ELISA and HPLC Methods for Analysis of Fumagillin and Its Decomposition Products in Honey

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Two sensitive methods for detection of the antibiotic fumagillin (winter honey bee medication used to control *Nosema apis*) in honey were developed. A reversed-phase high-performance liquid chromatography (HPLC) method could detect the presence of fumagillin in honey at levels of 100 ppb and was useful to identify decomposition products of fumagillin. An enzyme-linked immunosorbent assay (ELISA) was developed as an initial screening method for fumagillin with detection levels of at least 20 ppb of fumagillin in honey. Neofumagillin, which could also be detected by ELISA, was identified as the major product of light decomposition of fumagillin in honey. Fumagillin was very stable in honey at elevated temperatures (stable for at least 35 days at 80 °C). At the above detection limits, there was no evidence of fumagillin or its breakdown products found in any producer honey examined, including honey samples from a beekeeper who regularly used fumagillin.

INTRODUCTION

Fumagillin (FA, 1) is an antibiotic obtained from *Aspergillus fumigatus* (Girolami, 1963; Wilson, 1971). It is of interest because of its amebicidal action (Killough et al., 1952; Jaronski, 1972), and derivatives have recently shown effectiveness in suppressing tumor growth (Ingber et al., 1990). In the honey industry, fumagillin is fed to bees in winter to protect them from *Nosema apis*, a microsporidian protozoa that shortens the lifetime of bees and decreases honey production (Gojmerac, 1980; Woyke, 1984; Crane, 1990). Since there is concern with the use of chemicals in honey production, it is important to determine the fate of fumagillin fed to bees and whether the use of fumagillin results in residues in honey. In addition, fumagillin is known to be unstable in light (Garrett and Eble, 1954; Eble and Garrett, 1954) and in heat (Garrett, 1954), but there is no precise information available on the mechanism of decomposition of fumagillin or the identity of the products formed.

Several assay methods have been developed for fumagillin: thin-layer chromatography (TLC; Ochab, 1970; Isaaq et al., 1977), a microbial assay (Girolami, 1963), and a spectrophotometric assay (Girolami, 1963). However, most of these methods have disadvantages limiting their utility (Brackett et al., 1988).

An HPLC method for analysis of fumagillin was reported by Brackett et al. (1988), which is easier and more accurate than other available methods, although it has not been used for honey analysis. In addition, since HPLC analysis is costly, a sensitive and rapid screening method such as an enzyme-linked immunosorbent assay (ELISA) for detection of this antibiotic in honey would be useful.

MATERIALS AND METHODS

Instrumentation and Materials. Honey samples were provided by Alberta Honey Producers' Co-op. Ltd., Edmonton, AB, and by several Alberta beekeepers. Four honey samples from hives that had been treated with fumagillin the previous winter were supplied courtesy of Dr. Don Nelson, Agriculture Canada Research Station, Beaverlodge, AB. Samples of fumagillin dicyclohexylamine salt (FDCH) were obtained from Medivet Pharmaceuticals Ltd. (High River, AB). *N*-Hydroxysuccinimide, 1,3-dicyclohexylcarbodiimide, and *N,N*-dimethylformamide were purchased from Aldrich Chemical Co. *o*-Phenylenediamine tablets, goat antirabbit peroxidase conjugated antibodies, urea peroxide, and citric acid monohydrate were obtained from Calbiochem Co. Thimerosal, Tween 20, bovine serum albumin (BSA), and *Limulus polyphemus* hemolymph (LPH) were purchased from Sigma Chemical Co. Dynatech Immulon 1 microtiter plates and spectrapor dialysis tubing (12 000–14 000 MW cutoff) were purchased from Fisher Scientific. Freund's complete and incomplete adjuvants were purchased from Difco Laboratories. Water used in all reactions and ELISA tests was purified using a Milli-Q system from Millipore. All other reagents used were of reagent grade or better.

Absorbance values at different wavelengths were recorded with a Hewlett-Packard Model 8451 A diode array spectrophotometer. Optical densities in microtiter plate wells were measured with a Model EL 309 ELISA reader from Bio-Tek Instruments Inc. Centrifugation was performed in a Damon/IES Division Model HN-S II centrifuge from International Equipment Co. A Virtis freeze dryer (The Virtis Co. Inc.) was used to dry protein conjugates.

Nuclear magnetic resonance (NMR) spectra were measured on Bruker WM-360 or WH-400 instruments. Tetramethylsilane was used as the internal standard. Infrared (IR) spectroscopy was measured on a Nicolet 7199 FT-IR spectrometer. Mass spectra (MS) were obtained using a Kratos AEI-MS 50 (high resolution, electron impact ionization EI-MS) for exact mass determinations, MS-12 for chemical ionization (CI-MS) and MS-9 for fast atom bombardment with argon (POSFAB-MS). Microanalyses were measured using a Perkin-Elmer 240 CHN analyzer. All NMR, IR, MS, and elemental analyses were performed by Chemistry Services at the University of Alberta.

Flash chromatography was performed according to the method of Still et al. (1978) using silica gel, 70–230 mesh, from Aldrich Chemical Co. TLC plates used were PE SIL G/UV (20 × 20 cm) with polyester backing purchased from Whatman Ltd. Preparative TLC was performed on PLC silica gel 60 F_{254} S (20 × 20 cm) from Merck.

HPLC Analysis of Fumagillin. Honey (5.0 g) was dissolved in water (25 mL) and the total volume was made up to 50 mL using acetonitrile. The solution was filtered through a Millipore Swinney 25 syringe adapter containing Durapore [poly(vinylidene difluoride)] membrane filters (Millipore) and immediately injected through a 20-μL loop for analysis on a reversed-phase silica column (Phenomenex IB-SIL 5 C_{18} 150 × 4.6 mm) which was preceded by a guard column (Phenomenex IB-SIL 5 C_{18} 50 × 2.1 mm) and operated at ambient temperature. The mobile phase was acetonitrile/water/glacial acetic acid (500:500:1.5 v/v/v) pumped by a Scientific Systems Inc. (SSI) Model 300 at a flow rate of 1.0 mL/min, and detection was achieved using a UV

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detector (Bio-Rad UV monitor Model 1305) at 350 nm. The output was recorded on a Hewlett-Packard 3388A integrator.

Peak areas correlated directly with fumagillin amounts to a concentration of at least 0.62 mg/mL of acetonitrile/water (1:1). Samples were analyzed in triplicate with an average coefficient of variation of ±2.3%.

