Fumagillin and Dicyclohexylamine in Apiculture

by

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ABSTRACT

Nosema disease of the Western honey bee (*Apis mellifera* L.), is caused by two distinct microsporidian fungal species, *Nosema ceranae* Fries et al. and *Nosema apis* Zander. *N. apis* infection of *A. mellifera* was first documented in 1909, while *N. ceranae* infection of *A. mellifera* was described in 1996. *N. ceranae* infection has been implicated in colony collapse disorder (CCD) and decreased survival of overwintered colonies. There is currently only one registered chemical treatment available to control Nosema disease in apiculture in Canada, namely Fumagilin-B®, a potent fungal metabolite first isolated from *Aspergillus fumigatus* Fres. Fumagilin-B® (and the equivalent Fumidil-B®) has been extensively used against *N. apis* since its discovery in the early 1950's, and has more recently been used to control *N. ceranae* infections.

The toxicity of fumagillin, which has limited its use in human medicine, is also of concern for beekeeping, since any residues of fumagillin remaining in hive products pose a direct risk to the consumer. All analytical methods published to date measure only fumagillin and its decomposition products in honey, but overlook the fact that fumagillin is present in a 1:1 stoichiometric ratio with its dicyclohexylamine (DCH) counter ion in the commercial salt formulations (Fumagilin-B® and Fumidil-B®). DCH is almost five times more toxic than fumagillin to rats, and also exhibits genotoxic and tumorigenic properties. A reversed phase (RP) liquid chromatography tandem mass spectrometric (LC-MS/MS) method was developed to confirm and quantitate trace levels of fumagillin and DCH residues in honey. A labelled d_{10}-DCH internal standard was also synthesized and used to compensate for observed matrix affects when quantitating DCH in honey from different floral origins. While analyzing domestically

produced honey samples fumagillin was seldom detected at levels above 10 ng g^{-1} (method limit of quantitation), while DCH was detected in almost all of the samples at concentrations above 10 ng g^{-1}. The frequency and concentrations of DCH detected, even in the absence of any detectable amounts of fumagillin or its known degradation products, led to the design of an experiment to evaluate the relative stability of fumagillin and DCH in honey under a range of time-temperature exposures. During this experiment it was observed that DCH was significantly more stable in honey than fumagillin highlighting DCH as an important potential contaminant of honey. This further emphasizes the importance of evaluating all of the potentially active ingredients that may be present in a pharmaceutical formulation, since the latter may be more important than immediately apparent.

Finding alternative chemical treatments to fumagillin is necessary, as the sustained usage of this drug in apiculture for six decades may lead to the development of resistance in *Nosema* spp. In human medicine fumagillin has been tested against a variety of diseases, including cancer. Fumagillin inhibits angiogenesis (the formation of new blood vessels around a cancerous tumor) through covalently bonding to the methionine aminopeptidase 2 (MetAP-2) enzyme. This enzyme occurs ubiquitously in humans, honey bees and in both *N. apis* and *N. ceranae*. I hypothesized that the MetAP-2 structure-activity relationships discovered in human medicine could be extrapolated to apiculture. Several semi-synthetic and purely synthetic compounds were designed and synthesized to mimic this mode of action, and were subsequently tested on *N. ceranae*-infected bees in cage trial assays. Fumagillol, the basic hydrolysis product of fumagillin, as well as two semisynthetic fumagillin analogues and four other synthetic compounds exhibited activity against *N. ceranae*-infected caged bees. None of these compounds were, however, as effective as Fumagilin-B®. Commercially available thymol and enilconazole also exhibited

activity against *N. ceranae*, with thymol being the most promising chemical treatment other than Fumagilin-B®. In addition, high bee mortality was observed while evaluating Fumagilin-B®, and a series of cage trial experiments were thus conducted to evaluate the effect of fumagillin, Fumagilin-B® (fumagillin and DCH) and DCH on *N. ceranae*-infected bees. From these experiments it was observed that orally ingested DCH caused a statistically significant risk of increased bee mortality in *N. ceranae*-infected bees.

PREFACE

Some of the research for this thesis were conducted in collaboration with Dr. S.F. Pernal of Agriculture and Agri-Food Canada (AAFC) at the Beaverlodge Research Farm. He was assisted by Dr. A. Ibrahim, who was responsible for conducting the cage trial assays. All other research and activities toward this thesis are my own original work.

Dr. S.F. Pernal was my co-supervisor, with my main academic supervisor being Dr. J.M. Curtis. Dr. T.S. Thompson is a colleague who provided general advice and reviewed and critiqued all manuscripts that I wrote, prior to sending them out for review by the other co-authors.

Chapter 2 of this thesis has been published as:

> van den Heever, J.P., Thompson, T.S., Curtis, J.M., Ibrahim, A., Pernal, S.F. Fumagillin: An overview of recent scientific advances and their significance for apiculture, *J. Agric. Food Chem*. **2014**, *62*, 2728-2737.

I planned and wrote the entire manuscript, with the co-authors being responsible for review and approval of the manuscript before submission for publication.

Chapter 3 of this thesis has been published as:

> van den Heever, J.P., Thompson, T.S., Curtis, J.M., Pernal, S.F. (**2015**) Determination of dicyclohexylamine and fumagillin in honey by LC-MS/MS. *Food Anal. Methods* **2015**, *8*, 767-777.

I developed and validated the method as well as designed and synthesized the deuterated internal standard for DCH. I planned and wrote the entire manuscript, with the co-authors being responsible for the review and approval of the manuscript before submission for publication.

Chapter 4 of this thesis has been published as:

> van den Heever, J.P., Thompson, T.S., Curtis, J.M., Pernal, S.F. Stability of dicyclohexylamine and fumagillin in honey. *Food Chem*. **2015**, *179*, 152-158.

I designed and conducted the stability study experiment, with assistance in sample preparation from the following lab technicians: D. Tang, M. Siegenthaler, J. Kormanicki and R. Limanowka in the Chemistry Laboratory of the Agri-Food Laboratories Branch of Alberta Agriculture and

Rural Development in Edmonton. I planned and wrote the entire manuscript, with the rest of the co-authors being responsible for the review and approval of the manuscript before submission for publication.

Chapter 5 of this thesis is presented as originally submitted to Apidologie on 05 May 2015 for publication. The manuscript was subsequently divided into two parts, at the request of the journal, and accepted for publication as:

> van den Heever, J.P., Thompson, T.S., Otto, S.J.G., Curtis, J.M., Ibrahim, A., Pernal, S.F. Evaluation of Fumagilin-B® and other potential alternative chemotherapies against *Nosema ceranae*-infected honey bees (*Apis mellifera*) in cage trial assays. **2015**, *in press*

> van den Heever, J.P., Thompson, T.S., Otto, S.J.G., Curtis, J.M., Ibrahim, A., Pernal, S.F. The effect of dicyclohexylamine and fumagillin on *Nosema ceranae*-infected honey bee (*Apis mellifera*) mortality in cage trial assays. **2015**, *in press*

All cage trials, as well as preliminary statistical analysis, were conducted by Dr. S.F. Pernal and Dr. A. Ibrahim and their team at AAFC's Beaverlodge Research Farm, while the design of the experiments and synthesis of the semisynthetic and synthetic compounds were done by myself. The evaluation of the toxicity of DCH was also self-initiated, and I also supplied the chemicals required for this cage trial experiment. I planned and wrote the entire manuscript, while the final statistical analysis (**Chapter 5)** was conducted by Dr. S.J.G. Otto. The remaining co-authors being responsible for the review and approval of the two manuscripts before submission for publication.

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- Dr. Tom Thompson, my colleague, for his invaluable advice and support.
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- My current employment supervisor, Mrs. Norine Best, and my former supervisor, Mr. Don Noot, for their support and commitment to my research.
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Chapter 1 General Introduction

Honey has been a prized commodity since ancient times, with the oldest recorded evidence of honey collection being found in rock paintings from caves in Altamira in Northern Spain, dated between 8000 to 2000 BCE (Crane 1983). The earliest recorded evidence of beekeeping practices have been found on a stone bas-relief in the sun temple of Neuserre, situated in Abu Ghorab on the bank of the Nile River in Egypt, dated to 2400 BCE. This relief depicts honey being harvested and placed into containers (Crane 1983). The oldest known bee hives (1000 to 900 BCE) discovered during an excavation in the ancient town of Tel Rehov in Israel, were made from clay pots that formed part of a large apiary (Bloch et al. 2010). Modern commercial beekeeping however owes its existence to the development of the removable wooden frame that was developed by the Rev. L.L. Langstroth in 1851 (Johansson and Johansson 1967).

In addition to honey being a natural sweetener, it also possesses additional beneficial health and medicinal properties (Crane 1975). A recent example of a honey with proven *in vitro* antibacterial properties (ascribed to the chemical methylglyoxal) is Mānuka honey, which originates from the nectar of the Mānuka tree that is native to New Zealand and Southern Australia (Mavric et al. 2008). Mānuka honey was also approved for general wound management by the US Food and Drug Administration (FDA) in 2007 (Pieper 2009).

Current apicultural techniques have changed significantly since ancient times, keeping pace with scientific advances in chemistry and genetics, to name but a few. The bulk of honey produced today is through large-scale commercial beekeeping. Canada had 8,777 beekeepers with a combined number of 694,217 colonies. Canada produced 36,993,179 kg (81,556,000 lb) of honey during 2014 (Darrach and Page 2015). The province of Alberta was the largest honey producer, producing 41% of all of the honey in Canada. The total monetary value of honey alone amounted to CA $ 201,620,000 while the value of pollination is estimated at CA $ 4.4 billion. Canada was a net exporter of honey in 2014 with a value of CA $ 18,641,000. In total 64% of exported Canadian honey to the United States, followed by 33% to Japan and 3% to China (Darrach and Page 2015). The economic benefit of Canadian apiculture, including pollination, is therefore significant.

Current large-scale commercial apicultural practices have also created new challenges with regard to the control of disease outbreaks, which have the potential for significant financial loss to the beekeeper, as well as impacting the local economy. One important method used in controlling disease is through the use of pharmaceuticals. The major pharmaceutical treatments used in beekeeping worldwide, and the diseases they are used against, are summarized in recent research (Mullin et al. 2010; Reybroeck et al. 2012; Johnson et al. 2013). Chemical control of disease unfortunately also leads to the potential of residues of the applied chemicals being present in hive products, including honey. This is of concern to the consumer with regard to human health and the perceived quality of the honey.

The amount of chemical residues that are allowed to remain in a food product destined for human consumption, posing no adverse effects when consumed daily over the lifetime of an individual, is regulated by individual countries. Most, if not all countries have regulatory bodies that set maximum residue limits (MRL's) for known traces of agricultural pharmaceuticals and chemical contaminants in different food commodities. In Canada, MRL's are set by Health Canada, and are listed in parts per million (ppm). For consistency throughout, all MRL numbers were converted to ng g^{-1}, or parts per billion (ppb), to allow for ease of comparison. Health Canada also sets administrative maximum residue limits (AMRL's) for chemical contaminants that have been scientifically evaluated and for which a MRL has been established, but for which the official publication of the MRL has not yet been completed. Once the approved MRL is published, the AMRL becomes the MRL. Currently there are only two compounds registered for apicultural usage in Canada for which an MRL has been established in honey, namely oxytetracycline (**Figure 1**) which has an established MRL of 300 ng g^{-1} (Health Canada 2015a), and tylosin A (calculated as the sum of tylosin A and tylosin B) having a MRL of 200 ng g^{-1} (Health Canada 2015b). No Canadian MRL currently exists for fumagillin, which is registered for use in Canada under the trade name Fumagilin-B® (Medivet Pharmaceuticals Ltd., High River, Alberta, Canada; DIN 02231180). A Canadian MRL of 25 ng g^{-1} is being proposed for fumagillin only (Fishbein 2013). Fumagilin-B® however contains fumagillin as a salt of dicyclohexylamine (DCH), in a 1:1 stoichiometric ratio (**Figure 1**), which is not mentioned in this proposal. Worldwide no MRL has been established for either fumagillin or for DCH in honey.

