Table II. Recovery Test of Triforine and TF/2•HCl Metabolite from Peach, Strawberry, and Green Pepper[a]

	% recovery					
	triforine			TF/2·HCl		
sample	1	2	av	1	2	av
peach	98.5	102	100	66.0	69.8	67.9
strawberry	98.0	101	99.5	71.5	76.0	73.8
green pepper	97.0	97.3	97.2	68.9	70.0	69.4
average			98.9			70.4

[a] Fortification levels at 0.1 ppm for triforine and 0.4 ppm for TF/2·HCl.

Effects of reaction time and reaction temperature on yield of the product formed by the reaction of triforine with methanol were examined, and the results are shown in Figures 4 and 5, respectively. Ethyl acetate and pyridine were found to be effective for making the reaction time short as required for constant yield.

As can be seen in Figure 5, the higher the reaction temperature, the shorter the reaction time required for constant yield. However, the use of a reaction temperature above 180 °C is in danger of detonation of the closed glass tube. For these reasons, the reactive conditions with methanol were established as shown in procedure 1.

(3) Sensitivity, Reproducibility, and Linearity. The reproducibility of the thermal reaction for six replicate analyses of methanol sample solutions containing only standard triforine or TF/2·HCl was quite good. The relative standard deviation values were 2.2% (n = 6) for triforine and 2.3% (n = 6) for TF/2·HCl, respectively. Stability of the product (in acetone) at room temperature was evaluated by injection of the product to the GC again 1 month after the reaction. Relative sensitivity to an internal standard (a pesticide) was the same between the measurements just after the reaction and 1 month after the reaction. No new peaks other than that of the analyte was observed. The absolute detection limit (peak height equal to 3 times the noise) is 2 pg for triforine and 4 pg for TF/2·HCl, respectively. Both calibration plots for triforine and TF/2·HCl were linear from 0.1 to 0.5 ng.

(4) Application of Thermal Reaction to Residue Analysis. We have applied the thermal reaction to the residue analysis of triforine and TF/2 metabolite in the three crops, and the accuracy was evaluated by recovery experiment (Table II), in which known amounts of triforine or TF/2·HCl were added to peach, strawberry, and green pepper.

In triforine analyses, good recoveries were obtained in all samples. But only about 70% of TF/2·HCl was recovered. This low recovery of TF/2·HCl was probably due to incomplete recovery of the compound by the extraction with ethyl acetate in liquid–liquid partition, since the recovery of TF/2·HCl added to the ethyl acetate extract after the partition was complete. GC chromatograms on the analyses of spiked and nonspiked crops with triforine are shown in Figure 6.

LITERATURE CITED

Bourke, J. B.; Nelsen, T. R.; Eichler, D. Residues and Disappearance of Triforine from Various Crops. *J. Agric. Food Chem.* **1977**, *25*, 36–39.

Eichler, D. *Meded. Fak. Landbouwwet. Rijksuniv. Gent.* **1972**, *37*, 831.

Ishii, M., private communication, 1980.

Nagayoshi, E.; Osami, M.; Goto, S. Abstracts of Papers, 5th Meeting of Nouyaku Zanryu Bunseki Kenkyukai, 1981; p 43.

Newsome, W. H. Determination of Triforine in Fruit Crops as *N,N'*-Bis(pentafluorobenzoyl)piperazine. *J. Agric. Food Chem.* **1982**, *30*, 778–779.

Rouchaud, J. P. Analysis of Piperazine, of the Piperazine Generating Fungicide Triforine, and of Triforine's Metabolites in Barley Plants. *Bull. Environ. Contam. Toxicol.* **1977**, *18*, 184–189.

Rouchaud, J. P.; Decallonne, J. R.; Moyer, J. A. The Nature of Bound Residues Derived from Triforine in Barley Plants. *Pestic. Sci.* **1978**, *9*, 74–78.

Schicke, P.; Veen, K. H. *Proc. Br. Insect. Fungic. Conf.* **1969**, *5*, 569.

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Inhibition of Hepatic Mevalonate Biosynthesis by the Monoterpene, *d*-Limonene

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The adaptive increase in avian hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity following fasting and refeeding was muted when the monoterpene *d*-limonene was fed. The suppression of the induction of HMG-CoA reductase activity was dose dependent to 100 ppm dietary *d*-limonene and additive to that of dietary cholesterol. Noninduced hepatic HMG-CoA reductase activity in rats fed a diet containing 1.0% *d*-limonene was 55% of the activity in rats fed a control diet.

