hens fed the following experimental diets: (A) control (depletion white wheat) diet; (B) oleoresin paprika (OP) diet (4mg/kg feed, Kalsec Inc., Kalamazoo, MI); (C) OP (4mg/kg feed) plus α-tocopherol acetate (200mg/Kg feed, BASF, Co., Wyandotte, MI). The preparation of the dietary treatments was described in Chapter 4.

Extraction of Egg Lipids

Lipid extraction from egg yolk was performed using a slightly modified version of the Bligh and Dyer procedure (1959). Yolks of eggs from each group were separated from whites, with the adhering albumen being removed by rolling the yolks over moistened paper towels. The membranes surrounding the yolks were punctured, and the free flowing yolks were combined.

Egg yolk (10g) was mixed in a 250ml beaker with 19ml 2M NaCl solution prior to the addition of 60ml methanol and 30 ml chloroform. The mixture was homogenized for 1 min using an Ultra-Turrax type of homogenizer (Tekmar Co., Cinn., OH). Chloroform (30ml) was added to the homogenate and the mixture homogenized for another 15 seconds. Deionized water (30ml) was added to the mixture and after homogenizing for 15 seconds, the mixture was transferred to a 250ml

centrifuge bottle and centrifuged at 700xg for 20 min using an IEC centrifuge(International Equipment Co., Needham Hts., MA). The upper aqueous layer was removed by aspiration. The lower chloroform phase containing extractable lipids was then filtered through Whatman NO.4 paper and collected for use in the lipid model systems.

Preparation of Lipid/Celite Mixtures for the Egg Lipid Model System

Celite 545 (Analytical Filter Aids; Mansville Products Corp., Denver, CO) was used as a solid support for the lipid model system. The chloroform extract of egg lipids (from 20g egg yolk) were added to 12g Celite 545 in a 1000ml round-bottom flask and evaporated to dryness at 30^{o}C with a vacuum rotary evaporator (Büchi Rotavapor, Postfach, Switzerland). The lipid/Celite mixture was spread over aluminum foil to permit the evaporation of final traces of solvent. This was carried out in the dark at room temperature for 30 min. The lipid contents of the lipid/Celite samples were approximately 30-35%.

In addition to the lipids from eggs containing OP and α-tocopherol (through dietary supplemention), OP carotenoids (30μg/g egg lipids) were added directly to the lipid extracts from the control eggs as indicated below. This lipid system was also mixed with Celite as described above. A stock solution of OP (approximately 1%, w/w) was prepared in acetone. Concentration of paprika carotenoids in the

stock solution was obtained from the absorbance at 460nm (max. wavelength of capsanthin) using the experimentally determined absorptivity at 1% in acetone of 1922 (Fisher, 1993, personal communication). The stock solution of OP was then added to the chloroform extract of egg lipids in appropriate amounts to produce the desired final concentration of carotenoids.

Construction of the Lipid Model System

The apparatus shown in Figure 1 was developed to regulate the flow rate of gases, concentrations of NOx and temperature during the lipid model system oxidation . Swagelok fittings (H.E. Lennon, Inc., Farmington, MI) were used to connected the copper tubing, the three-way needle valve, needle valves, standard 65mm flowmeters (Cole Palmer, Chicago, IL) and the glass test cell (250ml 24/40 gas washing bottle, Ace Glass Inc., Vineland, NJ). Nitrogen, air and NOx gas were obtained from AGA Gas, Inc. (Maumee, OH). The NOx gas contained 100ppm NOx (50ppm NO and 50ppm NO_2) in nitrogen with 2% tolerance of errors. Air was mixed with appropriate amounts of NOx gas to prepare 0, 10, 20, 40ppm NOx in air at a constant flow rate of 100ml/min.

Oxidation of Egg Lipids

The oxidation reactions were carried out at 90^{o}C in a temperature-controlled oven (Model 17, Precision Scientific Co., Chicago, IL) with a gas flow of 100ml/min continually

Figure 1. Schematic of apparatus for the lipid model system.

A. Gas tanks (1, nitrogen; 2, air; 3, NOx gas)

B. Needle valves

C. Standard 65mm flowmeters

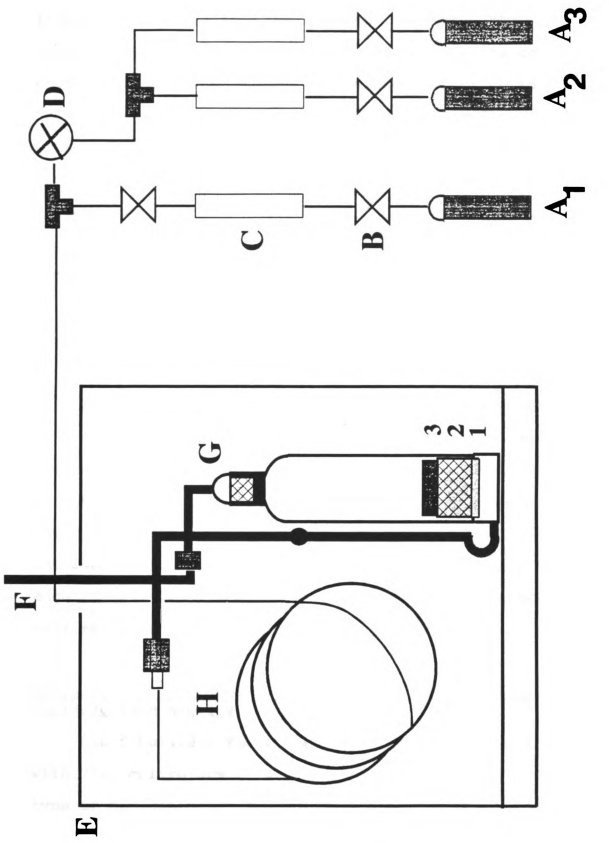
D. Three-way switching valve

E. Thermal controlled oven

F. Gas outlet (glass tube, 5/8 in. i.d.)

G. Test cell (1, fritted disc, 145-175μ; 2, glass wool; 3, sample)

H. Copper tube (1/8 in. i.d.; 2m inside the oven)


A3
A2
A1
D
C
B
G
3
2
1
F
H
E

flowed through the test cell and to the vent. The temperature of 90°C was chosen to approximate the conditions encountered in spray drying operations. Prior to testing the system at each NOx concentration, the air flow (with or without NOx gas) was flowed through an exhaust valve until the flow rate was stabilized (by visually reading the flowmeters, approximately 30 min). Once stabilized, the lipid/Celite mixture (10g) was placed in the test cell and the gas flow run through the test cell was switched from nitrogen to air/NOx. After flushing air/NOx for 5 min, the sample was removed from the test cell and cooled immediately in a freezer.