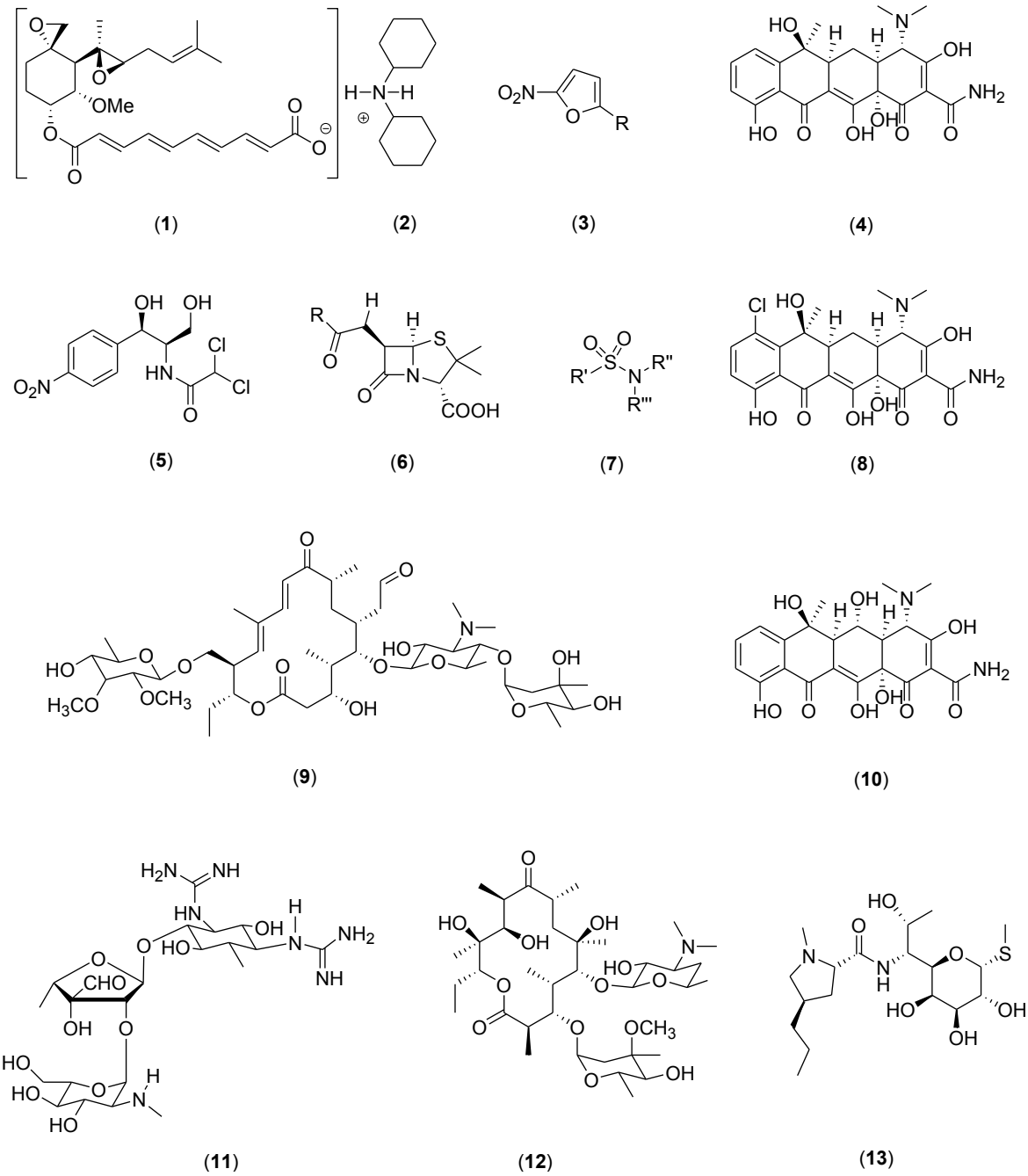


Figure 1 The chemical structures of fumagillin (**1**), dicyclohexylamine (**2**), 5-nitrofuran (**3**), tetracycline (**4**), chloramphenicol (**5**), penicillin (**6**), sulphonamide drugs (**7**), chlorotetracycline (**8**), tylosin (**9**), oxytetracycline (**10**), streptomycin (**11**), erythromycin (**12**) and lincomycin (**13**).

The concept of a Working Residue Limit (WRL) for chemical residues is a uniquely Canadian designation used for honey only (CFIA 2014). WRL's are established in collaboration with the

Canadian Food Inspection Agency (CFIA) and Health Canada to address the lack of requirements under the Food and Drugs Act and Regulations (FDAR) for drug submissions in minor species, which includes apiculture. WRL's are similar to MRL's, except that established chemical levels deemed safe for human consumption in other food producing animals (MRL's) are extrapolated to honey, where the same chemical compounds are expected to be found in honey, and for which no MRL exists in honey. These WRL's are not published and promulgated under the FDAR, and do not represent approval for their use, but serve only as a guide to the producers and the CFIA regarding usage levels. WRL's are only used within Canada. The current lowest Canadian AMRL's and MRL's in meats, along with the recommended WRL's in honey are given in **Table 1** (Health Canada 2015a), while the chemical structures of these compounds are displayed in **Figure 1**. These WRL's are lower for honey than in the meats, incorporating an additional safety margin, since actual data for apiculture is not currently available. The WRL's also apply to imported honeys, with testing and enforcement being conducted by the CFIA.

Table 1 Current lowest Canadian AMRL's or MRL's for antibiotics in meat, as well as the corresponding extrapolated WRL values for honey (Health Canada 2015a).

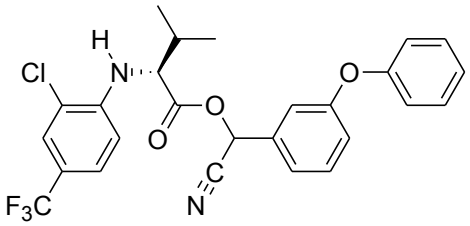
Drug Product	**Current Lowest AMRL/MRL ($ng\ g^{-1}$)**	**Recommended WRL ($ng\ g^{-1}$)**
Chlorotetracycline	100	30
Erythromycin	100	30
Lincomycin	100	30
Penicillin	10	3
Streptomycin	125	37.5
Sulphonamide drugs	100	30
Tetracycline	250	75
Tylosin[2]	200	60
Chloramphenicol	Banned substance	No WRL
5-Nitrofuran Compounds	Banned substance	No WRL

[2] Tylosin WRL values in this table are now superseded with the publication of a MRL of 200 ng g^{-1} for tylosin in honey on 28 Aug 2015 (Health Canada 2015b)

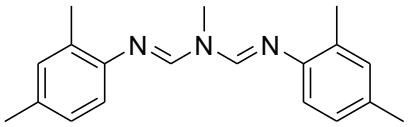
Within Health Canada there exists not only an MRL database for agricultural pharmaceuticals, but also a MRL database for pesticides in different food commodities (Health Canada 2015c). The pesticides with their respective established Canadian MRL's in honey are summarized in **Table 2**, while the chemical structures graphically displayed in **Figure 2**.

Table 2 A summary of Canadian pesticide MRL's in honey and related hive products (Health Canada 2015c).

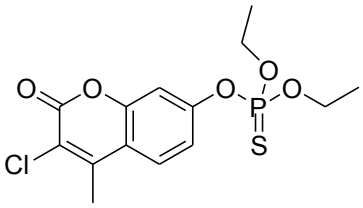
Pesticide Name	Food Commodity	MRL ($ng\ g^{-1}$)
tau-Fluvalinate	Honey	20
Amitraz	Honey	100
Coumaphos	Honey	20
	Honeycomb	100
	Beeswax	1000



(**14**)



(**15**)



(**16**)

Figure 2 The chemical structures of *tau*-fluvalinate (**14**), amitraz (**15**) and coumaphos (**16**), the allowable pesticide residues in Canadian honey.

Pesticides in honey and in other commodities are regulated under the Pest Control Products Act (PCPA), where the term Proposed MRL (PMRL) was previously used as the equivalent of the AMRL for the agricultural pharmaceutical residues that had established MRL values that have

not yet been officially published. PMRL's, upon being published then become Established MRL's (EMRL's). The term PMRL is still used, but the term EMRL was replaced with MRL. The different acts and terminology used, even within Health Canada, makes finding and evaluating residue information quite tedious and exceedingly difficult for producers and the public. The list of compounds that can be reasonably expected to occur as contaminants in honey is also not complete, which is understandable when considering the amount of work required to conduct risk assessments and to establish MRL's. The lack of suitable MRL's that encompass all possible chemical residues presents a significant problem for many agricultural commodities. Health Canada therefore employs a general MRL of 100 ng g^{-1} for agricultural residues that are not specifically defined for a given food commodity. Other countries such as Japan adopted a similar approach, with a general MRL value of 10 ng g^{-1} being used for undefined residues. It should be noted that this value is ten times lower than what is established for Canada. The EU does not have such a general MRL provision, although certain select compounds will have a general MRL specified in commodities not specifically mentioned. However, if a compound does not have a MRL in the honey, then that compound is considered to be a violation, and the honey may not be imported into the EU. Some countries, such as Japan, also have a MRL of 300 ng g^{-1} (The Japan Food Chemical Research Foundation 2014) for a combined class of compounds like the tetracyclines (tetracycline, oxytetracycline and chlorotetracycline).This practice is however not universally accepted, and the detection of an undefined or unknown chemical contaminant may lead to a product being refused entry into a jurisdiction that has a resulting "zero tolerance" policy. The recent change to a "zero tolerance" policy for tylosin-A and tylosin-B (desmycosin) residues in honey imported into Japan (The Japan Food Chemical Research Foundation 2014), as opposed to the previously acceptable limit of below 10 ng g^{-1} for combined tylosin-A and tylosin-B residues, is a good example this. This change effectively closes the Japanese market to any producer having detectable amounts of either tylosin-A or desmycosin in his honey.

The increasing sensitivity of analytical instrumentation used to detect trace levels of contaminants amplifies the problem of "zero tolerance" approaches, which may lead to artificial trade barriers that could unfairly exclude trade with other countries. This may then serve as a mechanism to help protect local industries. The difference in MRL requirements for jurisdictions

such as the European Union (Commission Regulation (EU) No 37/2010 2009), Canada, the USA and others complicate matters, because what is acceptable in one country may be in violation of another country's requirements.

The detection of any prohibited chemical contaminant in honey has serious long-term trade implications, as was witnessed by the suspension of honey imports from China into the European Union (EU) in 2002, upon the discovery of chloramphenicol and streptomycin residues resulting from their attempts to control American foulbrood disease (Reybroeck et al. 2012). The use of chloramphenicol and streptomycin in food producing animals was previously banned by the EU in 1994, thereby rendering imported honey containing these contaminants unsuitable for sale in the EU. The reputation of Chinese honey was severely damaged by the detection of these residues. Argentinian honey trade with the EU and North America also suffered a similar fate following the detection of 5-nitrofuran residues in Argentinian honey destined for the EU market (FAO/WHO 2004). In addition to antibiotics, pesticides like amitraz (100 ng g^{-1} Canadian MRL) are also applied against *Varroa destructor* Anderson and Trueman mites. There is also the possibility of environmental contamination of honey resulting from other agricultural or industrial sources. Testing for chemical contaminants in honey is therefore routinely conducted in almost all international trade in honey.

Another complication when monitoring chemical contamination of food commodities, including honey, is the importance of examining honey for the presence of other potentially bioactive degradation products or metabolites that may result from the application of the initial pharmaceutical formulation. The main biologically active compound of a formulated product may in some cases be unstable in the acidic honey, however their biologically active degradation products may be significantly more stable, and might be detectable for longer time periods. An example of this is tylosin-A in honey, where the degradation product, called desmycosin (tylosin-B), was reported to be more stable than tylosin-A in honey (Thompson et al. 2007a). Tylosin-B is reportedly detectable in honey, even when no trace of tylosin-A can be detected. Similarly, erythromycin reportedly rapidly decomposes in honey, affording several stable biologically active degradation products that can be detected in honey long after any trace of the parent compound is detectable (Thompson and van den Heever 2012). Recognizing the significance of biologically active degradation products, metabolites or pharmaceutical

excipients related to the commercial formulation of an applied product is therefore extremely important, as the resulting residues may have the potential to be even more significant than the main active ingredient in the commercial product (Kirkby et al. 1972). The synergistic effect of different chemical contaminants should also not be overlooked (Johnson et al. 2013; Zhu et al. 2014).

Even though there may be negative perceptions relating to the use and fate of agricultural pharmaceuticals in beekeeping and in other commodities, these compounds are indispensable to the success of modern apiculture. The main focus of my research was only on one of the many current bee diseases prevalent in apiculture, namely Nosema disease. Fumagilin-B® is currently the only effective treatment used to control Nosema disease, which is a microsporidian fungal infection of *Apis mellifera* L. caused by two distinct single cellular pathogens, namely *Nosema apis* and *Nosema ceranae*. It is important realize that fumagillin has been extensively used to control Nosema disease since its discovery in the 1950's. This extended usage of fumagillin makes the development of alternative chemical treatments desirable, should resistance to fumagillin develop in *N. apis* or *N. ceranae*. This inspired me to investigate alternative chemical treatments that could be used if fumagillin should fail.

An overview of the usage of fumagillin, as well as the importance of its related metabolites, degradation products and formulation is discussed in **Chapter 2**. While reviewing the available literature on fumagillin, I recognized the significance of the counter ion of the fumagillin salt, dicyclohexylamine (DCH), which is widely used in the commercial formulations (Fumagilin-B® and Fumidil-B®). This realization stimulated the further development of a LC-MS/MS method to detect DCH and fumagillin residues in honey, as described in **Chapter 3**. During subsequent analysis of domestically produced honey samples I observed that DCH appeared to be more stable than fumagillin in honey, which is evidenced by the higher concentration of DCH residues detected in honey compared to that of fumagillin. Both fumagillin and DCH are applied in equal amounts when using the commercial products. In order to investigate this, I designed and conducted an experiment to determine the relative stabilities of fumagillin and DCH in honey under a variety of conditions simulating storage, hive and shelf conditions, which is described in **Chapter 4**. I also attempted to find alternative chemical treatments against Nosema disease, as

described in **Chapter 5**. These alternative compounds were designed to mimic the mode of action of fumagillin against the MetAp-2 enzyme, known from human medicine.

Chapter 2 Fumagillin: An Overview of Recent Scientific Advances, and their Significance for Apiculture

2.1 Introduction

Fumagillin is a potent antibiotic isolated from *Aspergillus fumigatus* Fres. that has been applied against microsporidian infections and diseases in apiculture and in human medicine. In this review, in order to examine the importance and current usage of fumagillin in apiculture, it is necessary to first give a brief introduction to Nosema disease, the reason for the usage of fumagillin in beekeeping. The possible residues of fumagillin, its metabolites, degradation products and additives remaining in hive products destined for human consumption will then be discussed as they may have a significant impact on the health and safety of the consumer, as well as to apiculturists through occupational exposure. Furthermore, examination of the use of fumagillin and its analogues in human medicine reveals certain activities that may have relevance to apiculture. Finally, an overview of the toxicity and published analytical techniques for fumagillin will be presented to illustrate their importance to beekeeping, and possible deficiencies will be discussed.