Buffer Solutions. Phosphate-buffered saline (PBS) solution was prepared by dissolving sodium chloride (18.0 g), disodium hydrogen phosphate (Na_2HPO_4, 2.22 g), potassium dihydrogen phosphate (KH_2PO_4, 0.6 g), and thimerosal (0.2 g) in 1.9 L of water and the pH adjusted to 7.3. The total volume was then made up to 2.0 L with water. To prepare PBST, Tween 20 (1.0 g) was added before the final step.

Immunization. Two male rabbits (Flemish Giant × Lop Ear), both 4 weeks old, were injected (each 2 × 0.25 mL, subscapularly and 0.5 mL gluteal) with 1.0 mg of conjugate LPH–FA suspended in 1.0 mL of sterile PBS and emulsified with 1.0 mL of Freund's complete adjuvant. Boosters (containing 1.0 mg of LPH–FA in 1.0 mL of PBS emulsified with 1.0 mL of Freund's incomplete adjuvant) were given after 3 weeks and then again after 4 weeks. A test bleed at this stage revealed that the titer was low. Therefore, additional booster injections were made after 4 weeks and again after 3 weeks. Four weeks later, a final bleed out was performed (17 weeks after initial immunization). The blood was left at room temperature for about 2 h to coagulate and centrifuged at 1000 rpm for 5 min. The resulting clear yellowish serum was stored in 1.5-mL sealed containers at −20 °C.

Preparation of Honey Samples for ELISA. To prepare standard solutions of fumagillin in honey, a honey sample was obtained from a producer who does not use any chemicals or medications for his bees. This standard honey (25.278 g) was dissolved in 50 mL of water. Aliquots of the solution (5 mL) were placed in test tubes. To one of the test tubes was added 100 μL of ethanol/water (1:4), and this solution was used as a control. Fumagillin dicyclohexylamine salt (0.30 mg, equivalent to 0.22 mg of fumagillin) was dissolved in 1 mL of 95% ethanol, and 4 mL of water was added. Several dilutions of the fumagillin solution were made using ethanol/water (1:4) as solvent. To each test tube containing honey solution was added 100 μL of one of the diluted fumagillin solutions to make up honey solutions containing different levels of fumagillin. To analyze unknown honey samples, 5.0 g of the honey was dissolved in 10 mL of water and 200 μL of ethanol/water (1:4) added.

Indirect Competitive ELISA Procedure. The procedure of Sheth and Sporns (1990) was applied. The 96 wells on each ELISA microtiter plate were coated with the conjugate BSA–FA in PBS (1.0 mg/mL; 200 μL/well) or, for a blank, only PBS solution. The plates were stored overnight at 4 °C in a plastic bag containing a wet paper towel. The solutions were removed from the plates, and 1% BSA in PBS was added to all wells (200 μL/well). The plates were incubated in their plastic bags at room temperature for 1 h. The solutions in the wells were then shaken out and the wells washed with PBST (3 × 200 μL/well). Test samples, standards, or blanks were added to the wells (100 μL/well) and incubated with serum diluted 500 times using 0.05% BSA in PBST (100 μL/well). After 2 h at room temperature in the plastic bags, the plates were again emptied of their contents and washed with PBST (3 × 200 μL/well). The plates were incubated at room temperature for 2 h in the plastic bags and then washed with PBST (3 × 200 μL/well). A solution of the substrate, *o*-phenylenediamine (0.4 mg/mL), and urea peroxide (1 mg/mL) in 0.1 M citrate buffer (pH 4.75) was added to each well (200 μL/well). The plates were kept at room temperature for 30 min, and an ELISA reader was used to measure the difference in absorbance ($A_{450nm} - A_{660nm}$) in each well. The maximum percent relative standard deviation of three replicates on a plate was ±12% (average percent relative standard deviation for all analyses performed was ±6%).

Hapten Synthesis. *Fumagillin* (1). *[2,4,6,8-Decatetraenedioic Acid Mono[5-methoxy-4-[2-methyl-3-(3-methyl-2-butenyl)-oxiranyl]-1-oxaspiro[2.5]oct-6-yl] Ester.* Fumagillin dicyclohexylamine salt (1.46 g, 2.28 mmol) was suspended in water (200 mL) and acidified to pH 4 using dilute acetic acid. The solution was extracted with chloroform (3 × 50 mL), and the combined chloroform layers were washed with water (2 × 50 mL). The chloroform layer was dried (Na_2SO_4) and the solvent removed under vacuum at 30 °C to give a yellow oil. Recrystallization from 2-pentanone gave fumagillin (1.03 g, 98%) as a white powder: mp 187–188 °C (lit. 194–195 °C; Tarbell et al., 1955); IR (CH_2Cl_2 cast) 3240–2980, 1710, 1627, 1124, 1010 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 7.33 (m, 2 H, C=C*H*), 6.61 (m, 2 H, C=C*H*), 6.45 (m, 2 H, C=C*H*), 6.00 (q, 2 H, 3.5 Hz, 15 Hz, C=C*H*), 5.74 (s, 1 H, H_g), 5.23 (t, 1 H, 7.5 Hz, H_m), 3.73 (dd, 1 H, 3 Hz, 11 Hz, H_h), 3.48 (s, 3H, OCH_3), 3.04 (d, 1 H, 4.5 Hz, H_a), 2.68 (t, 1 H, 6 Hz, H_k), 2.59 (d, 1 H, 4.5 Hz, H_b), 2.42 (m, 1 H, H_l), 2.18 (m, 2 H, H_j, H_l), 2.04 (m, 2 H, H_e, H_f), 1.90 (t, 1 H, 14 Hz, H_c), 1.78 (s, 3 H, C=CCH_3), 1.70 (s, 3 H, C=CCH_3), 1.26 (s, 3 H, OCCH_3), 1.12 (d, 1 H, 13 Hz, H_d); exact mass 458.2303 (458.2305 calcd for $C_{26}H_{34}O_7$). Anal. Calcd for $C_{26}H_{34}O_7$: C, 68.10; H, 7.47. Found: C, 68.01; H, 7.87.

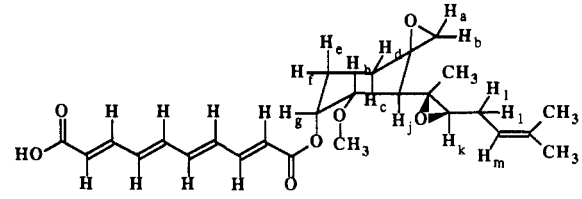

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Figure 1. Fumagillin.