Lipid/Celite samples were packed in polyethylene bags (2" x 5", 50μm thickness) without sealing and stored at 40°C for two weeks. The formation of COPS and the loss of carotenoids in the lipid/Celite samples were monitored immediately after NOx treatment and after storage for one and two weeks. Preparation and reaction of samples in this model system were repeated twice. Triplicate analyses were performed for each replicated experiment.

Extraction of Lipids and Quantification of Cholesterol in Lipids/Celite Samples

Lipid/Celite mixture (1g) was homogenized for 2 min with 75ml chloroform in a 150ml beaker using a Ultra-Turrax type of homogenizer. The extract was filtered through Whatman NO.4 paper to remove solid particles. Another 20ml

aliquot of chloroform was used to rinse the probe and reextract the residue. The extracts were combined and diluted to 100ml with chloroform.

Twenty μg of the internal standard, 5α-cholestane (Steraloids Inc., Wilton, NH), were added to 50μl of the chloroform lipid extract (or approximately 0.15mg lipids) in a 1/2 dram glass vial. After the solvent was evaporated under a stream of nitrogen, the extracts were redissolved in 100μl bis-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (BSTFA + 1% TMCS, Pierce Chemical Co., Rockford, IL), capped and mixed with a vortex mixer for 30 sec. This mixture was placed in the dark at room temperature for 50 min to form the trimethylsilyl (TMS) ether derivatives of cholesterol. Subsequently, the TMS reagent was removed under nitrogen and the residue was dissolved in 100μl hexane.

The TMS ether of cholesterol (2μl) was analyzed with a 15m x 0.25mm i.d. DB-1 (0.1μm film thickness) capillary column (J&W Scientific Inc. Folsom, CA) operated with a helium carrier gas (column flow rate of 1ml/min) in a Hewlett Packard (HP) 5890A gas chromatograph (Avondale, PA) equipped with a flame ionization detector. The oven temperature was programmed from 170^{o}C to 220^{o}C at a rate of 10^{o}C/min, then increased to 230^{o}C at a rate of 0.4^{o}C/min. The temperatures of the injection port and detector were held at 275^{o}C and 300^{o}C, respectively. Peak areas were integrated with a HP 3392A integrator (Avondale, PA) and

converted to cholesterol concentration using the internal standard method.

Quantification of Cholesterol Oxidation Products in Lipid/Celite Samples

Five COPS (cholesterol α- and β-epoxides, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol) in the lipid/Celite samples were quantified by the method described in Chapter 1. Lipids were extracted from the lipid/Celite samples with chloroform and the extracts applied to silica-packing tubes. COPS in the lipid extracts were isolated using solid phase extraction, derivatized to their TMS ethers, and subsequently determined by capillary gas chromatography using a 15m x 0.25mm i.d. DB-1 (0.1μm film thickness) column.

Determination of Total Carotenoids in Lipid/Celite Samples

A lipid/Celite sample (2g) was homogenized with 75ml acetone for 2 min using a Ultra-Turax type homogenizer. The extract was filtered through Whatman No.4 paper to remove solid particles. Another 20ml aliquot of acetone was used to rinse the probe and reextract the residue. The extracts were combined and diluted to 100ml with acetone. The absorbance of carotenoids extract was read at 460nm with a double beam Bausch and Lomb Spectronic 2000 spectrophotometer (Rochester, NY). Total carotenoid concentrations were obtained from the absorbance at 460nm

(maximum wavelength of capsanthin) using the experimentally determined absorptivity at 1% in acetone of 1922 (Fisher, 1993, personal communication).

Statistical Analysis

A one-way analysis of variance was performed for significant treatment effect on initial cholesterol and carotenoid contents of lipid/Celite samples. Bonferroni t statistics was used to analyze specific contrasts among treatments (Gill, 1978).

RESULTS AND DISCUSSION

Cholesterol and carotenoids are unsaturated lipids, and as such undergo oxidation in the presence of oxygen via a free radical reaction (Smith, 1981; Francis, 1985). The following equations explain the lipid oxidation reaction mechanism:

Initiation	$RH + X^{\cdot} \longrightarrow R^{\cdot} + HX$		k_i	(1)
Propagation	$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$	(fast)	k_o	(2)
	$ROO^{\cdot} + R'H \longrightarrow ROOH + R'^{\cdot}$	(slow)	k_p	(3)
Termination	$ROO^{\cdot} + ROO^{\cdot} \longrightarrow$ Non-radicals			(4)
	$ROO^{\cdot} + R^{\cdot} \longrightarrow$ Non-radicals			(5)
	$R^{\cdot} + R^{\cdot} \longrightarrow$ Non-radicals			(6)

where RH = unsaturated lipid (cholesterol or carotenoids)

$R^{\cdot}$ = alkyl radical

$X^{\cdot}$ = initiator

$ROO^{\cdot}$ = peroxyl radical

ROOH = hydroperoxide

k = rate constant

By applying "steady-state approximation" to the set of reactions given above, i.e., assuming the concentration of $R^{\cdot}$ and $ROO^{\cdot}$ do not change with time and assuming that the oxygen partial pressure and the amount of cholesterol are high enough to ignore the termination steps, the following expressions can be derived:

$$\frac{d[R^{\cdot}]}{dt} = k_i[RH][X^{\cdot}] - k_o[R^{\cdot}][O_2] = 0 \qquad (9)$$

$$[R^{\cdot}] = \frac{k_i[RH][X^{\cdot}]}{k_o[O_2]} \qquad (10)$$

$$\frac{d[ROO^{\cdot}]}{dt} = k_o[R^{\cdot}][O_2] - k_p[ROO^{\cdot}][R'H] = 0 \qquad (11)$$

$$[ROO^{\cdot}] = \frac{k_o[R^{\cdot}][O_2]}{k_p[R'H]} \qquad (12)$$

Rate determining step:

$$\frac{d[ROOH]}{dt} = k_p[ROO^{\cdot}][R'H]$$

$$= k_p[R'H] \frac{k_o[R^{\cdot}][O_2]}{k_p[R'H]}$$

$$= k_o[R^{\cdot}][O_2]$$

$$= k_o[O_2] \frac{k_i[RH][X^{\cdot}]}{k_o[O_2]}$$

$$= k_i[RH][X^{\cdot}] = \frac{-d[RH]}{dt} \qquad (13)$$

Although the kinetic mechanism outlined for lipid oxidation has been simplified, the "initiator" ($X^{\cdot}$) is still difficult to define. For example, $X^{\cdot}$ may be transition metal ions, radicals obtained by the decomposition of a hydroperoxide (e.g. $RO^{\cdot}$, $HO^{\cdot}$), radicals formed from an added initiator such as cumene hydroperoxide, or radicals existing in foods or which are produced during processing, e.g. NOx. The actual conditions of lipid oxidation in food system are even more complex, not only because the system is likely to be multiphasic, but also because there may be many different kinds of substrates in the system (Chan, 1987). Therefore, analysis of data based on curve fitting equations, i.e., using the slope of the appropriate quality versus time curve, is an effective approach to describe the reaction rate constants in food systems (Labuza and Ragnarsson, 1985; Özilgen and Özilgen, 1990).