2.2 Nosema disease in beekeeping

Nosema disease is one of the most prevalent diseases encountered in apiculture (Ellis and Munn 2005) and is now known to be caused by two species of single-cellular microsporidian parasites, *Nosema apis* Zander and *Nosema ceranae* Fries et al. (Genersch 2010). The phylum Microsporidia is comprised of more than 160 genera and about 1300 different species (Didier and Weiss 2008; Franzen 2008). Based on molecular evidence, microsporidia are now considered to be highly specialized parasitic fungi (Weiss et al. 1999; Sina et al. 2005).

The effects of *N. apis* are well documented (Fries 1993), with the organism first reported over a century ago (Zander 1909). In contrast, *N. ceranae* was described as a parasite of the Asian honey bee, *Apis cerana* Fab. as recently as 1996 (Fries et al. 1996). *N. ceranae* was suggested to be able to infect other *Apis* spp. (Fries 1997), with the first genetic confirmation of this reported when a *N. ceranae* isolate was obtained from *Apis mellifera* L. (Huang et al. 2007). The absence of host species boundaries for *N. ceranae* was further postulated after a study that compared

Nosema spp. isolates obtained from *A. mellifera* and from *A. cerana* across different geographical locations in Taiwan (Huang et al. 2008). While the earliest record of *N. ceranae* in *A. mellifera* appears to be from Africanized honey bees in Brazil (Weinstein Teixeira et al. 2013), discovery of this parasite in *A. mellifera* populations outside of Asia was first associated with samples collected in Spain in 2004 and 2005 (Higes et al. 2006). The parasite is now known to be commonly and widely distributed (Klee et al. 2007; Fries 2010; Higes et al. 2010; Martín-Hernández et al. 2012; Medici et al. 2012), and has subsequently been found in European samples of *A. mellifera* from as early as 1998 (Paxton et al. 2007). *N. ceranae* remained undetected in North America for several years after initial finds in Europe, however it has since been identified in analysis of historical samples dating from 1994 to 2007 (Klee et al. 2007; Chen et al. 2008; Currie et al. 2010) indicating that it may have been widespread and present much longer in this continent than first realized. Importantly, this "new" form of Nosema disease has been implicated in the large-scale loss of bee colonies not only in Europe, but also in North and South America (Cox-Foster et al. 2007; Martín-Hernández et al. 2007; Higes et al. 2008, 2009a, 2009b, 2009c; vanEngelsdorp et al. 2009; Botías et al. 2012a, 2012b; Martínez et al. 2012).

N. ceranae infections have also been detected in honey bee species other than *A. cerana* and *A. mellifera*. It was recently reported to occur in the dwarf Asian honey bee (*Apis florea* Fab.) and giant Asian honey bee (*Apis dorsata* Fab.) in Thailand (Chaimanee et al. 2010), as well as in *A. koschevnikovi* Enderlein in Borneo (Botías et al. 2012a). *N. ceranae* has also been detected in three native South American bumble bee species (Hymenoptera: Apidae), demonstrating its high virulence and ability to infect multiple bee species (Plischuck et al. 2009). Recent literature reports confirm the ubiquitous geographical presence of this invasive pathogen, its presence now being confirmed not only in Europe, Asia and North America (Williams et al. 2008b), but also in more remote geographical locations such as Australia (Giersch et al. 2009), South America (Plischuck et al. 2009) and North Africa (Higes et al. 2009b). Evidence suggests that *N. apis* is gradually being replaced by *N. ceranae* in *A. mellifera* (Martín-Hernández et al. 2012), though *N. apis* remains more prevalent in colder climates (Fries 2010; Forsgren and Fries 2013). Controversy regarding the greater relative virulence of *N. apis* compared with *N. ceranae* remains unresolved, with different results being obtained by different research groups

(Stevanović et al. 2010; Forsgren and Fries 2010, 2013; Martín-Hernández et al. 2012). Co-infection of *N. apis* and *N. ceranae* commonly occurs in *A. mellifera* (Paxton et al. 2007; Fries 2010) and has also been reported in *A. cerana* (Chen et al. 2009a).

N. ceranae infects the bee digestive tract midgut epithelial cells (ventriculus) of adult workers and queens (Higes et al. 2010; Traver and Fell 2012). Similar to *N. apis*, the mature spores burst forth by rupturing of the epithelial cells and spill into the midgut lumen, followed by defecation with the fecal matter. Infection spreads via fecal-oral route, where adult worker bees contract the infection while cleaning up fecal material originating from infected bees, or through trophallaxis of contaminated food (Higes et al. 2009a; Smith 2012). Infection with *N. ceranae* has been shown to increase precocious foraging in worker bees, resulting in reduced life expectancy by 9 days on average in cage trials when compared to control groups (Goblirsch et al. 2013a). Though outward signs of *Nosema* spp. infection cannot typically be seen, the inhibition of pollen digestion caused by the parasites leads to poor nourishment, smaller populations, reduced honey production and higher winter colony mortality (Bailey and Ball 1991). For more information on the biology of *N. ceranae* as honey bee pathogen, the reader is directed to recently published reviews (Fries 2010; Higes et al. 2010, 2013).

2.3 Fumagillin discovery and usage in apiculture, human medicine and in other agriculture

Fumagillin is a naturally occurring antibiotic compound that was first isolated in 1949 from an *Aspergillus* species, designated H-3 (Hanson and Eble 1949), later identified as *Aspergillus fumigatus* (Eble and Hanson 1951). The drug was also found to be a potent amebicide (McCowan et al. 1951). The structure of fumagillin was eventually elucidated (Tarbell et al. 1961) through an extensive series of chemical manipulations, including the hydrolysis of fumagillin to yield the alcohol, fumagillol (**Figure 3**).

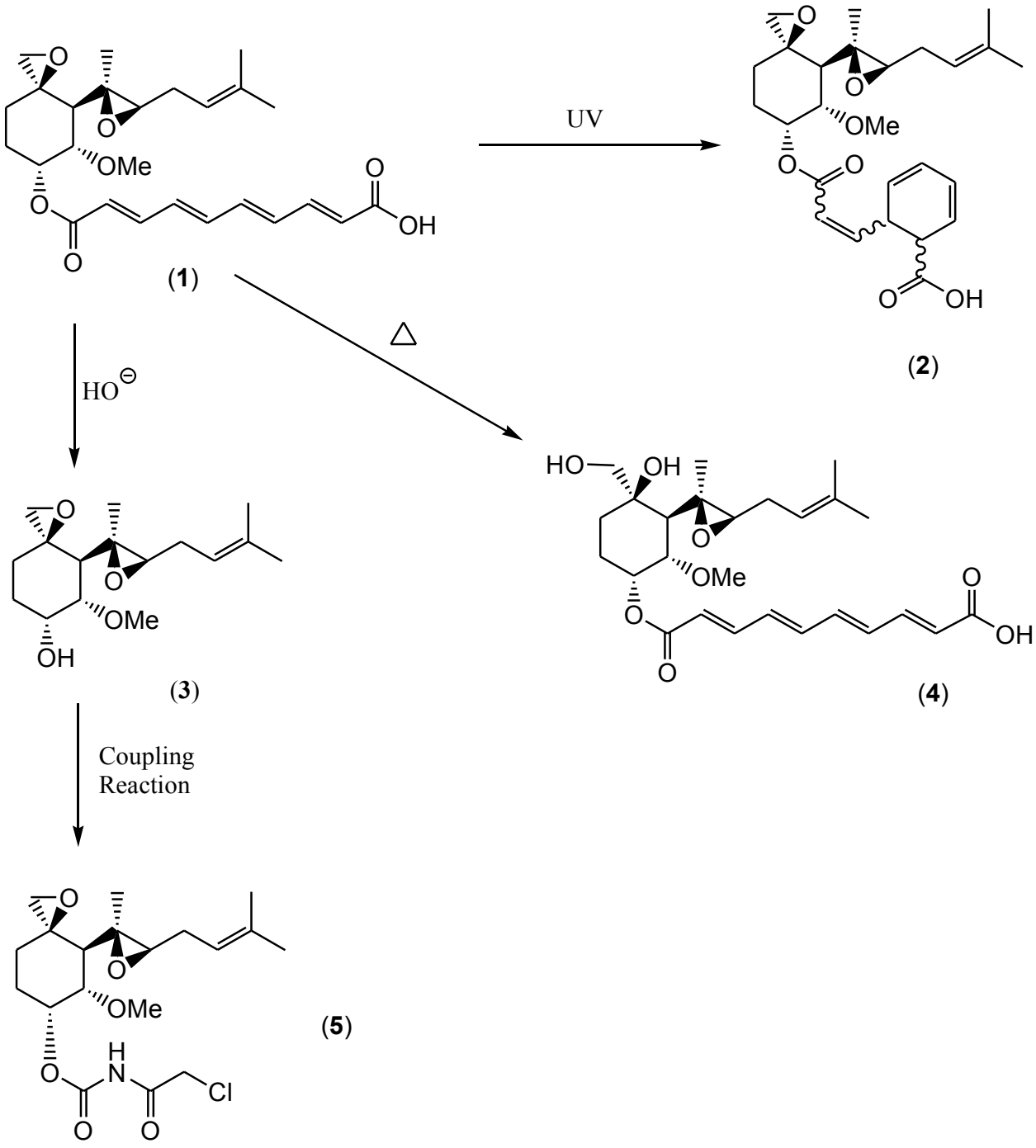
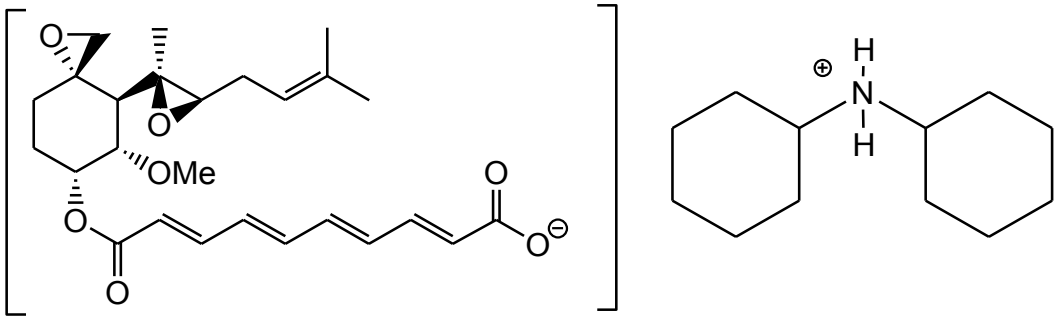


Figure 3 Fumagillin (**1**), UV-decomposed fumagillin (**2**), fumagillol (**3**), thermally decomposed fumagillin (**4**) and TNP-470 (**5**).

The importance of fumagillin as a treatment against the microsporidian fungal disease *N. apis* plaguing the European honey bee (*A. mellifera*) was soon recognized (Katznelson and Jamieson 1952; Bailey 1953). Fumagillin is also currently the only effective chemical treatment available to control *N. ceranae* (Williams et al. 2008a, 2011; Higes et al. 2011). The commercial formulation of fumagillin consists of the dicyclohexylamine (DCH) salt of fumagillin (**Figure 4**). The significance of the usage of fumagillin in the "salt" form is described in the section relating to the toxicity of fumagillin. Based on experimentation using cage bioassays it was nevertheless purported that fumagillin provides only short term suppression of *N. ceranae,* compared with *N. apis*, and that hyperproliferation of spores of the former results at specific and much degraded concentrations of the drug (Huang et al. 2013). Fumagillin was also tested for the treatment of microsporidian infections in fish (El-Matbouli and Hoffmann 1991; Kent and Dawe 1994; Molnar 1994; Rigos et al. 2000).



(**6**) (**7**)

Figure 4 Fumagillin, CAS 23110-15-8 (**6**) as the DCH CAS 101-83-7 (**7**) salt.

In human medicine, fumagillin is used as an inhibitor of microsporidian infections in patients with compromised immune systems due to acquired immunodeficiency syndrome (AIDS), or to relieve symptoms of intestinal microsporidiosis that may occur after organ transplant procedures (Molina et al. 2000, 2002; Lanternier et al. 2009). More interestingly though, fumagillin and its analogues are used to treat various cancers by inhibiting the formation of new blood vessels around growing tumors (angiogenesis) thereby limiting their blood supply (Ingber et al. 1990).

The treatment of cancer tumors by inhibition of angiogenesis was first proposed in 1985 (Folkman 1985; Folkman and Shing 1992). The discovery that fumagillin inhibits angiogenesis led to a renewed interest in fumagillin as a therapeutic drug (Ingber et al. 1990). The mechanism responsible for this activity was not clear, until the discovery of fumagillin binding to the methionine aminopeptidase type 2 (MetAP-2) protein (Griffith et al. 1997; Sin et al. 1997). The exact binding of fumagillin to the MetAP-2 enzyme was determined by crystallizing the enzyme with fumagillin covalently bound to the enzyme active site (Liu et al. 1998). A crystallographic study then revealed that fumagillin covalently binds to a histidine moiety (His^{231}) of the enzyme, resulting in an irreversible opening of the spiro-epoxide on the cyclohexane core skeleton of fumagillin (Sin et al. 1997; Liu et al. 1998; Zhang et al. 2002).