Brown and Goldstein (1980) described the multivalent feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in which a non-sterol, post-mevalonate product(s) regulates HMG-CoA reductase independently of, and in addition to cholesterol. Among the prospective exogenous, non-sterol modulators are *d*-limonene, the initial cyclic monoterpenoid product of the mevalonate pathway of secondary plant metabolism, and oxy- and hydroxy-substituted mono- and bicyclic monoterpenes that elicited a transient decrease in rat hepatic HMG-CoA reductase mass and activity when given by gavage (Clegg et al., 1980, 1982). *d*-Limonene (Elegbede et al., 1984, 1986) and other monoterpenes (Wattenberg, 1983; Maltzman et al., 1985) act as anticarcinogens when added to diets of rats treated with a chemical carcinogen,

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Table I. Effect of Short-Term Dietary Intake of *d*-Limonene on Lipid Metabolism of Fasted-Refed Pullets

	fed[a]		fasted-refed[c]	
parameter	control[b]	*d*-limonene	control	*d*-limonene
HMG-CoA reductase[d]	54 ± 3[g a]	50 ± 3[a]	323 ± 14[c]	198 ± 15[b]
serum cholesterol, mg/100 mL	137 ± 7[b]	133 ± 6[b]	120 ± 16[ab]	107 ± 8[a]
fatty acid synthetase[e]	83 ± 2[a]	79 ± 3[a]	190 ± 14[b]	197 ± 13[b]
malic enzyme[f]	247 ± 16[a]	242 ± 14[a]	329 ± 64[b]	387 ± 56[b]
serum triglyceride, mg/100 mL	119 ± 3[a]	148 ± 4[b]	110 ± 13[a]	203 ± 26[c]

[a] $n = 7$. The experimental diet, fed for 3 days, contained 20 ppm *d*-limonene. [b] The control diet consisted of ground corn (78.4%), 44% soybean meal (16.0%), 17% alfalfa meal (2.0%), meat and bone meal (1.1%), dicalcium phosphate (1.0%), calcium carbonate (0.5%), and vitamin and mineral mixture (0.5%). Grit (5%) was incorporated at the expense of feed. The vitamin and mineral mixture provides the following per kilogram feed: vitamin A, 3000 IU; vitamin D_3, 500 ICU; vitamin B_{12}, 0.005 mg; riboflavin, 3 mg; MnO_2, 50 mg; $ZnSO_4$, 110 mg. The 14-week-old pullets were caged by group with 24-h artificial light and free access to water. [c] $n = 8$. The pullets were fasted for 48 h and refed for 72 h. The experimental diet contained 20 ppm *d*-limonene. [d] Picomoles of mevalonic acid synthesized per minute per milligram of microsomal protein. [e] Nanomoles of NADPH oxidized per minute per milligram of cytosolic protein. [f] Nanomoles of $NADP^+$ reduced per minute per milligram of cytosolic protein. [g] Mean ± SD. (a–c) Means within a line not sharing a common superscript are different at $p < 0.05$.

7,12-dimethylbenz[*a*]anthracene. Analogous to the carcinogenic action of 7,12-dimethylbenz[*a*]anthracene is its atherogenic action, an action expressed in the absence of a promoter (e.g., phorbol esters) (Bond et al., 1981) and of an elevated cholesterol level (Albert et al., 1977; Revis et al., 1984). Atherosclerosis may have roots in a neoplastic process (Benditt and Benditt, 1973; Ross and Glomsett, 1973). We propose that isoprenoid constituents of plant products suppress cholesterogenic, atherogenic, and neoplastic processes (Qureshi et al., 1985). As background for our ongoing effort to delineate these actions of dietary *d*-limonene and of orange peel oil (Wattenberg, 1983), the commerical source of *d*-limonene (Shaw, 1977), we have conducted studies to determine under what feeding regimens they will influence heptatic HMG–CoA reductase activity.

MATERIALS AND METHODS

d-Limonene (99%) was purchased from the Aldrich Chemical Co., Milwaukee, WI. Orange peel oil was a gift of Golden Gem Grower's Inc., Umatilla, FL. Single-comb White Leghorn (WLH) chicks were purchased from a local hatchery and Sprague–Dawley rats from Harlan Sprague–Dawley, Madison, WI. Components of the chick diet were provided by the University of Wisconsin Poultry Research Laboratory. The sources of the remaining chemical substrates, labeled substrate enzymes, and diagnostic kits were those identified by Qureshi et al. (1983). All chemicals used in the studies were of analytical grade.