A first order reaction has been reported to be satisfactory for cholesterol oxidation in lard (Yan and White, 1990) and for the loss of β-carotene in a microcrystalline cellulose model system and dehydrated carrots (Glória et al., 1992). If a first order rate equation fits the data for cholesterol oxidation and carotenoid degradation in the egg lipid model at 40°C in presence of excess oxygen, then:

$$\underset{(C)}{\text{cholesterol or carotenoids}} \xrightarrow{k_{obsd}} \text{products}$$

$$\frac{-dC}{dt} = k_{obsd}\, C \qquad (14)$$

$$\frac{-dC}{C} = k_{obsd}\, dt \qquad (15)$$

$$\ln \frac{C}{C_0} = -k_{obsd}\, t \qquad (16)$$

where C_0 is the concentrations of cholesterol or carotenoids in the egg lipid model system at the beginning of storage, C is the concentration of cholesterol (C_0 - [COPS]) or carotenoids in the egg lipid model system at a given time during storage at 40°C, and k_{obsd} is the overall rate constant calculated from data and $k_{obsd} = k_i[X^{\cdot}]$ (from Equation 13).

Cholesterol Oxidation and Carotenoid loss in an Egg Lipid Model System

The lipid contents of the lipid/Celite samples ranged from 31% to 35% and are slightly smaller than the average lipid content in egg powders (approximately 40%). The average cholesterol content of the lipid/Celite samples was 30.65mg/g lipids with standard errors for means of ± 0.96mg/g lipids. The mean values of cholesterol contents did not differ significantly ($p < 0.01$) among samples from different egg treatments. The initial concentrations of carotenoids (immediately after reacting with NOx) in the samples were measured because the percent loss of carotenoids in the samples during storage was based on the

initial concentrations of carotenoids. These were 15.39, 22.71, 23.20 and 47.29μg/g lipids for treatments A (control); B, OP; C, OP/α-tocopherol; and D, OP/added, respectively.

Figure 2 shows the relationship between the extent of cholesterol oxidation and storage time at 40°C in the samples flushed with 0, 10, 20 and 40ppm NOx before storage. The first order-semilog plots for cholesterol oxidation and carotenoid loss indicate that cholesterol oxidation and carotenoid loss in the samples with different egg treatments or exposure to varying concentrations of NOx can be described by a first order reaction because of the high correlation for linear regression (Tables 1 and 2).

Calculated first order rate constants for carotenoid loss in egg lipids reacted with various concentrations of NOx and stored at 40°C are listed in Table 1. Carotenoids in egg lipids with dietary α-tocopherol were the most stable during storage. The protective effect of α-tocopherol for carotenoids was observed in all samples including those that were not exposed to NOx. The rate constants of carotenoid loss during storage in egg lipids with dietary α-tocopherol and treated with 40ppm NOx before storage were even smaller than those of samples without dietary α-tocopherol (treatment B) and without NOx exposure.

Dietary supplementation of α-tocopherol has been found to greatly improve the stability of carotenoids in eggs during direct gas-fired spray drying and subsequent storage

Figure 2. Formation of cholesterol oxidation products (COPS) in egg lipids reacted with various concentrations of NOx and stored at 40°C for 14 days (A, control; B, OP; C, OP/α-tocopherol; D, OP/added).