This result prompted numerous research efforts aimed at modulating the mode of action (Han et al. 2000; Weiss et al. 2003) by preparing analogues of fumagillin via modification of the alkene side chain. First, fumagillin was hydrolyzed with a suitable base, thereby removing the side chain to yield fumagillol (**Figure 3**). Subsequently, a new chemical moiety could be coupled to fumagillol (Han et al. 2000; Baldwin et al. 2002; Lee et al. 2007), resulting in analogues like TNP-470 (**Figure 3**), which has shown promise as a potential new treatment for malaria (Zhang et al. 2002; Chen et al. 2009b). Another analogue of fumagillin, named fumarranol, with the spiro-epoxide opened, exhibited an 80-100 fold lower activity than TNP-470 against malaria (Chen et al. 2009b). The low activity of fumarranol indicates the importance of the intact epoxide for biological activity of the fumagillin family of analogues. A 1000-fold decrease of MetAP-2 inhibition by fumagillin upon the opening of this cyclohexane ring spiro-epoxide was observed (Griffith et al. 1997, 1998; Lu et al. 2006).

Fumagillin binds only to the MetAP-2 enzyme via the epoxide group located on the cyclohexane core ring structure, and not to the MetAP-1 enzyme (Sin et al. 1997). The other remaining epoxide on the molecule is not crucial for the binding to take place, and is therefore considered dispensable (Griffith et al. 1998).

MetAP-2 enzymes are found ubiquitously in all organisms (Zhang et al. 2002). Fumagillin acts against microsporidian as well as the mammalian MetAP-2 enzyme, and the low selectivity of fumagillin between human and microsporidian MetAP-2 is the cause of its toxicity to humans, as

it also inhibits the human MetAP-2 enzyme necessary in protein maturation and post translation processes (Drahl et al. 2005; Huang et al. 2013). A similar observation was recently reported where it was shown that fumagillin is active against honey bee MetAP-2 at low concentrations while it has no therapeutic activity at those concentrations against *N. ceranae* MetAP-2 (Huang et al. 2013). The fact that fumagillin acts against both the disease and bee MetAP-2 may explain the significant bee mortality associated with fumagillin usage (Rada et al. 1997). An earlier study supports this increased mortality associated with fumagillin usage (Furgala and Boch 1970). In this study it was observed that fumagillin does not influence the mortality of healthy bees during cage trials, but when *N. apis*-infected caged bees are treated with fumagillin, at concentrations of 12.6-50 mg L^{-1} in sugar syrup, increased bee mortality was observed. Even though fumagillin degrades over time (Kochansky and Nasr 2004; Nozal et al. 2008), the repeated seasonal treatment with fumagillin that is required to control reestablishment of *N. ceranae* infections (not necessary for *N. apis* control) ensures that multiple generations of honey bees are exposed to low levels of fumagillin (Higes et al. 2011; Huang et al. 2013). It is postulated that this constant low level of fumagillin in the hive creates conditions conducive to hyperproliferation of *Nosema* spp. (Huang et al. 2013). In comparing the MetAP-2 fumagillin binding site and coordination site amino acid sequences among humans, honey bees, *N. apis*, *N. ceranae* and *Nosema bombi* (*Nosema* infection of bumble bees – *Bombus* spp.), sequences were found to be identical for honey bee and human MetAP-2, and were identical amongst the microsporidia . *Nosema* spp sequences differed from honey bees and humans at only two binding site amino acids (Huang et al. 2013). The authors also speculate that the MetAP-2 enzymes may not be the only factor influencing response to fumagillin.

Semisynthetic analogues of fumagillin exhibit different properties from fumagillin regarding their potency, selectivity and toxicity, and it has been shown that the potency of fumagillin against MetAP-2 depends not only on the covalent interaction with the spiro-epoxide, but also on more complex non-covalent interactions necessary for molecular recognition of the target drug by the enzyme (Arico-Muendel et al. 2009).

2.4 Toxicity of fumagillin

It is important to note that the key purpose in creating new analogues of fumagillin has been the need to limit the human toxicity associated with fumagillin, while retaining or enhancing the beneficial properties of the parent compound (Killough et al. 1952; Ingber et al. 1990; Didier 1997, Didier et al. 2006; Conteas et al. 2000).

Several contradictory findings have been reported in the literature regarding the toxicity of fumagillin. It is important to know that the commercial formulations of fumagillin (Fumagilin-B® and Fumidil-B®) contains the dicyclohexylamine (DCH, DCHA) salt of fumagillin in a 1:1 stoichiometric ratio with fumagillin. When evaluating the possible adverse effects of a compound, any additive or contaminant should also be taken into account, regardless of its concentration, as the contribution to properties such as toxicity or mutagenicity from the additive may be more significant than what is expected (Kirkby et al. 1972). The cited study actually used the potential carcinogenicity of DCH contamination in a cyclamate study as an example of such an effect.

It is also interesting to note that most studies on the toxicity of fumagillin itself were conducted using the fumagillin DCH salt as reagent, thereby introducing not only one, but two potentially toxic compounds (Didier et al. 2006; Stanimirović et al. 2006, 2007a, 2007b , 2010; Stevanović et al. 2006; 2008; Kulić et al. 2009). Experiments using the fumagillin analogue TNP-470 (AGM-1470) (**Figure 3**), appear to use only the pure TNP-470 in toxicity testing with no DCH included (Yamaoka et al. 1993a; Yanase et al. 1993; Didier 1997; Didier et al. 2006). In other studies it is unclear whether fumagillin was used as the DCH salt, free acid, or as an alternative formulation (Liu 1990; Kusaka et al. 1991; Rada et al. 1997), although one study was found that refers to the use of purified fumagillin with no DCH present (Ingber et al. 1990). It is important to realize this fact when the reported data on the toxicity of fumagillin, or the more frequently employed fumagillin DCH salt, is evaluated.

The toxicity of DCH in isolation has been investigated (Stoltz et al. 1970) and DCH was found to induce chromosomal damage in human leucocyte cultures at low concentrations of 10^{-3} to 10^{-5} M, over periods ranging from 5 to 25 hours. Stoltz et al. (1970) also reported that *N*-hydroxycyclohexylamine, a metabolite of DCH, exhibited equivalent toxicity. A study using a

Salmonella enterica serovar *Typhimurium* strain (TA1535/pSK1002) to verify the genotoxicity of DCH yielded negative results (Heil et al. 1996). Nevertheless, the same study yielded positive results when using an *in vivo* alkaline filter elution test (AFE) with freshwater clams as test subject to detect DNA damage. *Salmonella* mutagenicity tests also proved negative for DCH in another study (Mortelmans et al. 1986). Similar negative results were reported where DCH only exhibited a positive carcinogenic response in one out of six tests (Purchase et al. 1978). DCH was also reported to be a powerful inhibitor of spermidine synthesis catalyzed by extracts from *E. coli* and *P. aeruginosa in vitro* (Pegg et al. 1983). A national screening program in Sweden determined that DCH is toxic to rats by several modes of action, but no mutagenic properties were reported (Woldegiorgis et al. 2007). According to the MSDS (Sigma-Aldrich product 185841 v5.0 07/24/2012), DCH exhibits serious immediate toxic effects on rats with an LD_{50} of 373 mg kg^{-1} orally. Similar results were reported with a LD_{50} of 200-373 mg kg^{-1} (Greim et al. 1998). According to the MSDS, DCH is also extremely toxic to aquatic life and should not be released into the water system. It is also tumorigenic causing gastrointestinal and liver tumors (orally) as well as being subcutaneously carcinogenic in mice, causing tumors at the site of application (Pliss 1958). In contrast, the MSDS of pure fumagillin (Sigma-Aldrich product F6771 v4.5 08/04/2014), states a LD_{50} of 2000 mg kg^{-1} orally in mice, making DCH at least five times more toxic than fumagillin.

Fumagillin toxicity was extensively examined by Stanimirović et al. (Stanimirović et al. 2006, 2007a, 2007b, 2010; Stevanović et al. 2006, 2008, 2010; Kulić et al. 2009). In these reports it is sometimes unclear whether fumagillin DCH, or DCH alone was used in their experiments as their use of the terms fumagillin and dicyclohexylamine appear to be equivalent in earlier publications (Stanimirović et al. 2006; Stevanović et al. 2006). The authors also ascribed the dicyclohexylamine (DCH) toxicity data (Yamaoka et al. 1993b) as being that of fumagillin (Stanimirović et al. 2006; Stevanović et al. 2006; Kulić et al. 2009), while Stoltz et al. (1970) clearly only examined DCH, with no mention of fumagillin ever being made. These inconsistencies, however, should not detract from the value of their research, which is briefly summarized below.

Fumagillin was observed to exhibit significant negative chromosomal aberration effects at 50-75 mg kg^{-1} bodyweight in mice. Concentrations of 25, 50 and 75 mg kg^{-1} bodyweight were

administered by gavage (Stanimirović et al. 2006). All experimental dosages listed above induced significant antiproliferative and genotoxic potential in mice (Stevanović et al. 2006). Fumagillin exhibited clastogenic activity in human lymphocites at concentrations equivalent to the therapeutic dose in beekeeping (Stevanović et al. 2008). Genotoxicity to mouse bone marrow cells at concentrations of 10-20 mg kg^{-1} bodyweight administered *in vivo* to mice by gastric probe (5, 10 and 20 mg kg^{-1} concentrations tested), as compared to a control group was also observed (Kulić et al. 2009). These results were confirmed with fumagillin-induced chromosomal aberrations at 10–20 mg kg^{-1} in mouse bone marrow cells (Stanimirović et al. 2010). In summary, the Stanimirović group concluded that fumagillin DCH is a mutagenic formulation, both *in vitro* and *in vivo*.

Fumagillin was evaluated in the United Kingdom by the Committee on Mutagenicity in 1999, with the aim of establishing a maximum residue limit (MRL) in honey, after a submission by the patent holder, CEVA Animal Health (COM 2009a, 2009b). No MRL recommendation could be made by the Committee on Veterinary Medicinal Products because the available toxicity data at that time was considered to be insufficient to draw any conclusions on the risk. In 2011, it was concluded that fumagillin should be considered as an *in vitro* mutagen, but not an *in vivo* mutagen (COM 2011). Fumagillin is still not licensed for general use in beekeeping in Europe, except in exceptional circumstances where a temporary authorization to use it under veterinary supervision will be allowed (Higes et al. 2011). Fumagillin is registered for use in the USA and Canada to treat Nosema disease. Temporary authorization has been issued in exceptional conditions to use fumagillin in the UK, Spain, Belgium, Greece, Hungary and in Romania under veterinary supervision (Higes et al. 2011).

Although fumagillin is an extremely beneficial compound in human medicine and in apiculture, some undesirable side-effects cannot be ignored (Ingber et al. 1990; Yanase et al. 1993; Didier 1997; Conteas et al. 2000; Molina et al. 2000, 2002; Didier et al. 2006). Extended usage of fumagillin over prolonged periods of time, as required by chemotherapy, caused severe body weight loss of over 15% from the starting weight in the human test subjects (Yanase et al. 1993). In 1952, it was reported that fumagillin was essentially non-toxic to humans at oral doses of up to 50 mg daily for durations of two weeks of treating intestinal amebiasis (Killough et al. 1952), although no weight loss was observed in test subjects. A more recent study administered

fumagillin orally up to 60 mg daily for two weeks to treat microsporidiosis in patients with HIV infection (Molina et al. 2000). The authors acknowledged significant bone marrow toxicity of fumagillin, with 4 patients out of a group of 11 developing severe toxic side effects at the highest dosage administered (60 mg). These effects ceased within days of the treatment being terminated.

A common side-effect in human trials where fumagillin is administered orally is gastrointestinal-related cramping, diarrhea and significant loss of body weight (Yanase et al. 1993). This undesirable weight loss side effect prompted recent, perhaps ethically questionable, trial use of fumagillin as a chemical mitigation for obesity (Lijnen et al. 2010; Scroyen et al. 2010).

In beekeeping, potential toxic and undesirable consequences of fumagillin treatment have also been examined, with a limited number of effects being documented. For example, unique changes in the ultrastructure of the hypopharyngeal glands in worker bees have been observed after infection with *N. apis* followed by subsequent treatment with fumagillin (Liu 1990). Such alterations may influence protein secretions from these glands, though the role of fumagillin treatment on gland structure and function remain speculative from this descriptive study. Fumagillin was also noted to have a significant influence on bee mortality during a cage trial experiment where honey bees (150–200 bees per cage) were fed a sugar syrup solution for 7 days using a total dose of 140 mg of fumagillin per cage, compared with unmedicated controls (Rada et al. 1997). In contrast, a later study indicated few to no deleterious effects of fumagillin (50 mg L^{-1} fumagillin in sugar syrup) for caged *N. apis*-infected bees, also over a seven day period (Webster 1994). This concentration is also double that currently recommended for the only commercial formulation of the drug registered in North America for apiculture (Fumagilin-B®, Medivet Pharmaceuticals Ltd., DIN 02231180). Label directions for this product prescribe 9.5 g fumagillin DCH base to prepare 380 litres of syrup (25 mg L^{-1}) which is sufficient to treat 100-110 colonies (86-95 mg per colony) in the spring, or 50 overwintering colonies (190.5 mg per colony) in the fall.