Table II. Effect of Long-Term Dietary Intake of *d*-Limonene on Hepatic Lipid Metabolism in Fasted-Refed Cockerels

diet[a]				
limonene, ppm	cholesterol, %	HMG-CoA reductase[b]	fatty acid synthetase[c]	serum cholesterol, mg/100 mL
0	0	173 ± 14[d a]	81 ± 20[a]	102 ± 7[a]
25	0	112 ± 15[bc]	99 ± 12[a]	93 ± 4[ab]
50	0	129 ± 13[bd]	94 ± 20[a]	88 ± 6[ab]
100	0	94 ± 12[c]	99 ± 10[a]	83 ± 6[b]
200	0	105 ± 14[c]	115 ± 14[a]	89 ± 4[a]
0	1	143 ± 13[d]	62 ± 11[a]	143 ± 7[c]
200	1	76 ± 12[c]	82 ± 15[a]	123 ± 9[d]

[a] The diet (Table I) enriched with *d*-limonene and cholesterol was fed ad libitum to 9-week-old cockerels ($n = 8$) housed in floor pens with 24-h artificial light and free access to water for 26 days. The cockerels were fasted for 1 day and refed for 1 day prior to slaughter. [b] Picomoles of mevalonic acid synthesized per minute per milligram of microsomal protein. [c] Nanomoles of NADPH oxidized per minute per milligram of cytosolic protein. [d] Mean ± SD. (a–d) Means within a column not sharing a common superscript are different at $p < 0.05$.

Experimental Procedures. The effects of short- and long-term intake of *d*-limonene and orange peel oil, of fasting and refeeding, of sex, and of dietary cholesterol and their interactions on mevalonate and fatty acid biosyntheses were examined in WLH chicks. The long-term effect of dietary *d*-limonene on mevalonate biosynthesis was examined also in fed Sprague–Dawley female rats. Experimental details are described on Tables I–IV.

Preparation of Liver Homogenates. Liver homogenates were prepared in 0.1 M potassium phosphate buffer, pH 7.4, containing 4 mM $MgCl_2$, 1 mM EDTA, and 2 mM dithiothreitol. The tissue was chopped and suspended in the buffer (1:3 w/vl) and homogenized at 0–4 °C with a Polytron homogenizer for 15 s at setting 8. The homogenate was centrifuged for 10 min at 5000*g*. The supernatant was passed through cheesecloth and the volume of the supernatant recorded. A 10-mL aliquot of the supernatant was then centrifuged for 10 and 60 min at 20000*g* and 100000*g*, respectively. The supernatant after each centrifugation was passed through cheesecloth. The 100000*g* supernatant (cytosol) and precipitate (microsomes) were treated as described by Qureshi et al. (1983)

Table III. Effects of Short- and Long-Term Dietary Intakes of Orange Peel Oil and Cholesterol on Hepatic HMG-CoA Reductase Activity and Serum Cholesterol of Fasted-Refed Cockerels

dietary regimen[a]						
16-day feed			3-day refeeding			
orange oil, 100 ppm	cholesterol, 0.5%	2-day fast	orange oil, 100 ppm	cholesterol, 0.5%	HMG-CoA reductase[b]	serum cholesterol, mg/100 mL
–	–	–	–	–	373 ± 17[c a]	165 ± 15[a]
–	–	–	–	+	355 ± 15[b]	190 ± 19[c]
–	+	–	–	+	349 ± 14[b]	221 ± 17[d]
–	–	–	+	–	327 ± 19[c]	141 ± 9[b]
–	–	–	+	+	300 ± 11[e]	152 ± 10[a]
+	–	–	+	–	321 ± 18[c,d]	157 ± 9[a]
+	+	–	+	+	303 ± 16[d,e]	184 ± 14[c]

[a] The control diet consisted of ground corn (75.5%), 44% soybean meal (19.0%), 17% alfalfa meal (2.0%), meat and bone meal (1.9%), dicalcium phosphate (1.0%), calcium carbonate (0.5%), iodized salt (0.5%), and vitamin and mineral mixture (0.5%). The vitamin and mineral mixture provides the following per kilogram of feed: vitamin A, 3000 IU; vitamin D_3, 400 ICU; vitamin B_{12}, 0.01 mg; riboflavin, 2.0 mg; vitamin E, 5.0 IU; calcium pantothenate, 5 mg; pyridoxine, 3 mg; MnO_2, 40 mg; $ZnCO_3$, 50 mg. Groups of eight 1-day-old cockerels, housed in brooders with 24-h artificial light and free access to water, were fed as indicated on the table. [b] Picomoles of mevalonic acid synthesized per minute per milligram of microsomal protein. [c] Mean ± SD. (a–e) Means within a column not sharing a common superscript are different at $p < 0.05$.