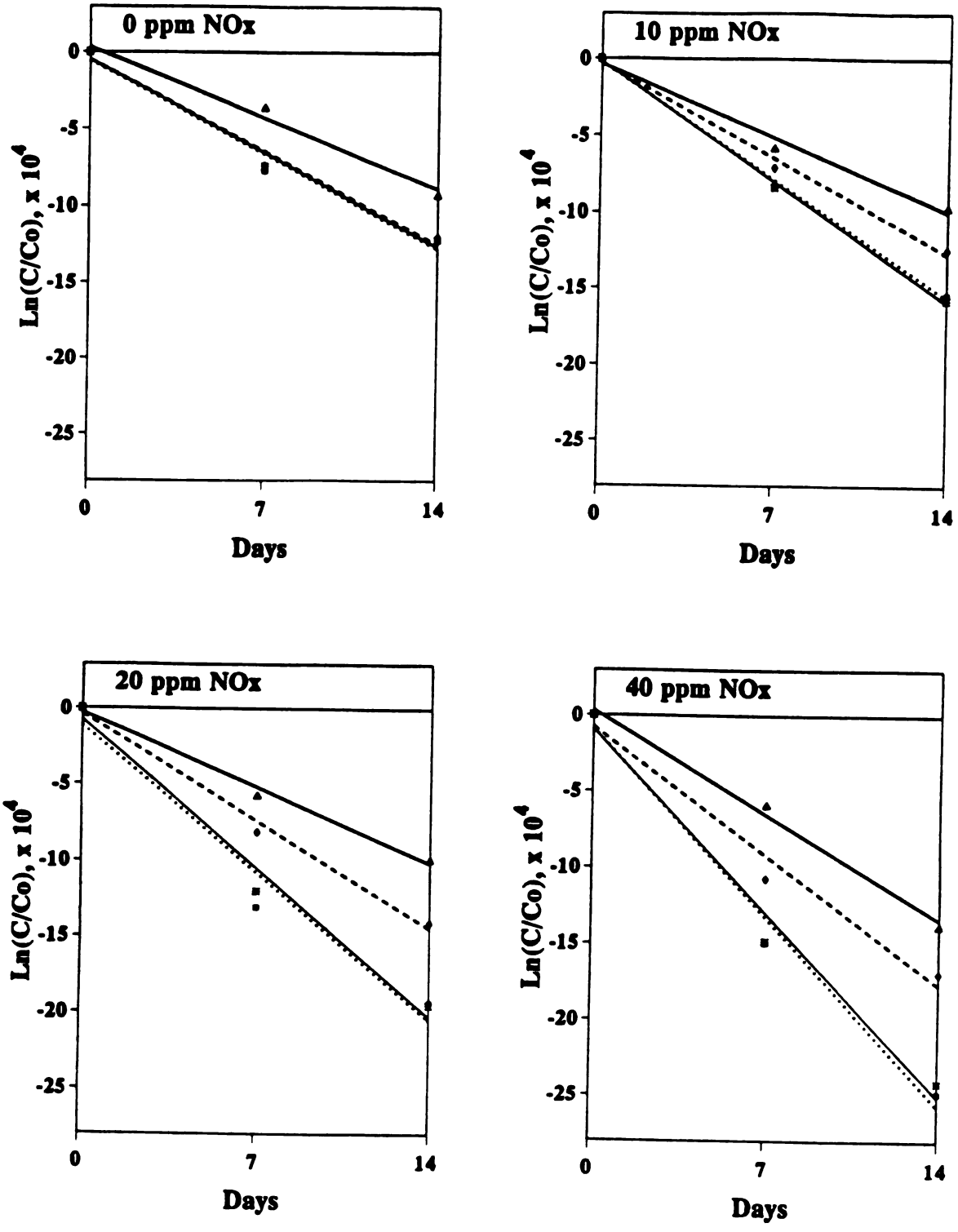

0 ppm NOx
10 ppm NOx
20 ppm NOx
40 ppm NOx
Ln(C/Co), x 10^4
0
-5
-10
-15
-20
-25
0
7
14
Days
A
B
C
D

Table 1. First order rate constants for carotenoid loss in egg lipids reacted with various concentrations of NOx and stored at 40°C for 14 days[1,2].

Concentration of NOx in air	Rate constant (k, day^{-1})			
	A control	B OP	C OP/α-T	D OP/added
0ppm	$1.79x10^{-2}$ (0.932)	$1.74x10^{-2}$ (0.966)	$6.34x10^{-3}$ (0.950)	$1.08x10^{-2}$ (0.998)
10ppm	$2.40x10^{-2}$ (0.993)	$2.14x10^{-2}$ (0.947)	$9.09x10^{-3}$ (0.906)	$1.42x10^{-2}$ (0.999)
20ppm	$2.81x10^{-2}$ (0.960)	$2.68x10^{-2}$ (0.829)	$1.27x10^{-2}$ (0.988)	$1.96x10^{-2}$ (0.997)
40ppm	$4.23x10^{-2}$ (0.985)	$3.20x10^{-2}$ (0.984)	$1.56x10^{-2}$ (0.983)	$2.45x10^{-2}$ (0.999)

[1] OP, oleoresin paprika; α-T, α-tocopherol.
[2] Correlation coefficients are shown in parentheses

Table 2. First order rate constants for the formation of cholesterol oxidation products in egg lipids reacted with various concentrations of NOx and stored at 40°C for 14 days[1,2].

Concentration of NOx in air	Rate constant (k, day^{-1})			
	A contro	B OP	C OP/α-T	D OP/added
0ppm	$8.68x10^{-5}$ (0.992)	$8.71x10^{-5}$ (0.988)	$6.57x10^{-5}$ (0.992)	$8.54x10^{-5}$ (0.991)
10ppm	$1.12x10^{-4}$ (0.999)	$1.10x10^{-4}$ (0.999)	$6.88x10^{-5}$ (0.993)	$8.84x10^{-5}$ (0.996)
20ppm	$1.39x10^{-4}$ (0.991)	$1.38x10^{-4}$ (0.980)	$7.04x10^{-5}$ (0.996)	$1.00x10^{-4}$ (0.996)
40ppm	$1.72x10^{-4}$ (0.991)	$1.77x10^{-4}$ (0.993)	$9.82x10^{-5}$ (0.996)	$1.21x10^{-4}$ (0.988)

[1] OP, oleoresin paprika; α-T, α-tocopherol.
[2] Correlation coefficients are shown in parentheses

(Chapter 4). The concentrations of NOx in the drying air of direct gas-fired spray dryer are 8-10ppm (Kelly et al., 1989). Results from this model system demonstrated that dietary α-tocopherol protected carotenoids from NOx-initiated oxidation regardlessly the level of exposure to NOx.

The rate constants for carotenoid loss in egg lipids with low carotenoid (A, control) and moderate carotenoid (B, OP) contents during storage were similar, and both increased when the samples were exposed to NOx. Although the rate constants for carotenoid loss in egg lipids with high carotenoid content (treatment D) were smaller than those for treatments A and B, they were also affected by the concentrations of NOx.

Table 2 summarizes the calculated first order rate constants for cholesterol oxidation in egg lipids reacted with various concentrations of NOx and stored at 40^{o}C. The dietary α-tocopherol treatment (C) protected cholesterol as well as carotenoids from NOx-initiated oxidation. The rate constants for cholesterol oxidation during storage of samples containing low to moderate concentrations of carotenoids (treatments A and B) were doubled when the egg lipid samples were treated with 40ppm NOx before storage. On the other hand, addition of OP (30μg/g lipids) to the control eggs suppressed the rate constants for cholesterol oxidation resulting from NOx exposure.

Results of this lipid model study agree with the findings of the spray drying study (Chapter 4). Significantly smaller quantities of COPS were formed in eggs from hens receiving dietary α-tocopherol during direct gas-fired spray drying and subsequent storage. α-Tocopherol functions as an antioxidant by donating a hydrogen from the chromanol ring to a lipid free radical, thus terminating the free radical chain reaction and protecting unsaturated lipids, including cholesterol and carotenoids from oxidation (Mahoney and Graf, 1986). The protective effect of dietary α-tocopherol toward cholesterol has been also demonstrated in poultry (Asghar et al., 1989), pork (Monahan et al., 1992) and veal (Engeseth et al., 1993).

Although carotenoids, particular β-carotene, have been established as effective singlet oxidation quenchers and can prevent photooxidation in vegetable oils in cooperation with tocopherol (Terao et al., 1980; Warner and Frankel, 1987), the antioxidant effects of carotenoids on free radical-induced oxidation are still inconclusive (Krinsky, 1989). Burton and Ingold (1984) found that β-carotene functions as an antioxidant at low oxygen partial pressures by a mechanism in which the chain propagating peroxyl radicals are trapped by the conjugated polyene system of β-carotene rather than by the mechanism of hydrogen donation. The resulting carbon-centered radical is resonance-stabilized because of the delocalization of the unpaired electron in the conjugated polyene system, leading to chain termination.