The majority of other reported cage experiments that evaluated bee mortality associated with fumagillin usage has found few effects. No statistically observable increase in mortality was observed when feeding sugar syrup at concentrations of 12.6, 25.2 and 50.4 mg L^{-1} *ad libitum*

over 17 days to bees not infected by *N. apis* (Furgala and Boch 1970). Field trials on overwintering bee colonies infected with *N. apis* in Ontario, Canada showed that fumagillin treatments in the fall (25 mg L^{-1}, 440 mg per colony) significantly increased colony survival, and did not harm bees at the colony level (Furgala and Boch 1970). In a Polish study, fumagillin (56.8 mg L^{-1}, 71 mg per colony) increased the unit honey productivity (+19%), brood production (+20%), surplus honey production (+58%) and the lifespan of worker honey bees (+20%), with no observable negative effects for *N. apis*-infected colonies in the spring (Woyke 1984). A study from Alberta, Canada recommended fall treatments with fumagillin at increased amounts of fumagillin (22.2 mg L^{-1}, 300-400 mg per colony), to effectively treat *N. apis* infection in the fall with no noted harmful side-effects (Szabo and Heikel 1987). Improvements in colony survival were similarly noted for fall sugar syrup treatments containing fumagillin at lower dosages (26.4 mg L^{-1}, 200 mg per colony), also under Canadian wintering conditions (Furgala and Gochnauer 1969a). Sugar syrup applications of the drug require a longer period for bees to consume, and hence have a longer duration of treatment, compared with dusting or pollen patty applications which are more suitable as spring treatments (Furgala and Gochnauer 1969a).

Though previous studies have concluded that fumagillin has suppressed infections and reduced mortality of *Nosema* spp. infected bees in cages (Furgala and Boch 1970; Webster 1994) and colonies (Furgala and Gochnauer 1969b; Furgala and Boch 1970; Woyke 1984; Szabo and Heikel 1987) a recent report suggests that fumagillin has negative effects on bee health and leads to the hyperproliferation of *N. ceranae* spores (Huang et al. 2013). It is difficult to directly compare this with older studies because of differences in methods used to evaluate the effects of fumagillin on the parasites and bees. Previous research has evaluated bee mortality specifically at high therapeutic concentrations of fumagillin using spore counts as an indicator of the efficacy of treatment, while harmful effects on bees have been measured as differential survival of bees or colonies compared with infected, untreated controls. In Huang et al. (2013), deleterious effects on bees were confirmed by alterations in structural and metabolic midgut proteins, notably at concentrations that did not suppress microsporidia reproduction. Though the study did use spore production as a measure of fumagillin efficacy, concentrations at which hypoproliferation of spores were seen were at very low, sub-therapeutic levels of fumagillin that were not examined in other studies. Such levels were estimated to represent the degraded concentrations of

fumagillin 2 to 5.5 months after cessation of treatment to a colony (Huang et al. 2013). Previous work examining the effects of degraded fumagillin-medicated syrup, after 44 months of storage, showed clear suppression of *N. apis* infections in caged bees with no significant deleterious effects on bee mortality (Furgala and Gochnauer 1969a).

The difference between test methods employing controlled environments as in cage trials, and field trials where colonies are exposed to other environmental influences, also make direct comparison amongst studies difficult. Field trial studies are more prone to the effects of environmental contamination by pesticides for example, making bees more susceptible to *N. ceranae* infection (Pettis et al. 2013). Furthermore, synergistic effects exhibited by some pesticides on fumagillin (Johnson et al. 2013) have been observed. The effect of other stress factors (Williams et al. 2011) and co-infection by pathogens, such as the deformed wing virus, further complicates the interpretation of results (Martin et al. 2013). A recent publication that details a comprehensive standard approach to Nosema disease research is of interest, as it will simplify the comparison of results obtained by different research groups (Fries et al. 2013).

2.5 Chemical analysis and stability of fumagillin in the field and under laboratory conditions

Mammalian toxicity and mutagenicity of fumagillin is well reported, and therefore it is important that the amount of fumagillin residues in honey intended for human consumption be established. The proposed structures of the main thermal and UV degradation products of fumagillin are shown in **Figure 3**. UV degradation products involve isomerization and cyclization of the alkene side chain, while the epoxide on the main cyclohexane skeleton of the active moiety remains intact (Kochansky and Nasr 2004; Nozal et al. 2008). Thermal degradation results in the opening of the epoxide situated on the cyclohexane ring. A combination of thermal and UV degradation is likely to occur during use. It is important to note that the UV decomposition products of fumagillin retain their biological activity (Kochansky and Nasr 2004), while the thermally degraded fumagillin does not (Higes et al. 2011). The hydrolyzed product (fumagillol) also retains some biological activity, albeit only about 10% of that of fumagillin (Gochnauer and Furgala 1962).

Several analytical methods for the detection of fumagillin have been reported. In 1988 a reversed-phase HPLC-UV method was reported to analyze fumagillin in acetonitrile solutions using a UV detection wavelength of 351 nm, which corresponds to the maximum UV absorption of the alkene side chain of the molecule, and a detection concentration range of 0.000-0.035 mg mL^{-1} (Brackett et al. 1988). Interestingly, it was found that 254 nm UV light does not cause fumagillin to degrade, but that 336 nm and 351 nm UV light (fumagillin λ_{max} 351 nm) and fluorescent room lights does cause degradation. Samples in that study were prepared in acetonitrile, and it is not clear if pure fumagillin, or a salt form of fumagillin was used. An ELISA method with a limit of detection (LOD) of 20 ng g^{-1} and a HPLC UV method (350 nm detection wavelength) with a LOD of 100 ng g^{-1} for quantitation of fumagillin and its degradation products in honey were developed (Assil and Sporns 1991). Honey samples were prepared by dissolving 5.0 g of honey in equal amounts first of water (25 mL), and then dilution with acetonitrile (25 mL). Assil and Sporns (1991) also describe the isolation of pure fumagillin with the elimination of the DCH and other formulation compounds from the commercial product. The authors also identify the UV-cyclized product which they called “neofumagillin”, as a further potential contaminant of honey.

Fumagillin was quantitated in rainbow trout muscle tissue, where it was tested to control Myxosporidiae, by using ion-pairing liquid chromatography with UV (351 nm detection wavelength) detection (Guyonnet et al. 1995). Fish muscle tissue (1 g) was macerated in the presence of 8mL acetonitrile-water mixture (2:6 v/v) with sample cleanup and concentration done using Bond Elut Octyl C_8 cartridge columns to give a method that has linear range of 20 – 1000 ng g^{-1}.

The photostability of fumagillin was investigated, and a HPLC method reported using a UV method for detection at 350 nm (Kochansky and Nasr 2004). The investigators found fumagillin to be extremely unstable when exposed to sunlight or fluorescent laboratory lighting in 50% ethanol solutions, with half-lives of fumagillin determined as being in the range of seconds to minutes. Nevertheless, the activity of UV-irradiated fumagillin in syrup solutions did not reduce its efficacy against Nosema disease significantly, with *N. apis* being identified as the microsporidian used in the efficacy experiments.

Fumagillin and its UV and thermal decomposition products were quantitated by LC-diode array-electrospray ionization mass spectrometry (Nozal et al. 2008). The method that the researchers developed to purify and concentrate fumagillin from the honey matrix using a polymeric solid phase extraction (SPE) technique forms the basis for most of the subsequent published analytical methods (Higes et al. 2011). Method detection limits ranging from 1 – 24 ng g^{-1} (for light coloured honeys), to 4 – 45 ng g^{-1} for darker coloured honeys are reported. The researchers also note that honey samples to be analyzed for fumagillin can be stored at room temperature for one month in the dark without influencing the fumagillin results significantly.

A multiclass LC-MS/MS method to determine a variety of antibiotics in honey, including fumagillin, was developed (Lopez et al. 2008). It was noted that fluorescent lights and sunlight accelerate fumagillin decomposition, and that sodium laboratory lights were preferred for fumagillin analysis. The method LOD for fumagillin was reported as 60 ng g^{-1}, and was used to determine several other classes of compounds including tetracyclines, fluoroquinolones, macrolides, lincosamides, aminoglycosides, sulfonamides and phenicols. Sample cleanup was done using the same SPE cartridges described earlier (Nozal et al. 2008), but sample elution consisted of a very laborious process with multiple elutions of the samples off the SPE cartridges, followed by concentration and reconstitution steps, making this very time consuming.

A rapid LC-MS/MS method quantitating fumagillin using a QuEChERS (quick, easy, cheap, effective, rugged and safe) sample preparation technique in conjunction with an Oasis mixed-mode weak anion-exchange SPE cartridge for concentrating fumagillin was reported, with a reported LOQ of 0.1 ng g^{-1} (Kanda et al. 2011). The additional QuEChERS step adds 30 minutes to the extraction, since it is still followed by an SPE extraction.

The stability of fumagillin under various laboratory and field conditions pertaining to its thermal and UV degradation was reported (Higes et al. 2011). It was shown that higher temperatures (40 ^{o}C) caused rapid thermal degradation of fumagillin in sugar syrup application solutions, with resulting loss of its activity. Fumagillin was undetectable in sugar syrup solutions after 20 days at this temperature. A stability study of fumagillin under various temperature conditions in the presence and absence of light was also conducted, confirming the sensitivity of fumagillin to light that is amplified by higher temperatures (Higes et al. 2011).

The most recent published method of fumagillin analysis in honey by LC-MS/MS (Dmitrovic and Durden 2013) is an optimized version of a previously published method (Nozal et al. 2008). This study provides useful information for laboratory analysis, demonstrating that fumagillin solutions stored in acetonitrile at 4 ^{o}C are stable for 6 months (Dmitrovic and Durden 2013). The authors report a LOD of 0.368 – 0.522 ng g^{-1}, and a LOQ of 1.564 – 1.112 ng g^{-1} in honey, depending on the brand of analytical instrumentation used. They also observed that performing the SPE cleanup and sample concentration step under gravity elution yielded better sample recoveries.

None of the analytical methods for fumagillin published to date recognize the importance of the presence of DCH as a contaminant in honey or other hive products (like wax), although the presence of DCH in solvent samples originating from the formulation is recognized in a recent report (Dmitrovic and Durden 2013).

2.6 Future outlook

It could be hypothesized that the mode of action of fumagillin against the two *Nosema* spp. in question is similar to that observed in humans against the MetAP-2 enzyme. It is important to note that fumagillin will also act in a similar fashion on the MetAP-2 enzyme of the host honey bee (Huang et al. 2013). The intact epoxide on the cyclohexane ring of fumagillin is crucial for its observed activity, but the rest of the molecule is also important for enzymatic recognition. This observation is supported by the reports in the beekeeping literature (Kochansky and Nasr 2004; Higes et al. 2011). A potential strategy for the design of simpler and more cost effective compounds that mimic the chemical behavior of fumagillin (Balthaser et al. 2011) using the cyclohexane spiro-epoxide as a scaffold should be considered. The development of new compounds to treat Nosema disease in apiculture is of great importance, owing to the fact that fumagillin has been extensively employed in human medicine and in apiculture almost since its discovery in the early 1950s. Although there is no proof in apiculture to indicate that *Nosema* spp. have developed resistance to fumagillin, it has been reported that *Nosema* spp. infecting diamondback moth, *Pluetella xylostella* L., have developed resistance to fumagillin (Fumidil-B®), although the identity of the *Nosema* spp. was not established (Idris et al. 2000, 2001). Given this finding, it can be speculated that *Nosema* spp. resistance to fumagillin in

apiculture may develop in the future, owing to the prolonged and intensive utilization of fumagillin dating back to the 1950's.

Numerous different fumagillin toxicity evaluations, employing direct *in vitro* testing against isolated cancer cell lines and *in vivo* testing using test organisms such as mice and rabbit with the DCH salt form of fumagillin have been reported, as discussed in the section relating to the toxicity of fumagillin. In early human trials no observable side-effects are noted in some studies, while significant weight loss and toxic effects were observed in others. A recent report (Huang et al. 2013) proposes that fumagillin may influence the MetAP-2 enzymes in the host and in the pathogen differently due to the slight differences in the amino acid sequence of the binding site portion of the enzymes in *Nosema* spp. as compared to that in honey bees. It remains unclear whether the reduced toxicity of fumagillin and its analogues is due to inherent properties of these analogues themselves, or to the fact that the toxicity contribution arising from the DCH is not accounted for in reports to date.

It is also important in the evaluation of toxicity studies to take into account the inter-species differences when extrapolating animal and other data to humans (Bajić et al. 2004). *In vitro* testing is also not equivalent to *in vivo* human testing when using human and other cell cultures or bacteria such as *Salmonella* spp. When all the currently available information is evaluated, it is evident that fumagillin is indeed cytotoxic and mutagenic *in vitro*, and that it has undisputed negative effects in humans such as short term toxicity, weight loss and diarrhea. The establishment of a MRL for fumagillin and DCH in honey and other hive products is needed. Caution should be exercised by beekeepers during fumagillin sugar syrup solution preparation, since it could lead to unnecessary exposure of beekeepers to elevated levels of fumagillin and DCH through dermal contact, and through breathing of airborne fumagillin dust.

However, a significant concern is not only the fumagillin toxicity, but also that of DCH, which is present in equimolar amounts to fumagillin in the commercial products. As discussed in the fumagillin toxicity section, it is evident that DCH is at least five times as toxic as fumagillin, comparing only the LD_{50} values, and disregarding any additional negative effects. All published analytical methods to date only evaluate the presence of fumagillin, and in a few instances one or two of the major metabolites or degradation products, in honey. It is known that fumagillin

degrades within days to weeks in honey (Higes et al. 2011), and that there is a low likelihood of fumagillin being detected in significant quantities in honey if it is applied well before the extraction process.