Table IV. Effect of Long-Term Dietary Intake of *d*-Limonene on Hepatic HMG-CoA Reductase Activity and Serum Lipid of Fed Rats[a]

	control	1% *d*-limonene
HMG-CoA reductase[b]	159 ± 63[d a]	88 ± 24[b]
LDL-cholesterol[c]	30 ± 6[a]	21 ± 5[b]
HDL-cholesterol[c]	80 ± 8[a]	79 ± 10[a]
triglycerides[c]	65 ± 8[a]	80 ± 25[a]

[a] Two groups (n = 4) of 12-week-old Sprague–Dawley rats were fed powdered Wayne Laboratory Animal Diet with or without 1% *d*-limonene for 3 months. The rats, housed individually in suspended cages with a 12-h photoperiod, had free access to diet and water. The rats were killed 4 h into the light period. [b] Picomoles of mevalonic acid synthesized per minute per milligram of microsomal protein. [c] Milligrams/100 mL of serum. [d] Mean ± SD. (a, b) Means on lines not sharing a common superscript are different at $p < 0.05$.

and stored at −20 °C until they were assayed for enzymatic activities.

Hepatic Enzyme Activities. The assays for microsomal HMG-CoA reductase (Shapiro et al., 1974), malic enzyme (Hsu and Lardy, 1963), and fatty acid synthetase (Muesing and Porter, 1975) have been described. Protein concentrations were estimated by a modification of the Biuret method using bovine serum albumin as a standard (Gornall et al., 1949).

Serum Cholesterol and Triglyceride Concentrations. Cholesterol and triglyceride concentrations in serum samples were estimated with use of Cholesterol Reagent and Triglyceride Reagent kits, obtained from Worthington Diagnostics Division of Millipore Corp., Freehold, NJ.

RESULTS

Fasting and refeeding under the conditions listed on Table I resulted in significant increases in avian hepatic HMG-CoA reductase (6-fold), fatty acid synthetase (2.3-fold), and malic enzyme (1.3-fold) activities. The effects of dietary *d*-limonene (20 ppm) in these 3-day studies of 14-week-old WLH pullets were 2-fold; namely the monoterpene attenuated the effect of fasting/refeeding on HMG-CoA reductase activity (a 4-fold rather than 6-fold increase) and elevated the serum triglycerides of both fed and fasted/refed birds.

The second protocol (Table II) differs from the first in several respects. The hepatic HMG-CoA reductase and fatty acid synthetase activities and serum cholesterol level of 13-week old cockerels fed experimental diets for 26 days and then fasted and refed for 1-day periods were lower than those present in the pullets under the conditions described in Table I. There is a general dose–response pattern that suggests that increasing dietary *d*-limonene to 100 ppm elicits a maximum suppression of reductase activity ($p < 0.05$) and lowering of serum cholesterol ($p < 0.05$). The effect of dietary *d*-limonene on fatty acid synthetase activity was not significant. This study also examined the effect of dietary cholesterol on HMG-CoA reductase activity. Under the conditions employed, the sterol elicited a 40% increase in serum cholesterol ($p < 0.05$) and a 17% decrease in hepatic HMG-CoA reductase activity ($p < 0.05$); the effects of 1% cholesterol and 200 ppm *d*-limonene were additive in producing a 66% decrease in reductase activity ($p < 0.05$); *d*-limonene attenuated the effect of dietary cholesterol on serum cholesterol.

The differences in the control values presented on Tables I and II could be attributed to the sex of the birds, the length of the feeding trial, or the length of the refeeding regimen. Cockerels, 21 days of age at slaughter, had induced hepatic HMG-CoA reductase activities (Table III) similar to those reported on Table I. The 2/3-day regimen appears to be more effective than the 1/1-day regimen for the induction of HMG-CoA reductase activity. The higher serum cholesterol level of control birds (Table III) compared to values in Tables I and II may reflect a carryover effect from the cholesterol present in the egg yolk. Both the short- and long-term dietary effects of orange peel oil (95% *d*-limonene; Shaw, 1977) and cholesterol on reductase activity and serum cholesterol were examined. Under the condition where orange peel oil (100 ppm) and/or cholesterol (0.5%) was fed only during the refeeding phase, orange peel oil elicited a 12% decrease in reductase activity, cholesterol a 5% decrease; and in combination the dietary treatment reduced the activity by 20%. Orange peel oil blocked ($p < 0.05$) the dietary cholesterol initiated increase ($p < 0.05$) in serum cholesterol. Similar conclusions can be drawn from the results of the studies in which orange peel oil and/or cholesterol were fed both prior to and following the fasting period.