More recently, Terao (1989) studied the antioxidant activity of carotenoids in a solution model system with excess air and found that the reaction of carotenoids with the peroxyl radicals competes with the formation of lipid hydroperoxides via a chain reaction. This may explain why the addition of OP (30μg/g lipids) to the low carotenoid sample (control) reduced the rate of cholesterol oxidation in the lipid model study.

Influence of NOx Concentrations on the Rate Constants for Cholesterol Oxidation and Carotenoid Degradation

It was observed that the rate constants for cholesterol oxidation and carotenoid loss in the lipid model system were affected not only by the different egg treatments, but also by exposure to different concentrations of NOx before storage. Therefore, the rate constants for carotenoid loss and cholesterol oxidation were plotted against the concentrations of NOx (Figures 3 and 4) and the regression lines derived from these relationships are listed in Tables 3 and 4. The high correlation coefficients for these regressions suggest that the first order rate constants observed from data analysis, i.e., k_{obsd}, can be expressed as follows:

$$k_{obsd} = k_0 + k_2[NOx] \quad (17)$$

where k_0 is the rate constant for carotenoid loss or cholesterol oxidation in the sample without NOx treatment

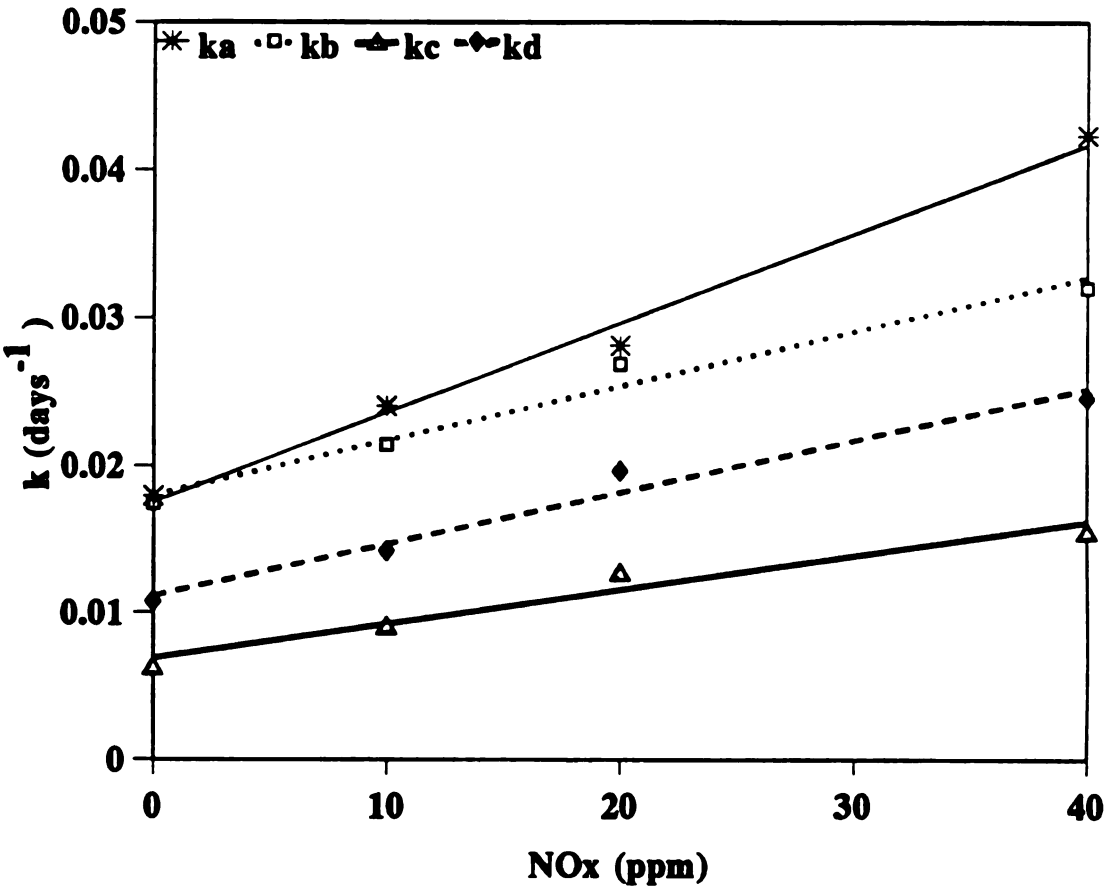


Figure 3. The dependence of the first order rate constants for the carotenoid loss in egg lipids on the concentrations of NOx (ka, kb, kc, kd are rate constants for A, control; B, OP; C, OP/α-tocopherol; D, OP/added).