Fumagillol (2) *and 2,4,6,8-Decatetraenedioic Acid* (3). Fumagillin (299.1 mg, 0.652 mmol) was dissolved in 0.1 N NaOH (16 mL). The yellow solution obtained was stirred for 4 h at 25 °C. Flocculent crystals formed were filtered, and the filtrate was extracted with ether (3 × 15 mL). The ether layer was dried over Na_2SO_4 and solvent removed under vacuum. A yellow oil was obtained from which the alcohol fumagillol (2) was crystallized using petroleum ether (122 mg, 66%): mp 49–51 °C (lit. 54–56 °C; Landquist, 1956); IR (CH_2Cl_2 cast) 3400–3500, 2976, 2928, 2885, 2824, 1445, 1378, 1103 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$) δ 5.25 (t, 1 H, 7.53 Hz), 4.45 (br, 1 H, OH), 3.72 (m, 1 H), 3.58 (s, 3 H), 3.04 (d, 1 H, 4.5 Hz), 2.68 (t, 1 H, 6.5 Hz), 2.64 (d, 1 H, 4.5 Hz), 2.46 (m, 2 H), 2.28 (m, 2 H), 2.60 (m, 1 H), 1.86 (s, 3 H), 1.76 (s, 3 H), 1.33 (s, 3 H), 1.10 (m, 1 H); CI-MS base peak 283 (MH^+). The yellow aqueous solution was acidified with acetic acid, yielding a greenish yellow oily precipitate which was dried over P_2O_5 to give 2,4,6,8-decatetraenedioic acid (3) as a yellow powder: mp 293–295 °C (lit. 295–297 °C; Schenck et al., 1953); IR (KBr) 3420, 3000–2830, 1672, 1620, 1422, 1308 cm^{-1}; ^1H NMR (360 MHz, DMSO-d_6) δ 7.27 (q, 2 H, 11 Hz, 14.5 Hz), 6.84 (m, 2 H), 6.65 (m, 2 H), 6.04 (d, 2 H, 14.5 Hz); exact mass 194.0578 (194.0579 calcd for $C_{10}H_{10}O_4$).

Fumagillyl Hemisuccinate (4). Fumagillol (21.9 mg, 0.078 mmol), succinic anhydride (41.4 mg, 0.411 mmol), and (dimethylamino)pyridine (2.0 mg, 0.016 mmol) were dissolved in anhydrous pyridine (1.0 mL) and stirred at 22 °C for 3 days. The solvent was removed under vacuum, and the resulting residue was dissolved in chloroform (15 mL) and extracted with acidified water (pH 3.5, 3 × 15 mL). The combined water layers were washed with chloroform, and the combined chloroform layers were dried (Na_2SO_4). The solvent was removed under vacuum to give a yellow oil (25.4 mg, 85%); IR (CH_2Cl_2 cast) 2929, 1734, 1160, 1105 cm^{-1}; exact mass 382.1978 (382.1992 calcd for $C_{20}H_{30}O_7$).

BSA–Fumagillin Conjugate (BSA–FA). Fumagillin (5.9 mg, 13 mmol), dicyclohexylcarbodiimide (DCC, 6.1 mg, 30 mmol), and *N*-hydroxysuccinimide (2.6 mg, 23 mmol) were dissolved in anhydrous dimethylformamide (DMF, 0.1 mL). The solution was stirred at 4 °C for 24 h. The solution was filtered (to remove the needlelike crystals of dicyclohexylurea) directly into a solution of bovine serum albumin (BSA, 110.5 mg, 1.67 mmol) in 2 mL of phosphate buffer solution (pH 7.6). A cloudy solution was obtained which was stirred at 4 °C for 24 h. The suspension was then transferred to a dialysis bag and dialyzed for 24 h in each of the following solutions successively: 8 M urea (1 L), 50 mM ammonium bicarbonate (4 L), 25 mM ammonium bicarbonate (4 L). The dialyzed solution was freeze-dried to give the BSA–fumagillin conjugate (BSA–FA) as an off-white flocculant powder. UV absorption for the conjugate (37 mg/mL of PBS) at 350 nm

was 0.0755. From standard curves for absorption of pure fumagillin, it was determined that the conjugate contains approximately two fumagillin groups per BSA molecule.

LPH–Fumagillin Conjugate (LPH–FA). Fumagillin (19.3 mg, 42.1 mmol), DCC (13.8 mg, 66.9 mmol), and *N*-hydroxysuccinimide (11.8 mg, 102.5 mmol) were dissolved in anhydrous DMF (2 mL) and stirred at 4 °C for 24 h. The solution was filtered into 2 mL of PBS (pH 7.6) containing LPH (84.1 mg, 1.27 mmol). A thick precipitate formed and was stirred at 4 °C for 24 h. The resulting suspension was dialyzed (as for BSA–FA conjugate) and then freeze-dried. Anal. found for pure LPH: N, 15.76. Found for LPH–FA conjugate: N, 13.42. Therefore, the LPH–FA conjugate contains approximately 18 fumagillin molecules per LPH molecule.

BSA–Fumagillyl Hemisuccinate Conjugate (BSA–FS). Fumagillyl hemisuccinate (4, 25.4 mg, 0.07 mmol), DCC (28.5 mg, 0.14 mmol), and *N*-hydroxysuccinimide (16.7 mg, 0.15 mmol) were dissolved in anhydrous DMF (2.0 mL) and stirred at 4 °C for 24 h. The reaction mixture was filtered into a solution of BSA (306 mg, 4.6 mmol) in PBS (pH 7.6, 2.0 mL) and stirred at 4 °C for 24 h. A thick white precipitate formed immediately after addition of the active ester solution to the solution of BSA and remained after dialysis (performed as for the BSA–FA conjugate). After freeze-drying, the conjugate BSA–FS was obtained in the form of a flaky solid. Anal. found for pure BSA: N, 15.77. Anal. found for BSA–FS: N, 14.40. Therefore, the conjugate contained about 14 hapten molecules per BSA.

Products of the Decomposition of Fumagillin. *Decomposition of Fumagillin at 60 °C at pH 3.5.* Fumagillin (362 mg) was dissolved in 10% ethanol in water (200 mL) and protected from light. The pH of the solution was lowered to 3.5 using dilute acetic acid, and the solution was stirred at 60 °C for 42 days. The solvent was then removed under vacuum, and the resulting compound was dissolved in acidified water (pH 3.5) and extracted with dichloromethane. The dichloromethane layer was collected and the solvent removed under vacuum. The resulting oil was purified by flash chromatography using ethyl acetate/hexane (2:1). Two main compounds were isolated, one of which was found to be unreacted fumagillin. The second compound (5) was isolated as a white powder after recrystallization from ether: mp 161–163 °C; IR (KBr) 3431, 2940, 1710, 1628, 1277, 1237 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$–DMSO-d_6) δ 7.30 (m, 2 H, 4.5 Hz, 13 Hz), 6.63 (m, 2 H), 6.50 (m, 2 H), 5.97 (q, 2 H, 11.5 Hz, 15 Hz), 5.65 (d, 1 H, 3 Hz), 5.32 (m, 1 H), 4.29 (br, 1 H, OH), 3.87 (d, 1 H, 9 Hz), 3.80 (br, 1 H, OH), 3.73 (dd, 1 H, 3 Hz, 12 Hz), 3.62 (d, 1 H, 9 Hz), 3.39 (s, 3 H), 3.32 (q, 1 H, 2 Hz, 11 Hz), 2.41 (q, 1 H, 7 Hz, 14 Hz), 2.11 (m, 3 H), 1.89 (m, 1 H, 14 Hz), 1.72 (s, 3 H), 1.71 (m, 1 H), 1.64 (s, 3 H), 1.61 (d, 1 H, 4 Hz), 1.32 (s, 3 H); POSFAB-MS (Cleland) 477 (MH^+), 499 (M + 23). Anal. Calcd for $C_{26}H_{36}O_8$: C, 65.53; H, 7.61. Found: C, 65.31; H, 7.42.