The contribution of DCH present in the commercial fumagillin formulation to the overall toxicity and resulting food safety implications in products destined for human consumption is definitely not negligible (Kirkby et al. 1972). None of the analytical methods for analyzing fumagillin and metabolites in honey published to date even mention the presence of DCH as an analyte of concern. This is a significant oversight, since the potential presence of DCH in hive products is clearly of significance for human health and food safety.

The industrial use of DCH as a corrosion inhibitor and in the rubber industry (vehicle tires) could result in a background level of this compound being present in hive products, especially in hives located close to main roads and areas of higher vehicle use (Woldegiorgis et al. 2007). This potential background level of DCH contamination needs to be further investigated to determine whether it is a real cause of concern. More importantly, the stability and presence of DCH in bee products such as honey and wax also needs to be investigated. There is a clear need for the inclusion of the analysis of DCH relating to usage of fumagillin in hive products.

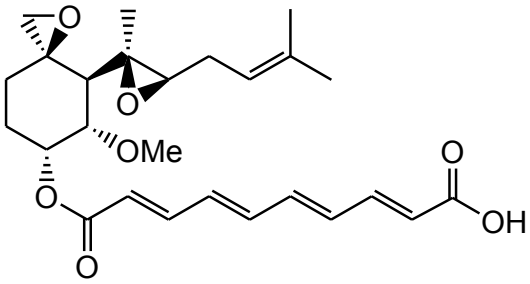
Despite all the potential shortcomings and detrimental aspects relating to fumagillin's usage in beekeeping, and their resulting implications for bee and human health, it is important to note that this is still the only effective chemical treatment currently available to treat Nosema disease. The potential contamination by DCH of hive products destined for human consumption could be eliminated by exploring alternative formulation methods of fumagillin, or through the development of alternative chemical treatments utilizing the current knowledge regarding the proposed mode of action of fumagillin against *Nosema* spp.

Chapter 3 Determination of dicyclohexylamine and fumagillin in honey by LC-MS/MS

3.1 Introduction

Fumagillin (**Figure 5**) is a fungal metabolite first isolated from *Aspergillus fumigatus* Fres. (Hanson and Eble 1949). Soon after its discovery, fumagillin was successfully used to control the microsporidian fungal disease caused by *Nosema apis* Zander infections (Katznelson and Jamieson 1952; Bailey 1953) plaguing the European honey bee (*Apis mellifera* L.). A related, emerging, but epidemiologically different disease caused by *Nosema ceranae* Fries et al. is implicated in the large-scale loss of bee colonies worldwide (Cox-Foster et al. 2007; Higes et al. 2008, 2009c; vanEngelsdorp et al. 2009; Fries 2010; Higes et al. 2010; Botías et al. 2012b; Martínez et al. 2012). Infections by *N. ceranae* and *N. apis* are collectively referred to as Nosema disease. Fumagillin is the only currently available effective chemical treatment against this parasite (Williams et al. 2008a, 2011; Higes et al. 2011).

The commercial formulation of fumagillin, Fumagilin-B® (Medivet Pharmaceuticals Ltd., High River, Alberta, Canada; DIN 02231180), consists of a salt of fumagillin, the counter ion of this salt being dicyclohexylamine (DCH). DCH is therefore applied in a 1:1 stoichiometric ratio with fumagillin (**Figure 6**). The importance of recognizing DCH as a possible contaminant having potential human health and food safety implications for consumers of honey was recently recognized in **Chapter 2** of this thesis, with DCH reportedly being at least five times more toxic than fumagillin when tested on rats (van den Heever et al. 2014). The DCH salt of fumagillin reportedly has genotoxic (clastogenic) potential to mammalian cells *in vivo*, and is reportedly clastogenic and cytotoxic to cultured human lymphocytes (Stanimirović et al. 2006, 2007; Stevanović et al. 2006, 2008, 2010; Kulić et al. 2009). There is currently no maximum residue limit (MRL) set for fumagillin in Canada (Dmitrovic and Durden 2013) or any other jurisdiction, but a proposed MRL of 25 ng g^{-1} is being considered by Health Canada, although this number is not official and might be subject to change (Fishbein 2013). To the best knowledge of the author no MRL exists for DCH in honey or other hive products.



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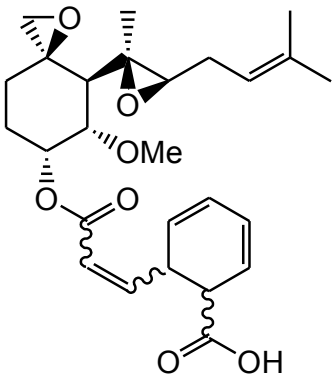

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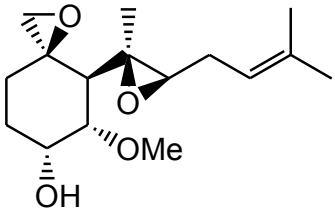

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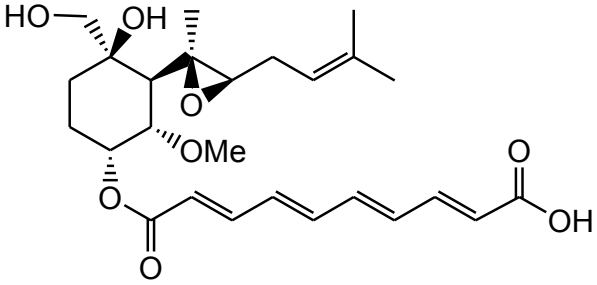


(**4**)

Figure 5 Chemical structure representation of fumagillin (**1**) and its biologically active UV decomposition (**2**) and semisynthetic hydrolysis product, fumagillol (**3**), as well as the biologically inactive thermal decomposition product (**4**).

Analytical methods to date describe the analysis of fumagillin and its UV degradation products in honey and other commodities such as fish, but none recognize the importance of DCH as an analyte (Gochnauer and Furgala 1962; Brackett et al. 1988; Assil and Sporns 1991; Guyonnet et al. 1995; Kochansky and Nasr 2004; Lopez et al. 2008; Nozal et al. 2008; Higes et al. 2011; Kanda et al. 2011; Dmitrovic and Durden 2013). Fumagillin can undergo decomposition in light, to yield biologically active UV decomposition products with similar activity as compared to that of fumagillin (Kochansky and Nasr 2004), or undergo hydrolysis under basic conditions in the

laboratory to yield the semi-synthetic fumagillol (**Figure 5**), which reportedly exhibits approximately 10% of the biological activity of fumagillin (Gochnauer and Furgala 1962). Thermal decomposition of fumagillin yields a biologically inactive compound (Kochansky and Nasr 2004), as shown in **Figure 6**. The lack of commercially available standards of fumagillol, as well as of the thermally degraded fumagillin, combined with the low reported biological

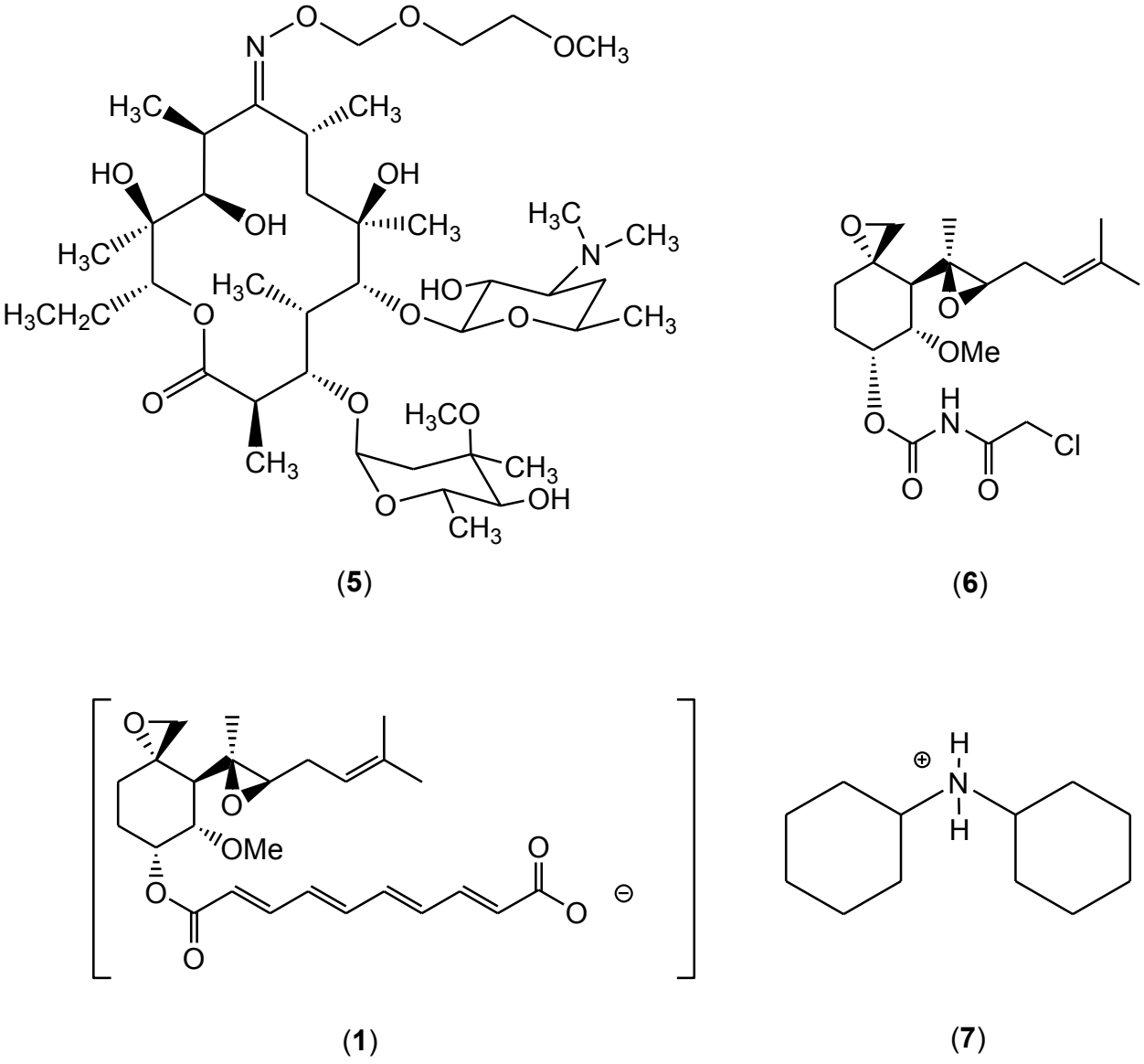


Figure 6 The current internal standard for fumagillin analysis, roxithromycin (**5**), TNP-470 (**6**), and the salt of fumagillin (**1**) with its counter ion, DCH (**7**) as found in the commercial formulation, Fumagilin-B®.

activity of fumagillol, resulted in the exclusion of these compounds from this method validation. The lack of an analytical method to accurately quantitate DCH (**Figure 6**) in honey destined for human consumption, led to the development of the LC-MS/MS method described here. Observed matrix effects in the analysis of DCH, combined with the absence of a suitable isotopic labelled analogue of DCH which could be used to counteract these observed matrix effects associated with analysis of honeys from different floral origins, was overcome by the preparation of a deuterium labelled analogue of DCH, namely d_{10}-DCH (**Figure 7**). The use of this easily prepared labelled compound could benefit other laboratories wishing to include DCH as an analyte of concern in honey.

3.2 Materials and Methods

3.2.1 Reagents and Materials

Fumagillin (F6771), roxithromycin (R4393), formic acid (33015; ≥98%), borane-pyridine complex (179752; ~8M BH3), sodium chloride (S9625), cyclohexylamine (240648; ≥99%) and dicyclohexylamine nitrite (317837; 97% purity) were all purchased from Sigma-Aldrich (St. Louis, MO). Cyclohexanone-d_{10} (D-571; 99.3 atom %D) and cyclohexyl-d_{11}-amine (D-6305; 99.1 atom %D) were obtained from CDN Isotopes (Quebec, Canada). An extracted honey calibration standard was briefly exposed to sunlight for 20 s to induce partial UV degradation of fumagillin, in order to establish the elution profile of the UV degradation products. Petroleum ether (35-60 ^{o}C bp), acetonitrile, methanol and ammonium formate were obtained from Caledon Laboratory Chemicals (Ontario, Canada). Glacial acetic acid was purchased from Merck (Darmstadt, Germany). Hydrochloric acid (Specific Gravity 36.5-38) was purchased from Anachemia Chemicals (Rouses Point, NY). Water was purified using a NANOPURE Diamond system from Barnstead Nanopure (Lake Balboa, CA). Amber coloured SuperClear^{TM} centrifuge tubes suitable for light sensitive samples (525-0437; 50 mL) were obtained from VWR International (Radnor, PA). Strata X (Part 8B-S100-FCH; 33 μm; 200 mg; 6 mL) polymeric reversed phase extraction cartridges were obtained from Phenomenex (Torrence, CA). Commercial honey from the Buram honey company in Turkey, that was tested in our laboratory

and determined to be free from both fumagillin and DCH, was used for preparing matrix matched standards and controls.