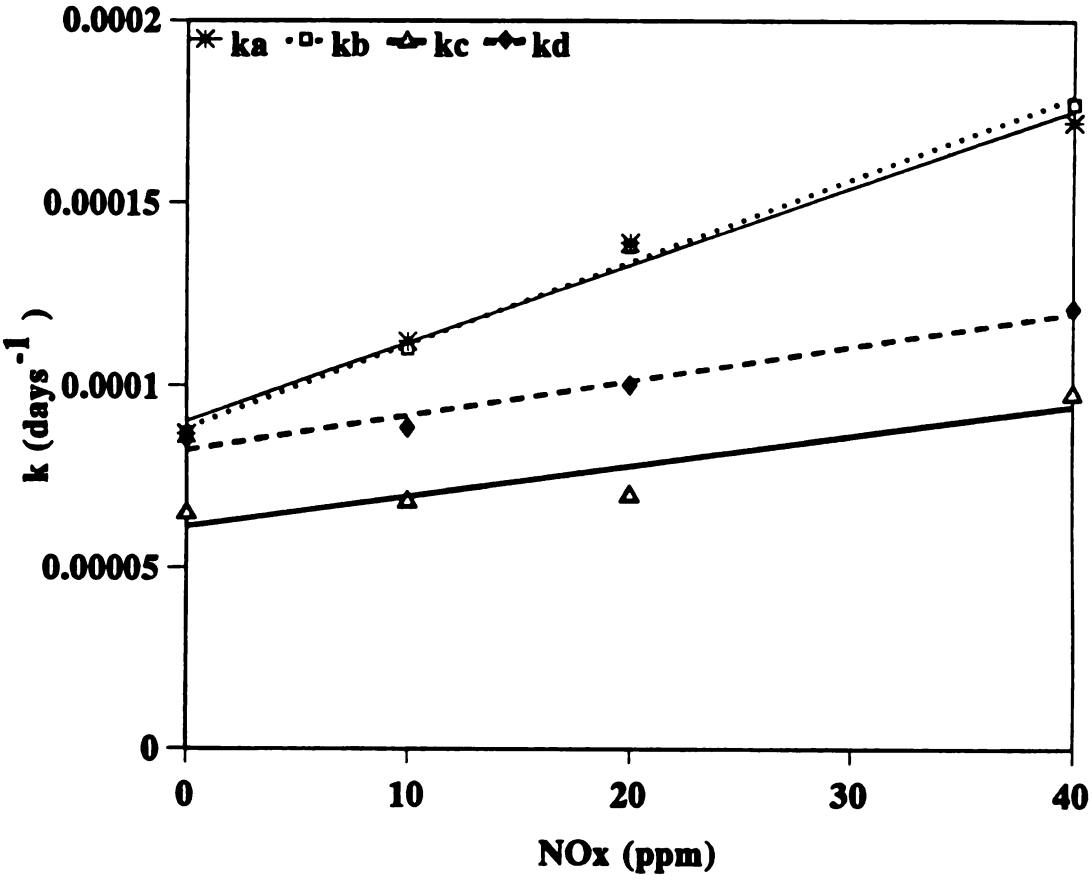


Figure 4. The dependence of the first order rate constants for the formation of cholesterol oxidation products in egg lipids on the concentrations of NOx (ka, kb, kc, kd are rate constants for A, control; B, OP; C, OP/α-tocopherol; D, OP/added).

Table 3. Effect of NOx concentration on the rate constant for carotenoid loss in an egg lipid model system[1,2].

Egg samples	Best fit regression line	r
A control	$ka = 1.75x10^{-2} + 6.03x10^{-4}$ [NOx]	0.995
B OP	$kb = 1.80x10^{-2} + 3.66x10^{-4}$ [NOx]	0.986
C OP/α-T	$kc = 6.87x10^{-3} + 2.32x10^{-4}$ [NOx]	0.978
D OP/added	$kd = 1.11x10^{-2} + 3.50x10^{-4}$ [NOx]	0.987

[1] OP, oleoresin paprika; α-T, α-tocopherol.
[2] r, correlation coefficient.

Table 4. Effect of NOx concentration on the rate constant for the formation of cholesterol oxidation products in an egg lipid model system[1,2].

Egg samples	Best fit regression line	r
A control	$ka = 9.03x10^{-5} + 2.12\ x10^{-6}$ [NOx]	0.992
B OP	$ka = 8.85x10^{-5} + 2.26\ x10^{-6}$ [NOx]	0.997
C OP/α-T	$ka = 6.14x10^{-5} + 8.23\ x10^{-7}$ [NOx]	0.932
D OP/added	$ka = 8.24x10^{-5} + 9.31\ x10^{-7}$ [NOx]	0.985

[1] OP, oleoresin paprika; α-T, α-tocopherol.
[2] r, correlation coefficient.

and k_2 is the rate constant responsible for carotenoid loss or cholesterol oxidation initiated by NOx.

From the best fitting lines, k_0 and k_2 for carotenoid loss or cholesterol oxidation were obtained. Data indicated that the carotenoids in samples from treatments B and D were almost equally sensitive to NOx exposure, while the carotenoids in samples with dietary α-tocopherol were the most resistant to NOx-initiated oxidation. On the other hand, cholesterol in the samples from treatment B (OP) was as susceptible to NOx-initiated oxidation as was cholesterol in samples from treatment A (control). Treatment D, addition of OP (30μg/g lipids) to the control, did not improve k_0, but provided a comparable protective effect for cholesterol against NOx-initiated oxidation as treatment C with dietary α-tocopherol.

Morgan and Armstong (1992) investigated the effect of NOx on the formation of COPS in egg yolk powders produced with direct gas-fired heating. They manipulated the levels of NOx in the combustion gas by delivering NO_2 to the gas burner where NO_2 dissociates into a mixture of oxidizing nitrogen oxides gases. They demonstrated that the quantities of COPS in spray-dried egg yolk powders increased proportionally to the NOx concentrations in the combustion gas. The patterns of cholesterol oxidation and carotenoid loss in the lipid model system were similar to those in egg powders processed by the direct gas-fired spray dryer. Therefore, this model system can be applied to future

studies to improve lipid stability in spray-dried foods (see section on future research).

In conclusion, the lipid model system developed in this study can be used effectively to describe the effects of NOx on the oxidation of carotenoids and cholesterol. Dietary supplementation of α-tocopherol greatly improved the stability of carotenoids as well as cholesterol in egg lipids subjected to NOx-initiated oxidation. The addition of carotenoids to egg lipids also protected cholesterol from NOx-initiated oxidation.

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SUMMARY AND CONCLUSIONS

A series of studies were conducted to investigate influence of NOx on the stability of lipids in spray-dried egg powders, with specific emphasis on cholesterol oxidation and carotenoid degradation. NOx, including NO and NO_2, are present in air as a result of combustion processes and are a possible cause for the high concentrations of COPS in eggs spray-dried with a direct gas-fired system.

An analytical procedure using SPE and capillary GC was developed for the rapid quantification of COPS in egg powders. Total lipid extracts were fractionated on disposable silica SPE tubes to isolate COPS from other lipids including cholesterol. The COPS were resolved as their TMS ether derivatives on a non-polar capillary column. Recoveries of COPS with this analytical technique were homogeneous and consistently high (~ 86%).

Factors influencing the formation of COPS in spray-dried egg powders, particularly the role of free radicals generated during combustion, were investigated. The concentrations of COPS in egg samples spray-dried with direct gas-fired heating were significantly ($p < 0.01$) greater than concentrations in samples dried with indirect heating. Addition of prooxidants (NOx and cumeme hydroperoxide) also greatly promoted (2 to 5-fold) cholesterol oxidation in the indirect heating system. The

pathway of cholesterol oxidation initiated by NOx appeared to be similar to the hydroperoxide-induced free radical chain reaction. This study confirmed that NOx are responsible for the rapid formation of COPS in egg powders produced by the direct gas-fired spray dryer.

Deposition of SP and OP in egg yolks as well as the dietary level for desired pigmentation were evaluated in a feeding trial. Supplementation of a low carotenoid diet (white wheat diet) with 4mg/kg OP or SP provided an egg yolk color equivalent to that of eggs in supermarkets. Results also showed that the color of egg yolks from hens fed similar concentrations of OP or SP were not significantly different ($p < 0.01$). Therefore, a level of 4mg/kg OP was selected to produce OP-supplemented eggs for studying the stability of cholesterol and carotenoids in eggs during processing and subsequent storage.