In the final study, we examined the effects of 1% dietary *d*-limonene on rat hepatic HMG-CoA reductase activity and on serum lipids using the conditions under which Elegbede et al. (1984) demonstrated the anticarcinogenic action of the monoterpene. Under noninduced conditions, 1% dietary *d*-limonene elicited a 45% decrease in hepatic HMG-CoA reductase activity. Although the dietary treatment failed to effect a lowering of serum cholesterol, it was effective in lowering the LDL cholesterol level. The impact of *d*-limonene on serum triglyceride of the rat was not significant (Table IV).

DISCUSSION

Short-term and long-term exposure to dietary *d*-limonene (0–100 ppm) or orange peel oil (100 ppm), the commercial source of *d*-limonene, muted the induction of hepatic HMG-CoA reductase activity during the refeeding phase of a fasting–refeeding regimen. Under the condition of maximum *d*-limonene-mediated muting of the induction of HMG-CoA reductase activity (100 ppm dietary *d*-limonene), the HMG-CoA reductase suppressive effect of dietary cholesterol was additive. This observation was confirmed with long-term and short-term exposure to orange peel oil and cholesterol.

Hepatic HMG-CoA reductase activity was significantly lowered when rats were fed a 1% *d*-limonene diet for 3 months. This diet provided the rats with a daily intake of approximately 150 mg of *d*-limonene (3–4 mmol of *d*-limonene/kg of body weight).

Although long-term exposure to dietary *d*-limonene lowered HMG-CoA reductase activity in the livers of rats and chickens, only the latter responded with a lowering of serum total cholesterol level. This unique resistance of the rat to the hypocholesterolemic action of monoterpenes (Imaizumi et al., 1985), compactin (Endo et al., 1979), and mevinolin (Maltese et al., 1985), all of which lower HMG-CoA reductase activity, has been reported. Imaizumi et al. (1985) found that after 14 days body weight gain and serum cholesterol and apo A-I levels of rats fed 1% *d*-limonene did not differ from control values, whereas serum triglycerides were variably elevated. Our results [Table IV and Elegbede et al. (1984)] confirm the prior work and extend it with the observations that the protocol also lowers both hepatic HMG-CoA reductase activity and serum LDL cholesterol.

Branched pathways leading to several physiologically relevant compounds originate from the synthesis of mevalonate. Suppression of growth following the addition of competitive inhibitors of HMG-CoA reductase (Alberts et al., 1980; Endo et al., 1979) to cultures of plant (Bach and Lichtenthaler, 1983; Ceccarelli and Lorenzi, 1984) and

animal (Fairbanks et al., 1986; Cohen et al., 1982; Brown et al., 1983) cells is reversed in each by the addition of mevalonate but not by the addition of other end products of the various pathways (Sinensky and Logel, 1985; Fairbanks et al., 1986). Brown et al. (1983), expanding on the multivalent feedback regulation concept of Brown and Goldstein (1980), point out that since all cells have a requirement for one or more of the isoprenoids, it is possible that two levels of regulation exist for HMG-CoA reductase, a basic regulatory scheme that reflects a need for isoprenoid intermediates/products (non-sterol products) and a second scheme unique for sterol synthesis. Both regulatory schemes are operative in sterologenic cells whereas only the former may be operative in sterol auxotrophs (Silberkange et al., 1983). Our results, namely that dietary cholesterol and *d*-limonene are additive in suppressing hepatic HMG-CoA reductase activity in fasted–refed birds, are consistent with the regulatory scheme proposed for sterologenic cells. *d*-Limonene, the first cyclic end product of mevalonate metabolism and oxygen-substituted acylic, monocyclic, and bicyclic monoterpenes adminstered as dietary constituents (Mangels et al., 1985) or by gavage (3 mmol/kg of body weight; Clegg et al., 1980), inhibits mevalonate biosynthesis. Whether these monoterpenes have a common action or a common metabolite that exerts the action on HMG-CoA reductase remains to be determined. The latter prospect is of interest since *d*-limonene has been associated with a diverse spectrum of biologically harmful activities, some of which are sex specific, others organ specific, and yet other species/strain specific (Evans et al., 1987; Kanerva et al., 1987; Kanerva and Alden, 1987; Roe and Pierce, 1960). On the other hand, the GRAS status of *d*-limonene suggests that for the human it is nontoxic.