Decomposition of Fumagillin in Light. Fumagillin (180 mg) was dissolved in acetonitrile/water (1:1, 10 mL) and placed under sunlight/fluorescent light at room temperature. After 7 days, the solvent was removed under vacuum and the residue was dissolved in chloroform (40 mL) and washed with water (3 × 20 mL). The chloroform layer was then dried (Na_2SO_4), and the solvent was removed under vacuum to give a yellow oil. Attempts to purify this product further by recrystallization led to some decomposition. Preparative TLC using toluene/methanol (3:1) gave 6 as a reddish oil: IR ($CHCl_3$ cast) 3260, 2951, 2927, 2860, 2659, 2250, 2124, 1710, 1617, 1480 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.17 (m, 1 H), 6.39 (m, 1 H), 6.16 (m, 2 H), 5.77 (m, 2 H), 5.56 (s, 1 H), 5.09 (t, 1 H, 6 Hz), 3.55 (m, 1 H), 3.28 (s, 3 H), 2.85 (d, 1 H, 4.5 Hz), 2.51 (t, 1 H, 6 Hz), 2.48 (d, 1 H, 4 Hz), 2.25 (m, 1 H), 2.05 (m, 2 H), 1.89 (m, 3 H), 1.77 (m, 2 H), 1.66 (s, 3 H), 1.52 (s, 3 H), 1.05 (s, 3 H), 0.98 (d, 1 H, 13 Hz); POSFAB-MS (Cleland) 459 (MH^+), 481 (M + 23); UV absorption maxima at 240 and 306 nm.

Experiments To Determine the Rate of Decomposition of Fumagillin in Honey. *Experiment A. Decomposition in the Dark at 37 °C.* A honey sample (25.807 g) was weighed out in a round-bottom flask. Fumagillin dicyclohexylamine (FDCH, 0.048 g, equivalent to 0.035 g of fumagillin) was dissolved in 95% ethanol (0.5 mL), and 1.2 mL of water was added. The fumagillin solution was added to the honey solution and stirred. The flask was sealed, protected from light, and placed in an oil bath maintained at 37 °C. A control sample of the honey (25.298 g), to which 95% ethanol (0.5 mL) and water (1.2 mL) had been added, was also placed in the same oil bath and subjected to the same conditions as the above sample.

Experiment B. Decomposition in the Dark at 80 °C. A flask containing a sample of the honey (25.236 g) and FDCH (0.034 g, equivalent to 0.024 g of fumagillin) dissolved in 95% ethanol (1.0 mL) and water (1.2 mL) was protected from light and stirred in an oil bath at 80 °C. The control sample contained honey (24.779 g), 95% ethanol (1.0 mL), and water (1.2 mL).

Experiment C. Decomposition in Light at 37 °C. A sample of the same honey (25.969 g) was weighed out in a round-bottom flask. FDCH (0.050 g, equivalent to 0.034 g of fumagillin) was dissolved in 95% ethanol (0.5 mL), and 1.2 mL of water was added. This solution was added to the honey, and the flask was sealed and stirred in an oil bath maintained at 37 °C. Light was directed to the sample (Elmo S-30 projector with a high-efficiency projection lamp, 120 V, 300 W, 30 cm away from the sample). Another flask containing another sample of the same honey (25.503 g) was used as a control. Ethanol (95%, 0.7 mL) and water (1.0 mL) were added to it, and it was placed in the same oil bath as the sample containing fumagillin.

RESULTS AND DISCUSSION

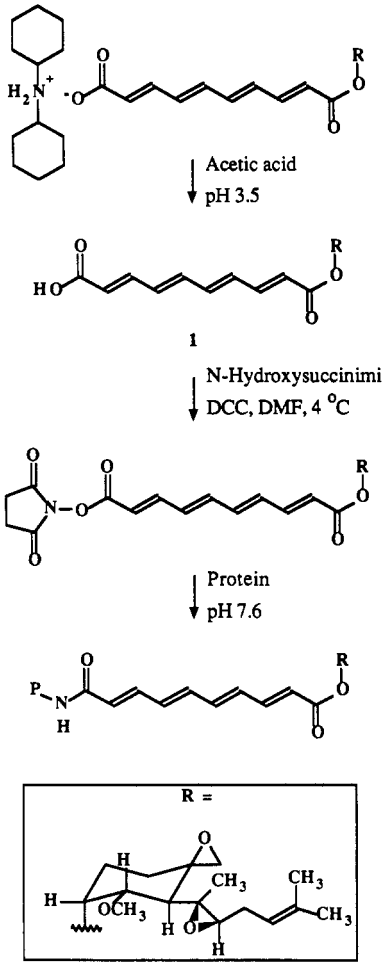
HPLC Analysis of Fumagillin in Honey. HPLC was found to be a useful method for detecting fumagillin in honey samples. For analysis, a honey was simply dissolved in acetonitrile/water (1:1, 5 g in 50 mL) and filtered through a membrane filter; 20 μL was injected into the HPLC system. When solutions of fumagillin in acetonitrile/water (1:1) were added to the honey samples (dissolved in the same solvent) and analyzed, the detection limit was found to be 0.1 ppm (100 ppb) of fumagillin in honey (gave peak height about 3 times noise level). In the UV spectrum, honey had a maximum absorbance at about 216 nm and there was little interference in UV detection between honey and fumagillin, which had a maximum absorbance at 351 nm.

Four honey samples, obtained from a research facility that regularly uses fumagillin in the winter as medication for its bees, were analyzed using the HPLC method. No fumagillin could be detected in any of the samples.