3.2.2 d_{10}-DCH synthesis

The synthesis (**Figure 7**) was carried out in accordance with published methodology (Pelter et al. 1984). Equimolar amounts (5 mmol) of cyclohexylamine and d_{10}-cyclohexanone were dissolved under a nitrogen atmosphere in petroleum ether (5 mL). The mixture was stirred at room temperature with slow drop-wise addition of 1.0 mL of glacial acetic acid. A cloudy white solution resulted, and this solution was stirred for an additional 2 h under nitrogen at room temperature. The borane-pyridine complex (6 mmol) was added drop-wise using a syringe, and the solution stirred for another 2 h. The nitrogen atmosphere was removed, and hydrochloric acid (5.0 M; 3.0 mL) was slowly added. A thick white precipitate formed, which was isolated on a Büchner funnel, and washed successively with sodium chloride (5 g) dissolved into hydrochloric acid (5.0 M; 3.0 mL), followed by petroleum ether (3 x 25 mL portions). The precipitate was dried under high vacuum overnight, to yield the d_{10}-DCH hydrochloric acid salt as a white powder (701 mg, 3 mmol, 60% yield) which did not need to be purified any further (**Figure 8**).

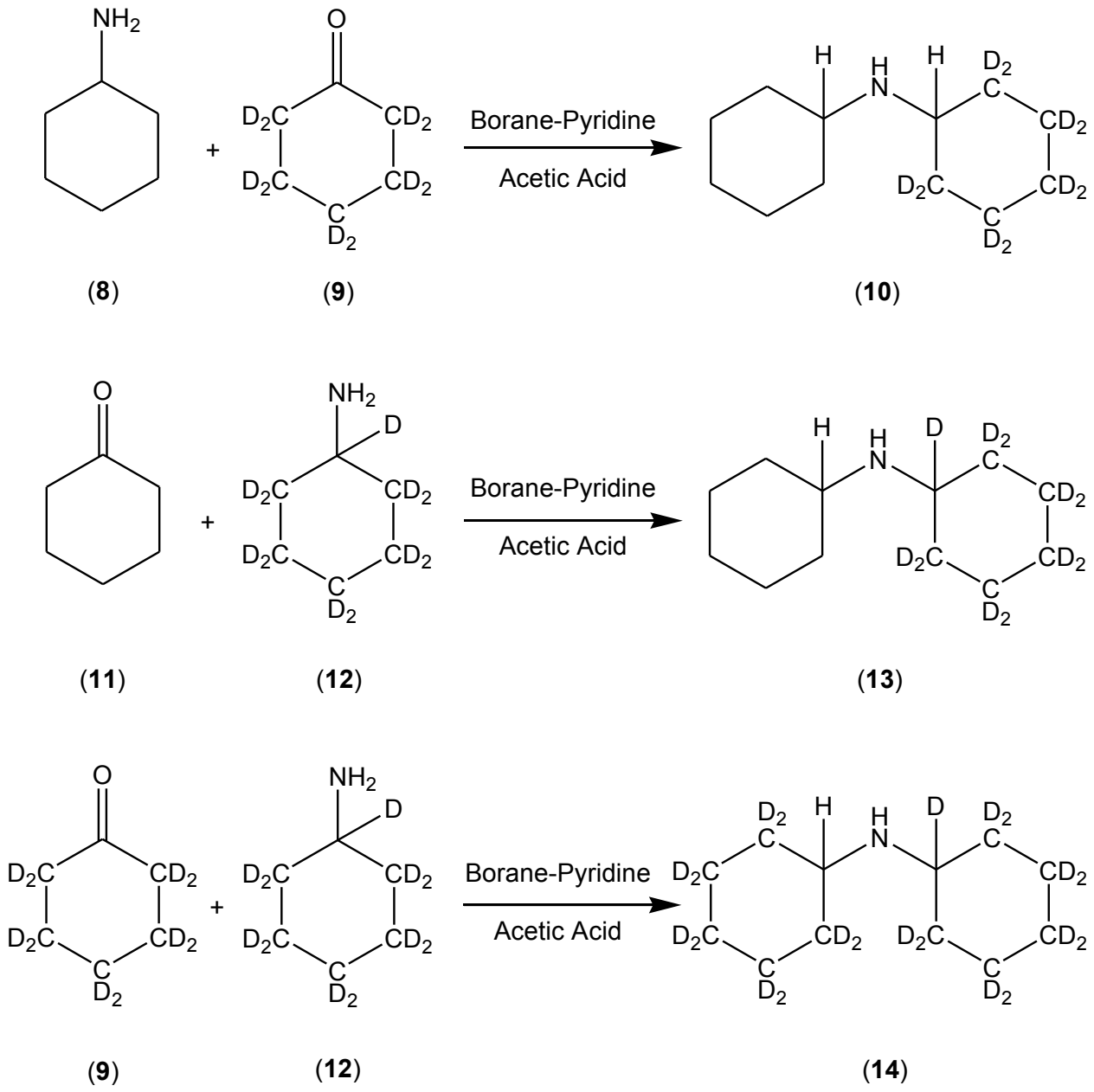


Figure 7 Schematic representation of the chemical synthesis of d_{10}-DCH (**10**), d_{11}-DCH (**13**) and d_{21}-DCH (**14**) from commercially available starting materials cyclohexylamine (**8**), d_{10}-cyclohexanone (**9**), cyclohexanone (**11**) and d_{11}-cyclohexylamine (**12**).

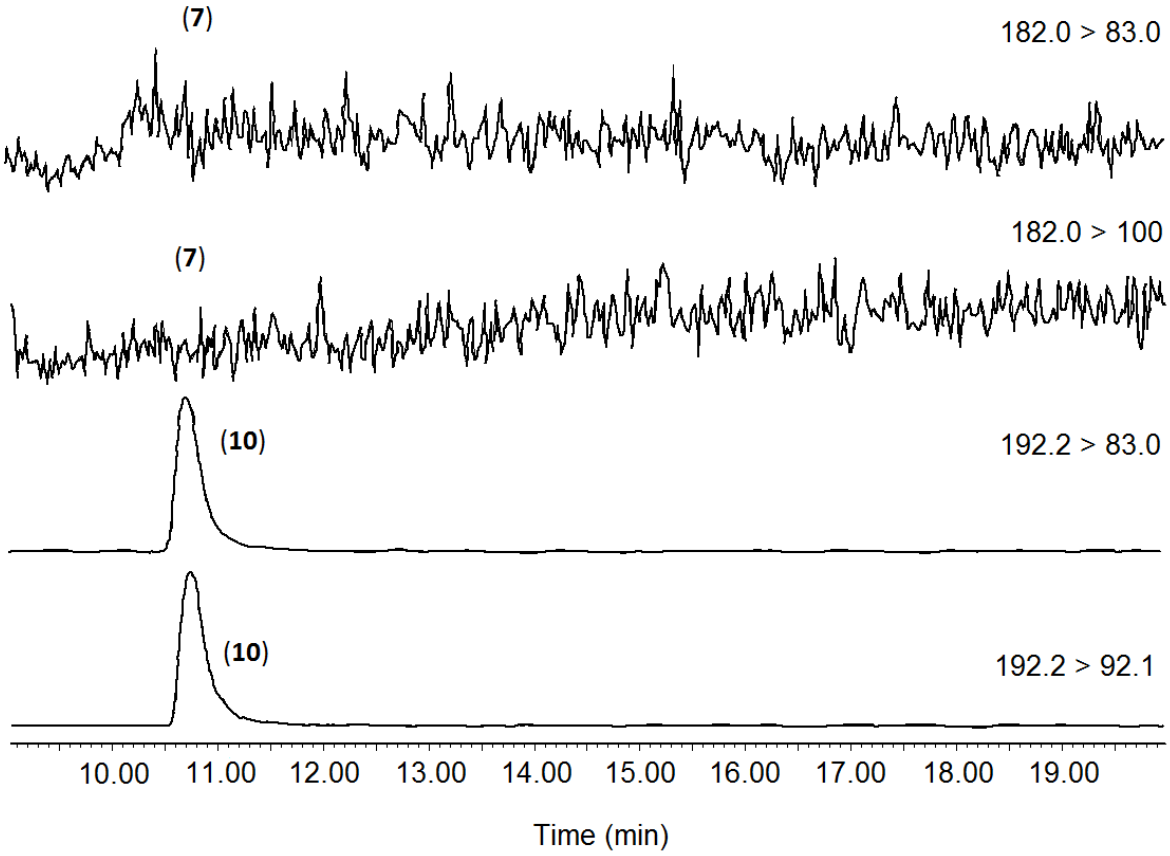


Figure 8 LC-MS/MS chromatograms at normal working concentration of 300 ng g^{-1}, showing that no detectable amounts of non-deuterated DCH (**7**) contamination is present in the synthesized d_{10}-DCH (**10**), indicating that it is suitable for use as an internal standard.

3.2.3 Preparation of Standard solutions

A fumagillin stock solution was prepared at 1000 μg mL^{-1} by dissolving 5 mg of fumagillin into 5 mL of acetonitrile. An intermediate solution was then prepared at a concentration of 50 μg mL^{-1} by dilution with acetonitrile. The absence of any DCH in this material (F6771; Sigma-Aldrich; (St. Louis, MO) was established by testing using the method described in this paper. A working solution was prepared at a concentration of 5 μg mL^{-1} by further dilution with acetonitrile. A DCH stock solution at 1000 μg mL^{-1} was prepared by dissolving 5 mg of DCH into 5 mL of methanol, after correction for using the nitrite salt form. The nitrite salt form of DCH was used, since pure DCH is a liquid that is volatile and difficult to handle safely and

accurately in the laboratory when used as an analytical standard. Then an intermediate solution was prepared at a concentration of 50 μg mL^{-1} by dilution with methanol, and a working solution was prepared at a concentration of 5 μg mL^{-1} by further 10 fold dilution with methanol.

Internal standard stock solutions were prepared at concentrations of 1000 μg mL^{-1} in acetonitrile for roxithromycin, and at 1000 μg mL^{-1} for d_{10}-DCH in methanol. A combined internal standard working solution consisting of roxithromycin (20 μg mL^{-1}) and d_{10}-DCH (300 μg mL^{-1}) was then prepared by dilution with acetonitrile.

3.2.4 LC-MS/MS Equipment

The liquid chromatography system used was a Waters 2695 separations module in tandem with a Waters Quattro Ultima Pt mass spectrometer. The column used for this analysis was a Waters Xterra MS-C18 with column internal dimensions of 4.6x100 mm with 3.5 μm packing particle size (Waters; Milford; MA), protected by a matching 4x2.0 mm guard column (Phenomenex; Torrance; CA).

3.2.5 Mobile phase preparation

A 1.0 M ammonium formate solution was prepared by dissolving ammonium formate (15.77 g, 0.25 mol) into distilled water (250 mL). Mobile phase A was prepared by adding 2.0 mL of the 1.0 M ammonium formate solution and 100 μL of formic acid into a 1000 mL volumetric flask, and diluting to volume with distilled water. Mobile phase B was prepared by adding 2.0 mL of the 1.0 M ammonium formate solution, 100 μL of formic acid and 100 mL of methanol into a 1000 mL volumetric flask, followed by dilution to volume with acetonitrile.

3.2.6 HPLC conditions

The mobile phase flow was kept constant at 0.250 mL min^{-1} with the column temperature maintained at 30 ^{o}C. The initial conditions were 90% mobile phase A (10% mobile phase B), linearly changing to 25% A (75% B) over 5 min. This condition was maintained for a further 15 min, followed by a linear return to 90% A over 5 min. The final condition was held for 5 min, for

a total runtime of 30 min. Injection volumes of 25.0 μL were used for all standards and samples. Sample temperature was maintained at 20ºC.

3.2.7 MS conditions

The source temperature used was 120 ºC, and the desolvation gas temperature was 350 ºC at a flow of 725 L h^{-1}. Cone gas flow was set at 55 L h^{-1} and a capillary voltage of 3.5 kV was used. Collision gas used was argon at a pressure of 2.7 x 10^{-3} mbar. All parameters and focussing potentials were adjusted for optimum detection of the protonated molecular ions $[M+H]^+$ and collision fragments for all compounds and internal standards of interest. Multiple reaction monitoring chromatograms were recorded in positive electrospray mode (ES+) for all compounds. Parameters for the relevant multiple reaction monitoring (MRM) transitions are given in **Table 3**, and typical MRM chromatograms for the major analytes of concern are shown in **Figure 9**.

Table 3 Details of the MRM MS/MS parameters.