Given the requirement for mevalonate end products in cell differentiation and division, and the elevated HMG-CoA reductase activity resistant to cholesterol feedback regulation that is characteristic of neoplastic tissues (Siperstein and Fagan, 1964), it is possible that both the anticarcinogenic action of dietary *d*-limonene as well as its cholesterol-suppressive action are expressed at the level of HMG-CoA reductase.

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LITERATURE CITED

Albert, R. E.; Vanderlaan, M.; Burns, F. J.; Nishizumi, M. "Effect of Carcinogens on Chicken Atherosclerosis". *Cancer Res.* **1977**, *37*, 2232–2235.

Alberts, A. W.; Chen, J.; Kuron, G.; Hunt, V.; Huff, J.; Hoffman, C.; Rothrock, J.; Lopez, M.; Joshua, H.; Harris, E.; Patchett, A.; Monagon, R.; Currie, S.; Stapley, E.; Albers-Shonberg, G.; Hensens, O.; Hirshfield, J.; Hoogsteen, K.; Liesch, J.; Springer, J. "Mevinolin: A Highly Potent Competitive Inhibitor of Hydroxy-methylglutaryl-coenzyme A Reductase and a Cholesterol-Lowering Agent". *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3957–3961.

Bach, T. J.; Lichtenthaler, H. K. "Inhibition by Mevinolin of Plant Growth, Sterol Formation and Pigment Accumulation". *Physiol. Plant* **1983**, *59*, 50–60.

Benditt, E. P.; Benditt, J. M. "Evidence for a Monoclonal Origin of Human Atherosclerotic Plaques". *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 1753–1756.

Bond, J. A.; Gown, A. M.; Yang, H. L.; Benditt E. P.; Juchau, M. R. "Further Investigation of the Capacity of Polynuclear Aromatic Hydrocarbons to Elicit Atherosclerotic Lesions". *J. Toxicol. Environ. Health* **1981**, *7*, 327–335.

Brown, M. S.; Goldstein, J. L. "Multivalent Feedback Regulation of HMG-CoA Reductase, a Control Mechanism Coordinating Isoprenoid Synthesis and Cell Growth". *J. Lipid Res.* **1980**, *21*, 505–517.

Brown, K.; Havel, C. M.; Watson J. A. "Isoprene Synthesis in Isolated Embryonic *Drosophila* Cells. II. Regulation of 3-Hydroxy-3-methylglutaryl Coenzyme-A Reductase Activity". *J. Biol. Chem.* **1983**, *258*, 8512–8518.

Ceccarelli, N.; Lorenzi, R. "Growth Inhibition by Competitive Inhibitors of 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase in *Helianthus tuberosus* Tissue". *Plant Sci. Lett.* **1984**, *34*, 269–276.

Clegg, R. J.; Middleton, B.; Bell, G. D.; White D. A. "Inhibition of Hepatic Cholesterol Synthesis and 3-Hydroxy-3-methylglutaryl-CoA Reductase by Mono and Bicyclic Monoterpenes Administered in Vivo". *Biochem. Pharmacol.* **1980**, *29*, 2125–2127.

Clegg, R. J.; Middleton, B.; Bell, G. D.; White, D. A. "The Mechanism of Cyclic Monoterpene Inhibition of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase in Vivo in the Rat". *J. Biol. Chem.* **1982**, *257*, 2294–2299.

Cohen, D. C.; Massoglia, S. L.; Gospodarowicz, D. "Feedback Regulation of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase in Vascular Endothelial Cells. Separate Sterol and Non-sterol Components". *J. Biol. Chem.* **1982**, *257*, 11106–11112.

Elegbede, J. A.; Elson, C. E.; Qureshi, A. A.; Tanner, M. A.; Gould M. "Inhibition of DMBA-Induced Mammary Cancer by the Monoterpene d-Limonene". *Carcinogenesis* **1984**, *5*, 661–663.