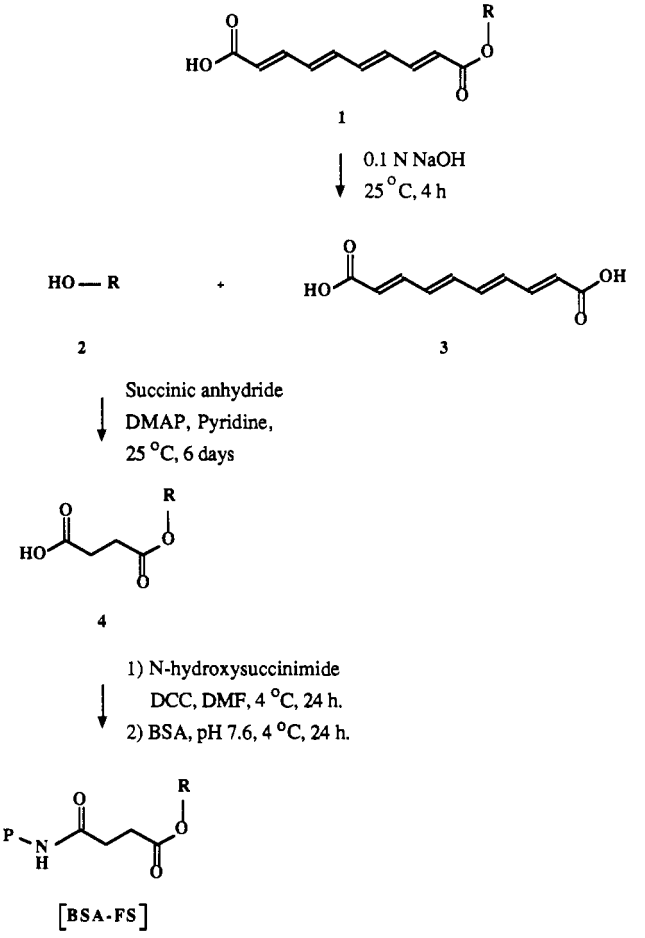
Enzyme-Linked Immunosorbent Assay (ELISA) for Fumagillin in Honey. *Preparation of Protein–Fumagillin Conjugates.* To develop the ELISA for fumagillin, the acid was prepared from the commercially available dicyclohexylamine salt (Scheme I). Fumagillin (FA, 1) was then attached to the protein via *N*-hydroxysuccinimide ester formation in the presence of dicyclohexylcarbodiimide (DCC; Anderson et al., 1964; Bauminger and Wilchek, 1980). BSA was used to prepare the fumagillin conjugate (BSA–FA) used for coating the ELISA plates, while an LPH–fumagillin conjugate (LPH–FA) was injected into the rabbits to produce the antibodies. Although this procedure gave satisfactory results, solubility problems were encountered during the reaction of the active ester with the protein solution. This precipitation problem was also encountered by Goodrow et al. (1990). Use of a water-soluble carbodiimide such as 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC) would have been an alternative method (Bauminger and Wilchek, 1980), although this could have led to protein cross-linking.

Purification of the protein–hapten conjugate was performed by the dialysis in 8 M urea to allow the protein to unfold to remove any small molecular weight compounds not covalently linked to the proteins. The urea solution was then replaced by an ammonium bicarbonate solution which evaporated during freeze-drying to leave the protein–fumagillin conjugate as a flaky material.

Scheme I. Preparation of BSA–FA Conjugate



Scheme II. Preparation of BSA–FS Conjugate



For ELISA techniques, bridge heterology (the use of different protein–hapten linking arms in the coating conjugate and the immunogen) is reported to significantly increase assay sensitivity (Tijssen, 1985; Gosling, 1990). We therefore decided to prepare a coating conjugate with a different linking arm to compare it to the BSA–FA conjugate which had the same linking arm as the immunogen (Scheme II). Fumagillin was hydrolyzed to the alcohol fumagillol (2) and the polyene decatetraenedioic acid (3) under basic conditions (Landquist, 1956). The alcohol was then reacted with succinic anhydride in the presence of (dimethylamino)pyridine (DMAP) to produce the fumagillyl hemisuccinate (4), which was then conjugated to BSA via the *N*-hydroxysuccinimide ester to form the conjugate BSA–fumagillyl hemisuccinate (BSA–FS).

To determine hapten to protein ratio in the conjugate, spectrophotometry and elemental analysis were used. Fumagillin absorbs in the ultraviolet range, and its spectrum does not overlap with that of BSA. Absorption of the BSA–FA conjugate indicated that about two molecules of fumagillin were attached to each BSA molecule. UV could not be used to analyze the LPH–FA conjugate because it was insoluble in all suitable solvents. In this case, elemental analysis was very useful, especially since fumagillin contained no nitrogen. From elemental nitrogen analysis, the LPH–FA conjugate was found to have about 18 fumagillin molecules per protein.

Comparison of BSA–FA and BSA–FS as Coating Conjugates. The immunization conjugate LPH–FA was injected into two male rabbits, and three booster injections were given before the rabbits were bled 17 weeks later. The sera, containing the required antibodies, were collected and tested according to a literature ELISA procedure (Sheth and Sporns, 1990) using either BSA–FA or BSA–FS as the coating conjugate on the microtiter wells.

Using BSA–FA conjugate (1 μg/mL in PBS) for coating, a 1000-fold serum dilution was needed to obtain about 1.0 OD (450 nm) after half an hour of peroxidase reacting on the substrate, *o*-phenylenediamine. A serum diluted 16 000 times gave 3 times the background absorbance. When the BSA–FS conjugate (1 μg/mL in PBS) was used for coating, about 1.0 OD was obtained with 2000-fold diluted serum and a serum dilution of 16 000 times gave 30 times the background absorbance. Therefore, it appeared that the antibodies bound more strongly to the succinyl conjugate than to BSA–FA.

When the sera were tested in an indirect competitive ELISA with different concentrations of free fumagillin, no significant reduction in absorbance could be detected with BSA–FS as coating conjugate, even with test samples containing up to 800 ppm of free fumagillin in solution (Figure 2). This result was surprising and could be due to the greater number of hapten molecules per protein in BSA–FS (14 vs 2 groups for the BSA–FA) resulting in avidity effects or greater hydrophobic interactions with antibodies. Another possibility is that the hemisuccinate (4), lacking the long rigid polyene chain, allows the shorter and more flexible methylene chain to orient more favorably in the antibody binding site. Further work with this conjugate was not pursued, since it was more difficult to synthesize than the BSA–FA which worked well in a competitive ELISA.