The fourth study was designed to investigate the influence of dietary α-tocopherol on the stability of cholesterol and carotenoids in eggs spray-dried by the direct gas-fired heating system. The carotenoids in eggs from hens fed a diet supplemented with OP alone oxidized rapidly when exposed to NOx in a direct gas-fired spray drying system. Dietary supplementation with 200mg/kg α-tocopherol acetate not only stabilized carotenoids but also protected cholesterol from oxidation in eggs during spray drying and storage.

Finally, a model system was designed to further investigate the mechanism of NOx-initiated lipid oxidation in eggs. The first order rate constants for cholesterol oxidation and carotenoid degradation in egg lipids during storage at 40°C were affected by egg treatments as well as the level of exposure to NOx before storage. Dietary supplementation with α-tocopherol improved the stability of cholesterol and carotenoids in egg lipids subjected to NOx-initiated oxidation. The addition of carotenoids to egg lipids also protected cholesterol from NOx-initiated oxidation. The patterns of cholesterol oxidation and carotenoid degradation in the lipid model system were similar to those in egg powders processed by the direct gas-fired spray dryer. Therefore, this model system can be applied to future studies to improve lipid stability in spray-dried foods.

FUTURE RESEARCH

The influence of NOx on the stability of lipids (cholesterol and paprika carotenoids) in spray-dried egg powders was studied. It was demonstrated that NOx formed during the combustion process are responsible for the initiation of cholesterol oxidation and carotenoid loss in eggs spray-dried by a direct gas-fired heating system. Supplementation of the diets of laying hens with α-tocopherol stabilized cholesterol as well as the carotenoids against oxidation during spray drying and storage. The addition of carotenoids (i.e., 30μg/g lipids) to eggs also protected cholesterol from NOx-initiated oxidation.

The antioxidant effect of carotenoids was not significant until the concentration of carotenoids in the eggs was 30μg/g lipids greater than the carotenoid content of the control eggs. This concentration can be achieved by supplementing the diets with at least 16mg/kg paprika carotenoids. A dietary supplementation of 4mg/kg paprika carotenoids produced egg yolk color equivalent to the color of eggs in supermarkets. The costs of adding α-tocopherol acetate (200mg/kg feed) and paprika carotenoids (4mg/kg feed) are $7.40 per ton of feed and $1.54 per ton of feed, respectively, based on current manufacturers' process. Dietary supplementation with 16mg/kg paprika carotenoids costs $6.16 per ton of feed. Although dietary

supplementation with 16mg/kg paprika carotenoids ($6.16/ton) seems more economical than the supplementation with 200mg/kg α-tocopherol acetate and 4mg/kg paprika carotenoids ($7.40 + $1.54 = $8.94/ton), the latter has superior antioxidant effects compared to the former.

In addition, the high concentration of paprika carotenoids gave the eggs a reddish color which may be not desired for every product. Therefore, more information on the dose-effect of α-tocopherol and carotenoids is needed for formulating the best combination of α-tocopherol acetate and carotenoids to achieved the best antioxidant and economic effects.

The lipid model system developed in this study can be used to study the optimal levels of antioxidants for desired lipid stability. Furthermore, possible interactions of cholesterol with carotenoids and other lipids in the NOx-initiated oxidation can be monitored in a slightly modified model system with cholesterol standards and individual carotenoids or other lipid components in a dispersion or coated on Celite.

Another area identified in this study which requires further investigation is the deposition of paprika carotenoids in eggs. Capsanthin, the predominant carotenoid in paprika and a red color contributorpa, was deposited with only a 17% efficiency in eggs, while the yellow pigments, zeaxanthin and lutein were deposited in eggs with efficiencies of 56% and 130%, respectively. These results

imply that transformation of carotenoids (e.g. lutein) *in vivo* during metabolism of carotenoids in hens or selective deposition of carotenoids in eggs may occur. Further research should be conducted to investigate the distribution of carotenoids by feeding the hens with individual radioactive-labeled carotenoids.

APPENDIX A

Resolution of TMS ethers of Cholesterol Oxidation Products in Egg Powders in GC analysis

	Cholesterol Oxidation Products	Peak Resolution[a] Standards	Eggs
1	cholesterol	2.0	2.2
2	7α-hydroxycholesterol (7α-OH)	12.5	12.3
3	cholesterol-5β,6β,epoxide (β-epoxide)	1.5	1.6
4	cholesterol-5α,6α,epoxide (α-epoxide)	1.7	1.8
5	7β-hydroxycholesterol (7β-OH)	5.8	15.1
6	20α-hydroxycholesterol (20α-OH)	1.5	---[b]
7	25-hydroxycholesterol (25-OH)	5.2	---
8	6-ketocholesterol (6-keto)	1.5	1.7
9	7-ketocholesterol (7-keto)	2.1	---
10	cholestan-3β,5α,6β-triol (triol)		

[a] Resolution for the peak and the one immediately followed.
[b] 20α-hydroxycholesterol, 25-hydroxycholesterol and cholestan-3β,5α,6β-triol were not found in egg powders.

APPENDIX B

Effects of Dietary Treatments on Pigmentation of Egg Yolks

DAY 0

A. control
B. OP Low
C. OP Med.
D. OP High

E. SP 1/2 Low
F. SP Low
G. SP Med.
H. SP High

DAY 4

A. control
B. OP Low
C. OP Med.
D. OP High

E. SP 1/2 Low
F. SP Low
G. SP Med.
H. SP High

DAY 8

A. control
B. OP Low
C. OP Med.
D. OP High

E. SP 1/2 Low
F. SP Low
G. SP Med.
H. SP High

DAY 12

A. control
B. OP Low
C. OP Med.
D. OP High

E. SP 1/2 Low
F. SP Low
G. SP Med.
H. SP High

APPENDIX C

Influence of Saponification on Deposition of Paprika Carotenoids in Egg Yolk

OP Low

Day 0 4 8 12 20

SP Low

Day 0 4 8 12 20

OP Med.

Day 0 4 8 12 20

SP Med.