Elegbede, J. A.; Elson, C. E.; Tanner, M. A.; Qureshi, A. A.; Gould, M. N. "Regression of Rat Primary Mammary Tumors Following Dietary d-Limonene". *JNCI, J. Natl. Cancer Inst.* **1986**, *76*, 323–325.

Endo, A.; Tsujita, Y.; Kuroda, M.; Tanzawa, K. "Effects of ML-236B on Cholesterol Metabolism in Mice and Rats: Lack of Hypocholesterolemic Activity in Normal Animals". *Biochim. Biophys. Acta* **1979**, *575*, 266–276.

Evans, D. C.; Miller, D. M.; Jacobsen, K. L.; Bush, P. B. "Modulation of Immune Responses in Mice by d-Limonene". *J. Toxicol. Environ. Health* **1987**, *20*, 51–66.

Fairbanks, K. P.; Barbu, V. D.; Witte, L. D.; Weinstein, I. B.; Goodman, D. S. "Effects of Mevinolin and Mevalonate on Cell Growth in Several Transformed Cell Lines". *J. Cell. Physiol.* **1986**, *127*, 216–222.

Gornall, A. C.; Bardawill, C. J.; David, M. M. J. "Determination of Serum Proteins by Means of the Biuret Reaction". *J. Biol. Chem.* **1949**, *177*, 751–766.

Hsu, R. H.; Lardy, H. A. "Pigeon Liver Malic Enzyme Isolation, Crystallization and Some Properties". *J. Biol. Chem.* **1963**, *245*, 520–526.

Imaizumi, K.; Hanada, K.; Mawatari, K.; Sugano, M. "Effect of Essential Oils on the Concentration of Serum Lipids and Apolipoproteins in Rats". Agric. *Biol. Chem.* **1985**, *49*, 2795–2796.

Kanerva, R. L.; Alden, C. L. "Review of Kidney Sections from a Subchronic d-Limonene Oral Dosing Study Conducted by the National Cancer Institute". *Food Chem. Toxicol.* **1987**, *25*, 355–358.

Kanerva, R. L.; Ridder, G. M.; Lefever, F. R.; Alden, C. L. "Comparison of Short-Term Renal Effects due to Oral Administration of Decalin or d-Limonene in Young Adult Male Fischer-344 Rats". *Food Chem. Toxicol.* **1987**, *25*, 345–353.

Maltese, W. A.; Defendini, R.; Green, R. A.; Sheridan, K. M.; Donley, D. K. "Suppression of Murine Neuroblastoma Growth in Vivo by Mevinolin, a Competitive Inhibitor of 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase". *J. Clin. Invest.* **1985**, *76*, 1748–1754.

Maltzman, T. H.; Tanner, M. A.; Elson, C. E.; Gould, M. N. "Anticarcinogenic Activity of Specific Orange Peel Oil Monoterpene". *Fed. Proc.* **1985**, *45*, 970.

Mangels, A. R.; Altmann, W. A.; Din, Z. Z.; Elson, C. E. "Suppression of Mevalonate Biosynthesis by Dietary Acyclic, Monocyclic and Bicyclic Monoterpenes". *Fed. Proc.* **1985**, *45*, 612.

Muesing, R. A.; Porter, J. W. "Fatty Acid Synthetase from Pigeon Liver". *Methods Enzymol.* **1975**, *35*, 45–59.

Qureshi, A. A.; Abuirmeileh, N.; Burger, W. C.; Din, Z. Z.; Elson, C. E. "Effect of AMO 1618 on Cholesterol and Fatty Acid Metabolism in Chickens and Rats". *Atherosclerosis* **1983**, *46*, 203–216.

Qureshi, A. A.; Burger, W. C.; Peterson, D. M.; Elson, C. "Suppression of Cholesterogenesis by Plant Constitutents. Review of Wisconsin Contributions to NC-167". *Lipids* **1985**, *20*, 817–824.

Revis, N. W.; Bull, R.; Laurie, D.; Schiller, C. A. "The Effectiveness of Chemcial Carcinogen To Induce Atherosclerosis in the White Carneau Pigeon". *Toxicology* **1984**, *32*, 215–227.

Roe, F. J.; Pierce, W. E. "Tumor Promotion by Citrus Oils: Tumors of the Skin and Urethral Orifice in Mice". *J. Natl. Cancer Inst. (U.S.)* **1960**, *24*, 1389–1404.

Ross, R.; Glomsett, J. A. "Atherosclerosis and the Arterial Smooth Muscle Cell". *Science (Washington D.C.)* **1973**, *180*, 1332–1339.