Figure 3 shows the results of a competitive ELISA using BSA–FA (1 μg/mL in PBS) for coating of the microtiter wells, using a 1000-fold dilution of antibody serum and

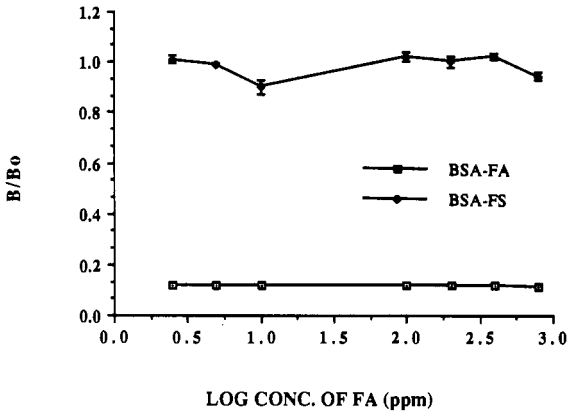


Figure 2. Competitive ELISA for fumagillin in solution (ethanol/water, 1:4) using BSA–FA (1.0 μg/mL in PBS) and BSA–FS (1.0 μg/mL in PBS) as coating conjugates. Serum was diluted 1000 times. Error bars denote standard deviation of triplicate analyses. B is the absorbance of a test sample; B_0 is the absorbance of the control free of fumagillin.

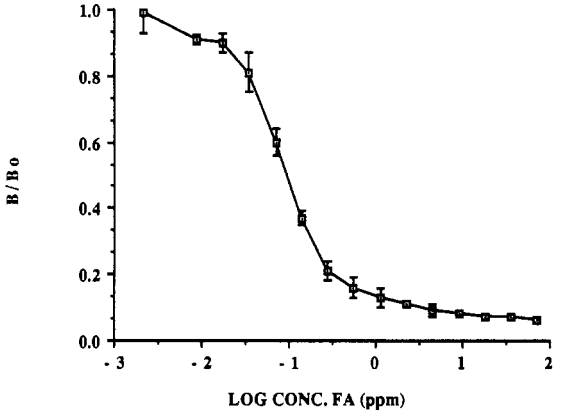


Figure 3. Competitive ELISA for fumagillin using only BSA–FA for coating (1.0 μg/mL in PBS). Other conditions were as in Figure 2.

free fumagillin in a solution of ethanol/water (1:4) at different concentrations. Under these conditions, levels of fumagillin down to about 35 ppb could be detected (20% decrease in absorbance), and about 100 ppb resulted in a 50% decrease in absorbance.

ELISA for Fumagillin in Honey. To determine the ideal concentration of honey to be used in the tests, a honey sample free of fumagillin was diluted to different concentrations with water and an ELISA was performed. The results (Figure 4) indicated that more concentrated solutions of this honey were less likely to exhibit wide variabilities between different samples. Table I shows the results for ELISA on this honey spiked with known amounts of fumagillin. It appeared that the use of a serum diluted 1000 times and a honey concentration of about 0.5 g/mL led to the most sensitive results. However, the absorbances obtained for such solutions were very low (an OD of only 0.1–0.4). Using a more concentrated serum (500-fold dilution) and the honey solution at a concentration of 0.5 g/mL gave higher absorbance values. In this latter case, the honey containing 10 ppb of fumagillin gave absorbance reductions of 50% (B/B_0 of 0.5). It was interesting to note that the ELISA test was about an order of magnitude more sensitive when honey was present in solution than when only solvent was used (Figure 3).

To analyze for fumagillin in honey, it was essential to account for the variability between different honeys. A wide range of honeys (samples from honey comb, combined producer samples, and processed honey) were tested by

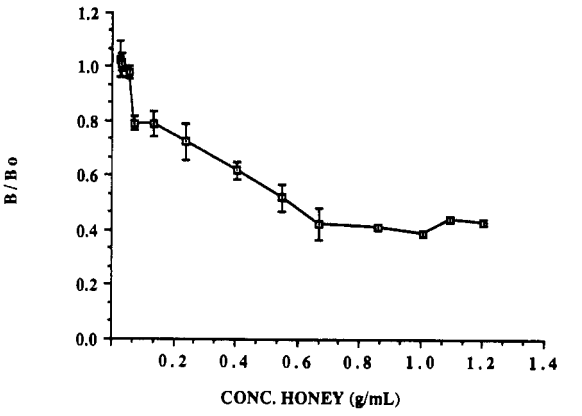


Figure 4. B/B_0 for a honey at different concentrations in ELISA using coating conjugate BSA–FA (1.0 μg/mL in PBS). Serum was diluted 1000 times. Error bars denote standard deviation of triplicate analyses. B is the absorbance of samples containing varying percentages of honey; B_0 is the absorbance of the control free of honey.

Table I. Detection Limit for Fumagillin in Honey Using ELISA

condition	serum dilution	honey dilution	concn of FA in honey, ppb, to give $B/B_0 \sim 0.5$
1	1/500	1/1	20
2	1/500	1/2	10
3	1/1000	1/2	5

Table II. ELISA of Honey Samples Produced in Alberta

	B/B_0		
honey[a]	condition 1[b]	condition 2[b]	condition 3[b]
A		0.38	0.48
B	0.98	0.89	1.06
C	0.88	0.64	0.72
D	0.77	0.55	0.67
E	0.73		0.48
F			0.48
G			0.62
H			0.50
I	0.77	0.58	0.73
J	0.96	0.82	0.61
K	1.06	1.02	
L	0.87	0.22	
M	1.08	1.04	0.93
N	0.94	0.94	0.77
O	0.78	0.94	0.77
P	0.73	0.88	0.25
range of B/B_0	0.7–1.1	0.4–1.0	0.3–1.1

[a] Honeys A–H were unprocessed honey samples isolated from individual combs obtained from individual producers in 1989; honey I was a processed honey and therefore a composite of honeys from different producers. Honeys J–L were unprocessed composites from individual producers; honeys M–P were produced by bees given fumagillin for medication. HPLC analysis of honeys L–P indicated no detectable (>100 ppb) fumagillin. [b] See Table I.

ELISA under different conditions (Table II). The control honey was the one used for the experiments noted above (that is, free of fumagillin). Honey samples M–P were provided by a honey producer that used fumagillin for his bees. These samples were analyzed by HPLC, and no fumagillin could be detected. It appeared that there was least variability in the different honeys when the serum was diluted 500 times and the honey was at a concentration of 1 g/mL (condition 1). Under these conditions, any honey resulting in a 50% reduction in absorbance compared to a control honey would be suspected of contamination with fumagillin and could be further analyzed, by HPLC for example, to confirm or deny this suspicion. However, there was some problem in dealing with a honey

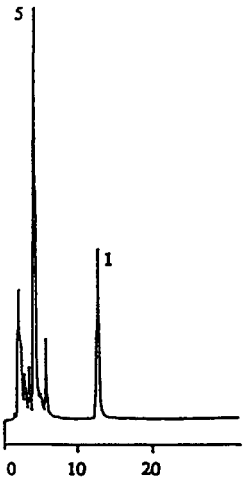


Figure 5. HPLC chromatogram for thermal decomposition of fumagillin in solution: column, Phenomenex IB-SIL 5 C_{18}; eluent, water/acetonitrile/acetic acid (500:500:1.5); detection, UV 350 nm; flow, 1 mL/min. Scale denotes retention time in minutes. 1, Fumagillin; 5, dihydroxyfumagillin.