Day 0 4 8 12 20

OP High

Day 0 4 8 12 20

SP High

Day 0 4 8 12 20

APPENDIX D

Calculation of Rate Constants for Chapter Five

First order reaction for loss of total carotenoids (TC)
$-d[TC]/dt = k[TC]$
$d[TC]/dt = dx/dt = k(a-x)$
$dx/(a-x) = kdt$
Integrate both sides (from 0 to x)
$Ln(a/(a-x)) = kt$

a= 100%
x= %TC loss

0 ppm NOx

sample	time days	x % TC	Ln(a/(a-x))		k, day^-1 at 40C
A. cont1	0	0.00	0.0000		
	7	5.45	0.0560	r=	0.932
	14	24.86	0.2858	k=	0.017934 day^-1
B. OP	0	0.00	0.0000		
	7	14.81	0.1603	r=	0.966
	14	20.16	0.2251	k=	0.017445 day^-1
C. OP	0	0.00	0.0000		
+Vit.E	7	2.38	0.0241	r=	0.950
	14	9.62	0.1011	k=	0.006335 day^-1
D. OP	0	0.00	0.0000		
added	7	6.67	0.0690	r=	0.998
	14	14.17	0.1528	k=	0.01075 day^-1

10 ppm NOx

sample	time days	x % TC	Ln(a/(a-x))	k, day^-1 at 40C
A. cont1	0	2.50	0.0253	
	7	14.98	0.1623	r= 0.993
	14	28.71	0.3384	k= 0.023975 day^-1
B. OP	0	0.93	0.0093	
	7	18.51	0.2047	r= 0.947
	14	23.81	0.2719	k= 0.021388 day^-1
C. OP +Vit.E	0	0.96	0.0096	
	7	2.38	0.0241	r= 0.906
	14	13.87	0.1493	k= 0.009094 day^-1
D. OP added	0	0.00	0.0000	
	7	9.17	0.0962	r= 1.000
	14	18.11	0.1998	k= 0.014193 day^-1

20 ppm NOx

sample	time days	x % TC	Ln(a/(a-x))	k, day^-1 at 40C
A. cont1	0	3.75	0.0382	
	7	12.50	0.1335	r= 0.960
	14	34.59	0.4245	k= 0.028071 day^-1
B. OP	0	2.78	0.0282	
	7	25.93	0.3002	r= 0.829
	14	27.35	0.3195	k= 0.026834 day^-1
C. OP +Vit.E	0	0.93	0.0093	
	7	7.14	0.0741	r= 0.988
	14	17.04	0.1868	k= 0.012729 day^-1
D. OP added	0	0.55	0.0055	
	7	11.67	0.1241	r= 0.997
	14	24.40	0.2797	k= 0.01959 day^-1

40 ppm NOx

sample	time days	x % TC	Ln(a/(a-x))		k, day^-1 at 40C
A. cntl	0	5.00	0.0513		
	7	22.50	0.2549	r=	0.985
	14	45.80	0.6125	k=	0.042282 day^-1
B. OP	0	4.63	0.0474		
	7	18.52	0.2048	r=	0.984
	14	36.67	0.4568	k=	0.031955 day^-1
C. OP	3	2.81	0.0285		
+Vit.E	7	11.90	0.1267	r=	0.983
	14	19.17	0.2128	k=	0.01555 day^-1
D. OP	0	1.16	0.0117		
added	7	15.67	0.1704	r=	0.999
	14	29.14	0.3445	k=	0.024529 day^-1

First order reaction for cholesterol oxidation

$-d[chol]/dt = d[cop]/dt = k[chol]$

$d[cop]/dt = dx/dt = k(a-x)$

$dx/(a-x) = kdt$

Integrate both sides (from 0 to x)

$Ln(a/(a-x)) = kt$

a= conc. of cholesterol = 30650 ug/g fat

0 ppm NOx

sample	time days	COP (x) ug/g fat	Ln(a/(a-x)) *10000		k, day^-1 at 40C
A. cntl	0	12.46	4.0661		
	7	35.24	11.5042	r=	0.992
	14	49.66	16.2154	k=	8.68E-05 day^-1
B. OP	0	14.01	4.5720		
	7	37.63	12.2849	r=	0.988
	14	51.33	16.7612	k=	8.71E-05 day^-1
C. OP	0	10.22	3.3350		
+Vit.E	7	21.11	6.8898	r=	0.992
	14	38.38	12.5299	k=	6.57E-05 day^-1
D. OP	0	11.41	3.7234		
added	7	33.98	11.0926	r=	0.991
	14	48.00	15.6730	k=	8.54E-05 day^-1

10 ppm NOx

sample	time days	COP (x) ug/g fat	Ln(a/(a-x)) *10000	k, day^-1 at 40C
A. cont1	0	20.12	6.5666	
	7	45.77	14.9443	r= 0.999
	14	68.20	22.2760	k= 1.12E-04 day^-1
B. OP	0	24.22	7.9052	
	7	49.73	16.2383	r= 0.999
	14	71.19	23.2538	k= 1.10E-04 day^-1
C. OP +Vit.E	0	13.19	4.3044	
	7	30.93	10.0964	r= 0.993
	14	42.68	13.9347	k= 6.88E-05 day^-1
D. OP added	0	18.39	6.0018	
	7	40.17	13.1146	r= 0.996
	14	56.29	18.3823	k= 8.84E-05 day^-1

20 ppm NOx

sample	time days	COP (x) ug/g fat	Ln(a/(a-x)) *10000	k, day^-1 at 40C
A. cont1	0	29.46	9.6164	
	7	66.26	21.6417	r= 0.991
	14	89.11	29.1158	k= 1.39E-04 day^-1
B. OP	0	31.28	10.2108	
	7	71.32	23.2963	r= 0.980
	14	90.41	29.5411	k= 1.38E-04 day^-1
C. OP +Vit.E	0	19.94	6.5078	
	7	37.38	12.2032	r= 0.996
	14	50.12	16.3658	k= 7.04E-05 day^-1
D. OP added	0	25.39	8.2873	
	7	50.28	16.4180	r= 0.996
	14	68.41	22.3447	k= 1.00E-04 day^-1

40 ppm NOx

sample	time days	COP (x) ug/g fat	Ln(a/(a-x)) *10000	k, day^-1 at 40C
A. contl	0	41.55	13.5655	
	7	86.94	28.4057	r= 0.991
	14	115.30	37.6892	k= 1.72E-04 day^-1
B. OP	0	44.28	14.4574	
	7	90.01	29.4103	r= 0.993
	14	120.12	39.2679	k= 1.77E-04 day^-1
C. OP +Vit.E	0	25.02	8.1665	
	7	42.99	14.0359	r= 0.996
	14	67.09	21.9131	k= 9.82E-05 day^-1
D. OP added	0	30.34	9.9038	
	7	63.22	20.6477	r= 0.988
	14	82.11	26.8255	k= 1.21E-04 day^-1