Shapiro, D. J.; Nordstrom, J. L.; Mitschelen, J. J.; Rodwell, V. W.; Schimke, R. T. "Micro Assay for 3-Hydroxy-3-methylglutaryl-CoA Reductase in Rat Liver and in L-Cell Fibroblasts". *Biochim. Biophys. Acta* **1974**, *370*, 369–377.

Shaw, P. E. *Citrus Science and Technology*; AVI: Westport, CT, 1977.

Silberkange, M.; Havel, C. M.; Friend, D. S.; McCarthy, B. J.; Watson, J. A. "Isoprene Synthesis in Isolated Embryonic *Drosophila* Cells. I. Sterol-Deficient Eukaryotic Cells". *J. Biol. Chem.* **1983**, *258*, 8503–8511.

Sinensky, M.; Logel, J. "Defective Macromolecule Biosynthesis and Cell Cycle Progression in a Mammalian Cell Starved for Mevalonate". *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 3257–3261.

Siperstein, M. D.; Fagan, V. W. "Feedback Control of Mevalonate Synthesis by Dietary Cholesterol". *Cancer Res.* **1964**, *24*, 1108–1115.

Wattenberg, L. W. "Inhibition of Neoplasia by Minor Dietary Constituents". *Cancer Res. (Suppl.)* **1983**, *43*, 24485–24535.

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Determination of Furazolidone in Eggs by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic (HPLC) method for the determination of furazolidone in eggs at levels as low as 1 ppb has been developed. Egg homogenate was acidified at pH 4 and extracted with dichloromethane. The extract, after solvent evaporation, was subjected to clarification by treatment with dry ice cooled acetone, partitioning in hexane–water, and back-partitioning in dichloromethane. HPLC determination was performed on a reversed-phase C_8 10-μm column. Peak characterization was based on simple sequential procedures, such as comparison of retention values, on-line UV–vis scanning and absorbance ratio technique, off-line thin-layer chromatography (TLC), and conversion of furazolidone to a fluorescent species. Precision data suggested an overall relative standard deviation of 8.3%. The accuracy was found to be 92.9 ± 2%, while the linearity was excellent ($\bar{R}^2$ = 0.999 81) in the range (1–104 ppb) examined.

Furazolidone [*N*-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone] is a widely used antimicrobial agent added in feed to control diseases in pig, poultry, and cattle (Leidahl, 1984). However, controversy regarding the carcinogenicity of furazolidone has arisen since 1976 (FDA, 1976). The compound has been found to be mutagenic in *Escherichia coli* WP2 (McCalla and Voutsinos, 1974), *Drosophila* (Blijleven et al., 1977), and *Salmonella typhimurium* TA100 (Tatsumi et al., 1978).

Furazolidone is extensively metabolized in animals after its absorption from the gastrointestinal tract (Tennent and Ray, 1971; Tatsumi et al., 1984); immediately after the last administration to chickens and pigs, residual concentrations in muscle, kidney, and liver tissues were found to be less than 0.5 ppb (Winterlin et al., 1984). In eggs, however, no further metabolism occurs during the long egg development time (Card, 1952). This fact has raised the possibility that considerable furazolidone levels might be present in eggs.

Two HPLC methods for the determination of furazolidone in eggs have been published recently (Petz, 1984; Beek and Aerts, 1985). However, both of them lack the sensitivity required to monitor the ultralow levels (<10 ppb) of furazolidone expected in eggs during the final stage of drug elimination.

In a study conducted in our laboratory on furazolidone concentrations in hen's eggs following feeding trials, a method capable of detecting in eggs as low as 1 ppb of the drug has been developed.

MATERIALS AND METHODS

Instrumentation. High-performance liquid chromatography was carried out on a Perkin-Elmer system consisting of a Series 3 modular chromatograph equipped with two reciprocating pumps controlled by microcomputer, a Model LC-100 column oven set at 35 °C, a Model LC-55-B single-beam variable-wavelength UV–vis spectrophotometer set at 365 nm, and a Model 023 variable-span recorder. A Perkin-Elmer digital scanner (Model LC-55-S) permitted monitoring corrected spectra of the eluted compounds on stop-flow conditions; flow could be stopped by simultaneously shutting off pump power and closing a valve located at the injector inlet. Injections were made on a Perkin-Elmer C_8 10-μm, 25 × 0.46 cm prepacked column, through a Rheodyne Model 7105 injector. The mobile phase used was a mixture of 0.01 M sodium acetate

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