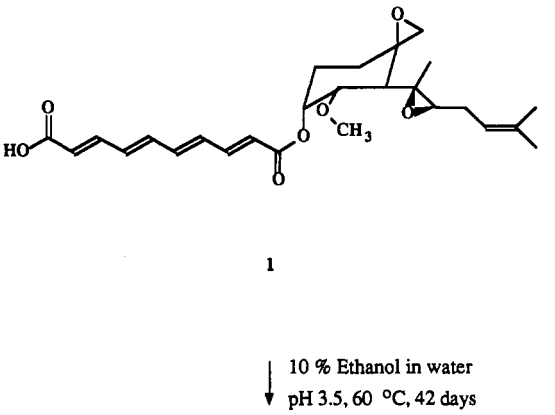
at such a concentrated level because of its high viscosity, which did not facilitate the handling and pipetting of the sample into the microtiter wells. Dilution of the honey by half made it easier to handle. A serum diluted 500 times gave average absorbances of about 0.6 when the honey concentration was about 0.5 g/mL. Under these conditions, normal honeys give B/B_0 ranging from 0.4 to 1.0. Again, a cutoff choice could be made. For example, a honey sample that caused over 90% reduction in absorbance with reference to a control honey would likely be contaminated with fumagillin.

A careful examination of Tables I and II indicates that a few of the honey samples presented in Table II could have contained 5–10 ppb of fumagillin (although we have no proof and the lower absorbances could have been due to other factors). However, not only would this level of contamination be very difficult to confirm with HPLC (100 ppb detection limit), but usually regulatory agencies consider levels of agricultural chemicals of less than 100 ppb tolerable in foods.

Decomposition of Fumagillin in Honey. Honey is a concentrated aqueous solution of sugars with a pH of about 3.5. During its production by the bees, it is stored in the hive, which is a dark enclosure maintained at a temperature of about 37 °C. Processing of honey exposes it to light and pasteurization temperatures as high as 77 °C (but only for a few seconds). Therefore, if honey did become contaminated with fumagillin during production, it would be useful to understand the route of decomposition of fumagillin in honey without light at elevated temperatures and in the presence of light.

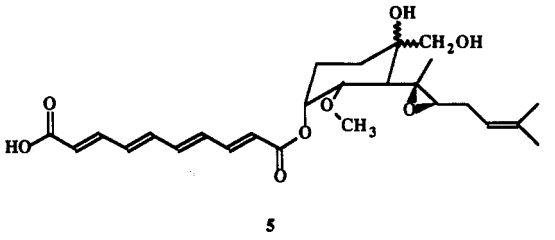
Thermal Decomposition. To determine the product of decomposition of fumagillin on heating, FDCH was dissolved in 10% ethanol in water (acidified with dilute acetic acid to pH 3.5), protected from light, and heated at 60 °C. After 42 days, some fumagillin could still be detected by TLC. HPLC of the reaction mixture (Figure 5) indicated the presence of one major product which was isolated by flash chromatography. The spectra of the product **5** were compared to those of fumagillin and fumagillol. It appeared that, on heating, the unstable epoxide in the molecule was hydrolyzed to give the dihydroxy product **5** (Scheme III). No fumagillol or decatetraenedioic acid was detected by TLC or HPLC, indicating that ester hydrolysis was not an important reaction under these conditions.

Scheme III. Thermal Decomposition Product of Fumagillin in Solution



1

10 % Ethanol in water
pH 3.5, 60 °C, 42 days



5

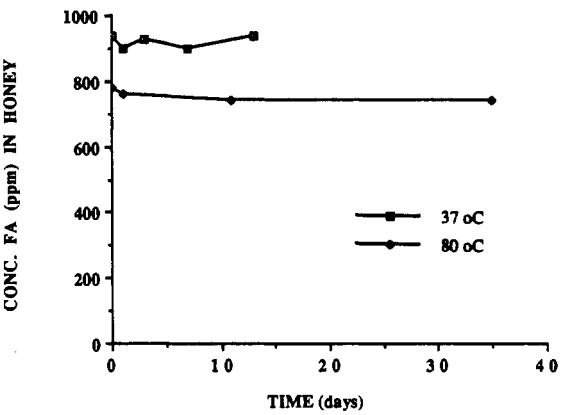
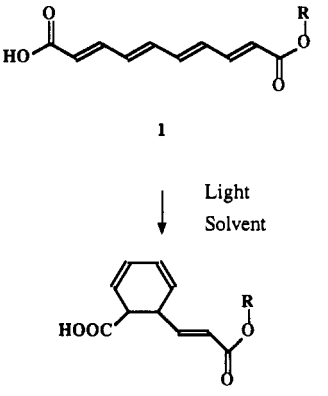


Figure 6. Thermal decomposition of fumagillin in honey with time at 37 and 80 °C measured by HPLC.

Tarbell and co-workers (Tarbell et al., 1955, 1961; Cross and Tarbell, 1958) has reported the isolation of a crystalline compound resulting from the action of 10% sulfuric acid on fumagillol (**2**) with boiling under reflux. They observed that their product did not undergo hydrogenation and, therefore, must have lost the double bond in the side chain. They proposed two possible ring structures for their product resulting from the rearrangement of the dihydroxyfumagillol as an intermediate. However, in our case, the conditions used for the thermal decomposition of fumagillin were much milder than the conditions of Tarbell and co-workers (Tarbell et al., 1961).

To determine the rate of decomposition of fumagillin in honey, two experiments were prepared at different temperatures. Flasks containing honey spiked with fumagillin and control samples of honey were protected from light and heated at 37 or 80 °C. Samples of these solutions were removed at different intervals and tested by HPLC and ELISA. As indicated in Figure 6, the amount of fumagillin present in the solution, as determined by HPLC, showed very little change over a period of up to 35 days

Scheme IV. Decomposition Product of Fumagillin in Light



at temperatures of 80 °C. This same trend was obtained when the solutions were tested by ELISA. This indicated that fumagillin was very thermally stable in honey even at temperatures higher and maintained for much longer than during production and processing. It was interesting to note that honey seemed to have a protective effect on fumagillin when heated, since milder temperature conditions did cause fumagillin breakdown in the original experiments with aqueous ethanol. This probably, at least in part, was due to limiting the availability of reactant water with the high sugar concentrations found in honey. ELISA was performed on the decomposition product 5, and even a solution of 1.6 μg/mL of 5 gave only 25% reduction in absorbance with respect to the solvent blank. The same percent reduction in absorbance was observed for a solution of fumagillin containing about 50 ppb (Figure 3, about 32 times lower concentration). Obviously the antibodies were very sensitive to structural changes in the ring portion of fumagillin and would not recognize product 5 at reasonable concentrations.

Photolytic Decomposition. A solution of fumagillin in acetonitrile/water (1:1) was placed under sunlight and fluorescent light. After 7 days, one major product could be detected by TLC. However, attempts to isolate and purify the compound led to some decomposition, and the compound was purified by preparative TLC. The spectral data of the product 6 indicate that some rearrangement of the polyene chain had occurred (Scheme IV). This was in accordance with the observation made by Garrett and Eble (1954) that the destruction of fumagillin under light was primarily photolytic and occurred in the presence or absence of oxygen. They named the product "neofumagillin" and observed that it absorbed at wavelengths of less than 345 nm. Garret and Eble's observations were based on kinetic and UV studies of the photolytic decomposition of fumagillin in ethanol and not on isolation or spectral analysis of products. Compound 6 had an NMR almost identical to that of fumagillin except in the region of olefin protons, where only six vinyl protons appear in the region δ 7.2–5.8, instead of the eight protons of the polyene chain in fumagillin, and two extra protons appeared in the region for hydrocarbon protons (δ 2.1–1.8). The IR spectrum of 6 lacked the peak at 1627 cm^{-1} which was present in fumagillin (for four conjugated double bonds) and exhibited a new peak at 1617 cm^{-1}, which could be assigned to the new configuration of double bonds. In ELISA, the antibodies recognized the decomposition product 6. A solution containing 2.0 μg/mL of 6 caused

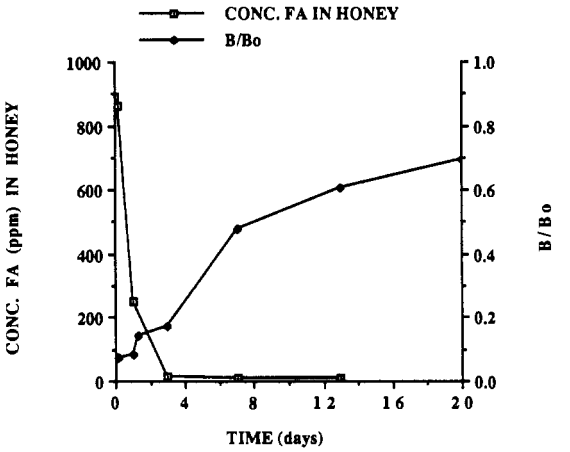


Figure 7. Decomposition of fumagillin in honey under intense light measured by HPLC and ELISA.

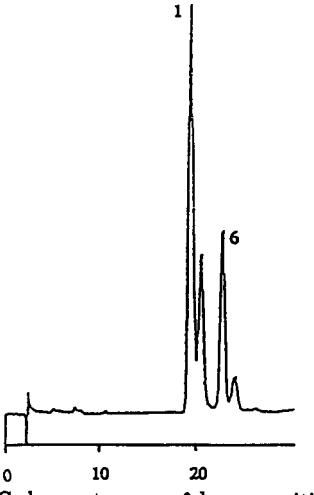


Figure 8. HPLC chromatogram of decomposition of fumagillin in honey in strong light after 1 day. HPLC conditions were as in Figure 5, except flow was 0.7 mL/min. 1, Fumagillin; 6, neofumagillin.

a 92% reduction in absorbance with respect to a solvent blank. A fumagillin solution containing about 1 ppm (Figure 3) caused about the same percent reduction in absorbance [the antibodies were about twice as sensitive for fumagillin as neofumagillin (6)].

An experiment was also performed to determine the rate of photolytic decomposition of fumagillin in honey, where a flask containing honey and fumagillin and another flask containing control honey were left at 37 °C under intense light. Samples from the experiment and the control were checked periodically by both HPLC and ELISA. The disappearance of fumagillin with time, as detected by HPLC and ELISA, is shown in Figure 7. The HPLC chromatogram for the honey containing fumagillin after 1 day is given in Figure 8. After 1 day, HPLC indicated that only about one-third of the fumagillin remained. The major new peak was neofumagillin (6). Of course, since the UV maximum for 6 was at 240 nm rather than 350 nm (used for the HPLC chromatogram and optimum for fumagillin), there was considerably more neofumagillin present than indicated by just the relative peak areas (1 vs 6) in Figure 8. The ELISA absorbance (Figure 7) increased less rapidly than the decrease in fumagillin noted by HPLC because of the orders of magnitude greater sensitivity for fumagillin in ELISA and because the ELISA was sensitive to the presence of at least the major decomposition product, neofumagillin.

Experiments on photolytic decomposition of fumagil-

lin in solution and honey were also performed in daylight/fluorescent light (less intense light conditions than above) and tested periodically by HPLC. The results for both experiments were similar and indicated that fumagillin decomposed quickly in the first few hours. After about 5 h (with about 60% of the fumagillin remaining), the decomposition rate slowed markedly, and after 11 days, the amount of fumagillin in the solution had decreased to about 30% of its original level. Despite the rapid initial degradation, the UV absorption spectra of the decomposing solutions, alone, were a poor indicator of what was occurring since the major product, neofumagillin, also had considerable UV absorbance.

We were unable to detect fumagillin (at levels >100 ppb) or any of its decomposition products (photolytic products such as neofumagillin) in any commercial Alberta honey sample. These samples included four from a beekeeper who regularly used fumagillin treatments for his hives. Although a wider survey would be necessary, it seemed that there was no evidence for concern of contamination of honey when fumagillin was properly used.

ABBREVIATIONS USED

BSA, bovine serum albumin; BSA–FA, BSA–fumagillin conjugate; BSA–FS, BSA–fumagillyl hemisuccinate conjugate; CI-MS, chemical ionization mass spectrum; DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; EI-MS, electron impact mass spectrum; ELISA, enzyme-linked immunosorbent assay; FA, fumagillin; FDCH, fumagillin dicyclohexylamine salt; FS, fumagillyl hemisuccinate; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; LPH, *Limulus polyphemus* hemolymph; LPH–FA, LPH–fumagillin conjugate; MS, mass spectra; mp, melting point; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing Tween 20; POSFAB-MS, positive fast atom bombardment mass spectrum; TLC, thin-layer chromatography; Tween 20, polyoxyethylene sorbitan monolaurate.

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