Compound	MRM Transitions (CV, CE, Q or C)
Fumagillin and UV-decomposed fumagillin	459.20>177.00 (45V, 30eV, Q) 459.20>102.80 (45V, 30eV, C)
DCH	182.00>83.00 (45V, 20eV, Q) 182.00 >100.00 (45V, 20eV, C)
d_{10}-DCH	192.20>83.00 (45V, 20eV, Q) 192.20>100.00 (45V, 20eV, C)
Roxithromycin	837.40>158.00 (45V, 40V, Q & C)

MRM MS/MS parameters used for all the analytes of interest, namely fumagillin and UV-decomposed fumagillin, DCH, d_{10}-DCH and roxithromycin. Transitions used for quantitation (Q) and confirmation (C) are indicated, along with their respective cone voltages (CV) and collision energies (CE)

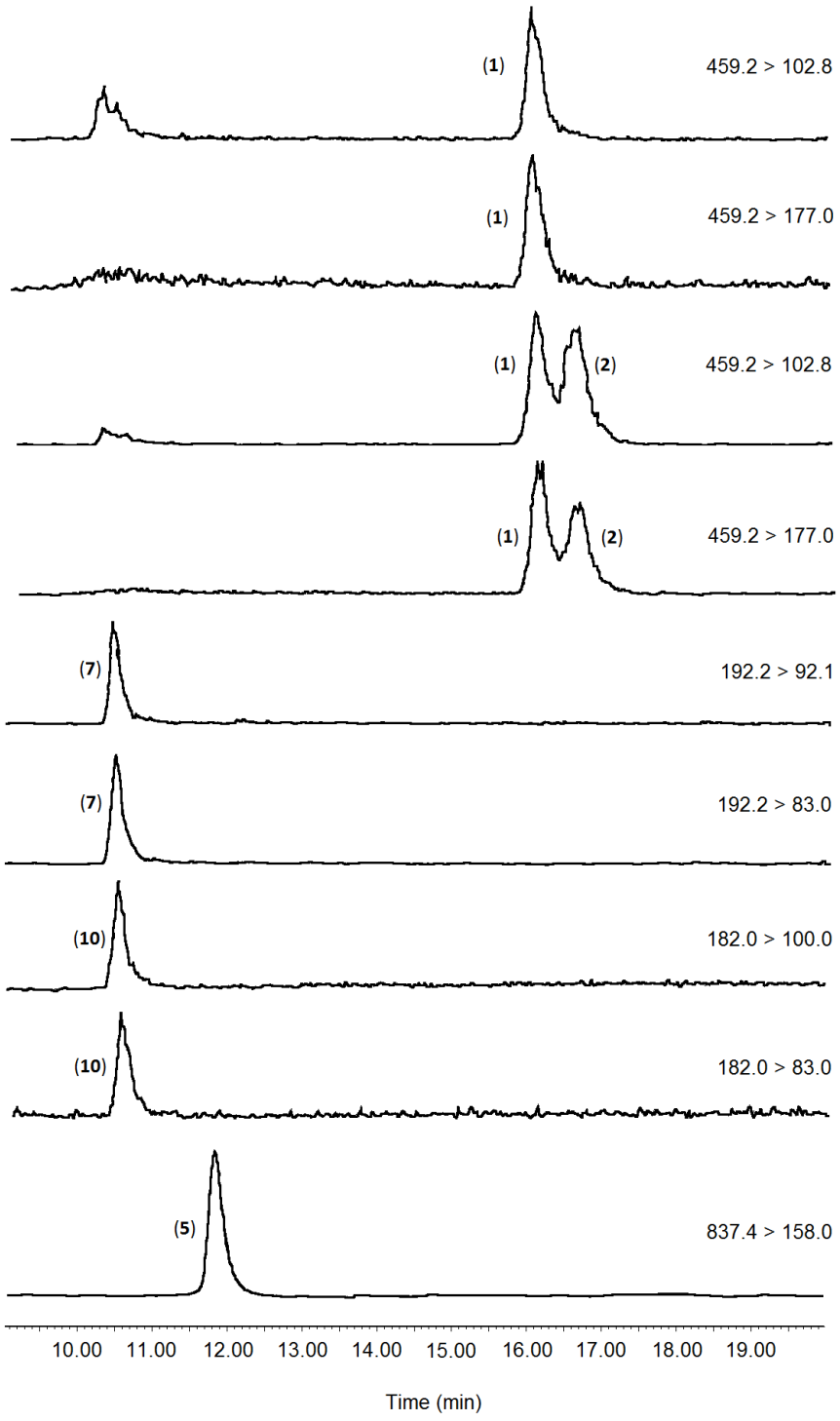


Figure 9 Unprocessed LC-MS/MS chromatograms at 10 ng g^{-1} for the analytes (not all to the same scale) that show the elution of fumagillin (**1**), UV decomposed fumagillin (**2**) and DCH (**7**). The internal standards d_{10}-DCH (**10**) and roxithromycin (**5**) are shown at operational concentrations.

MRM parameters were established by infusion, followed by optimization of all other instrumental parameters using injection of analytes dissolved in acetonitrile, and elution by the using the stated liquid chromatographic conditions. The UV degradation products of fumagillin were not validated for analysis, owing to the lack of commercially available standards and the difficulty in producing these compounds in house in sufficient purity and quantities, combined with the observed non-detection of these compounds in samples and standards during method development. The chromatographic elution profile of the UV decomposed fumagillin products relative to that of fumagillin was established by spiking fumagillin into a honey sample, followed by partial decomposition of fumagillin under fluorescent light, before sample extraction. Subsequent sample extraction and analysis enabled detection of the UV decomposition products eluting as a single peak slightly later than fumagillin (**Figure 9**).

This allowed the detection UV decomposed fumagillin for screening purposes. It was previously reported that photodecomposition products were rarely observed in honey-sugar mixtures, even when there is a notable decrease in fumagillin concentration resulting from induced photo-decomposition (Higes et al. 2011). The authors also noted that no other relevant decomposition peaks appear in the chromatograms, and speculated that this explained the non-detection of fumagillin and its decomposition products in bee hives treated with fumagillin as reported in another study (Assil and Sporns 1991). During our method development, the presence of the UV decomposition products of fumagillin could not be detected after 30 days in honey spiked with fumagillin, and then placed under fluorescent laboratory light conditions. Based on this observation, and on other research (Assil and Sporns 1991; Kochansky and Nasr 2004; Nozal et al. 2008; Higes et al. 2011; Kanda et al. 2011) the method described in this paper was not validated for analysis of the fumagillin UV degradation products, but only allows for screening to detect the presence of these compounds in honey. Roxithromycin was used as the internal standard to quantitate fumagillin, while the synthesized d_{10}-DCH was used as the internal standard to quantitate DCH.

3.2.8 Sample Extraction

The sample extraction procedure is based on previously published methods (Lopez et al. 2008; Nozal et al. 2008; Dmitrovic and Durden 2013). Care was taken not to expose the samples or

standards to fluorescent or other light sources that would cause or accelerate decomposition of fumagillin (Lopez et al. 2008; Nozal et al. 2008; Dmitrovic and Durden 2013). A sample consisting of 5 g of honey was weighed into an amber coloured 50 mL centrifuge tube, and 50 µL of the internal standard working solution was added. After addition of 10 mL of distilled water, the tube was vortexed for approximately 20 s to loosen the honey, followed by shaking on a mechanical shaker for one hour. The tubes were briefly centrifuged at 3500 rpm (2964 rcf) for 5 min in order to improve the flow through the SPE cartridge by isolating any suspended solid particles in the diluted honey. The Strata SPE cartridges were conditioned by sequential elution with methanol (5 mL) and water (5 mL) at a flow rate of 1 mL min^{-1}. The contents of the amber centrifuge tubes were then run through the SPE cartridges at a flow rate of 1 mL min^{-1}, with the waste being discarded. The SPE tubes were then washed once with 10 mL of a water/methanol solution (7:3 v/v) at a flow rate of 1 mL min^{-1}. The filtrate was discarded, and the SPE tubes were dried by pulling air through them for 5 min at a higher vacuum than that used for elution. Clean collection tubes were then placed in the vacuum manifold and the analytes of interest were eluted off the SPE cartridges by using 2 mL of acetonitrile containing 5% of formic acid (using vacuum to remove all possible eluent from the SPE tubes). The eluent was transferred into an amber 1.5 mL sample vial for analysis, without any further processing.

3.2.9 Matrix matched calibration standards, negative and positive control standards

Honey samples (5 g) free from both fumagillin and DCH were weighed into amber coloured 50 mL centrifuge tubes, and 50 µL of the internal standard working solution was added. A positive control sample was spiked at 200 ng g^{-1} with both DCH and fumagillin, while a negative control was only spiked with the internal standard working solution. Calibration standards were spiked at 10, 20, 50, 100 and 500 ng g^{-1} respectively. All standards were processed in the same manner, as described for the sample preparation above, by adding 10 mL of water to all and following the remainder of the procedure as described.

3.2.10 Method validation

The method validation entailed analysis of three replicate analyte concentrations (fortified at 10, 100 and 500 ng g^{-1} respectively), each fortified concentration comprised of six different samples being analyzed on three different occasions, using matrix matched calibration standards. The matrix matched calibration standards had a linear dynamic range of 10-500 ng g^{-1} (10, 20, 50, 100 and 500 ng g^{-1} respectively) with observed correlation coefficients of greater than 0.995 for both fumagillin and DCH being observed on all 3 days. The accuracy and precision data from the analysis of the fortified samples are presented in **Table 4**. The Limit of Detection (LOD) values were calculated using a signal-to-noise (*s/n*) ratio of 3:1 as criteria to analyze the combined 18 replicate honey samples fortified at the lowest concentration of 10 ng g^{-1}. The Limit of Quantitation (LOQ) values were calculated using a 10:1 *s/n* ratio as criteria. No interference was observed for either DCH, fumagillin or its associated UV degradation products in any sample or matrix matched standard during the entire method validation and development, confirming the specificity of the LC-MS/MS analysis for all these compounds. The validated method was applied to the analysis of 16 domestically produced honey samples available in our laboratory (**Table 5**).

Table 4 Intra- and inter day accuracy and precision data for the validation of fumagillin and DCH in fortified honey samples.

Compound	Fortification level (ng g^{-1})	% Recovery (% RSD)			
		Day 1 (n=6)	Day 2 (n=6)	Day 3 (n=6)	Day 1 to 3 (n=18)
Fumagillin	10	108.5 (8.6)	109.2 (8. 9)	99.5 (13.8)	105.7 (10.8)
	100	100.3 (6.4)	108.9 (7.9)	93.2 (6.4)	100.8 (9.3)
	500	101.4 (3.3)	115.2 (4.6)	96.3 (3.6)	104.3 (8.7)
Dicyclohexylamine	10	105.5 (5.0)	102.2 (8.3)	104.3 (4.4)	104.0 (5.9)
	100	101.4 (3.5)	102.3 (5.7)	91.0 (4.2)	98.3 (6.9)
	500	103.2 (4.9)	112.9 (7.7)	95.8 (8.4)	104.0 (9.7)

3.3 Results

Sample extraction was achieved using reversed phase polymeric solid phase extraction (SPE) with recoveries (±RSD%) at 10, 100 and 500 ng g^{-1} for 18 replicates at each respective concentration calculated as 105.7±10.8, 100.8±9.3 and 104.3±8.7 for fumagillin and as 104.0±5.9, 98.3±6.9 and 104.0±9.7 for DCH (**Table 4**).

The linearity fit (R^2) was observed to be greater than 0.995 for both DCH and fumagillin over the linear range of 10-500 ng g^{-1} for all instances. Limits of detection (LOD) were calculated as 1.2 ng g^{-1} for fumagillin and as 0.24 ng g^{-1} for DCH, using the *s/n* criteria of 3:1. A quantitation limit (LOQ) of 10 ng g^{-1} was chosen, based on the lowest matrix-matched calibration standard, which is well above the calculated LOQ of 4.03 ng g^{-1} for fumagillin and 0.79 ng g^{-1} for DCH.

A total of 16 domestically produced honey samples were analyzed for fumagillin and DCH, while the samples were screened to detect the presence of the UV degraded fumagillin residues (**Table 5**). The UV decomposition products of fumagillin elute as one peak under the chromatographic conditions used (**Figure 9**). No UV decomposed fumagillin could be detected during screening of the 16 samples. Fumagillin was detected and quantitated in 2 samples at levels above the LOQ at concentrations of 11.9 and 11.6 ng g^{-1} respectively. No traces of fumagillin were detected in 5 samples, however it was detected at concentrations below 10 ng g^{-1} in 9 samples. DCH was detected in all of the 16 samples, with only 1 sample having a concentration lower than 10 ng g^{-1}. Observed DCH concentrations higher than 10 ng g^{-1} ranged from 20.0 to 234.6 ng g^{-1}, with a median value of 49.4 ng g^{-1}.

Table 5 Results of 16 random domestic honey samples analyzed for fumagillin and DCH

Sample Number	Fumagillin (ng g^{-1})	DCH (ng g^{-1})	UV-decomposed fumagillin (ng g^{-1})
1	11.6	234.6	ND
2	<LOD	20.0	ND
3	Trace	116.4	ND
4	Trace	72.8	ND
5	11.9	124.4	ND
5	Trace	76.8	ND
7	Trace	116.0	ND
8	<LOD	Trace	ND
9	Trace	59.6	ND
10	<LOD	21.7	ND
11	<LOD	39.1	ND
12	<LOD	28.8	ND
13	Trace	64.5	ND
14	Trace	28.9	ND
15	Trace	27.5	ND
16	Trace	25.9	ND

Limits of quantitation (LOQ) were set as 10 ng g^{-1} and higher for both fumagillin and DCH, while limits of detection (LOD) was calculated as 1.2 ng g^{-1} for fumagillin, and as 0.24 ng g^{-1} for DCH. The presence or absence resulting from the screening for the UV decomposed fumagillin is indicated as detected (D) or not detected (ND), while "Trace" denotes levels greater than the LOD but below the LOQ for fumagillin and DCH.

3.4 Discussion

LC-MS/MS is the analytical method of choice for trace level analysis of agricultural pharmaceutical and other organic contaminants in honey, owing to its high specificity and sensitivity. A complication associated with this technique is however the observed matrix effects for certain compounds, due to the presence of co-eluting compounds or impurities that are not observed owing to the specificity of the LC-MS/MS technique when operating in the MRM mode. The consequence of these co-eluting "invisible" compounds may be either signal suppression or enhancement of the LC-MS/MS detector response. These matrix effects are usually analyte specific (Chambers et al. 2007). Matrix effects can be assessed by various post-column infusion or post-extraction spike methods, and can sometimes be reduced by optimizing the sample extraction, or by modifying the chromatography to alter elution times. The use of stable isotopically labelled analogues of the analytes of interest (Chambers et al. 2007; Van Eeckhaut et al. 2009) is preferred, but is often limited by their commercial availability.

Current methods for fumagillin analysis have employed some of these mitigation strategies mentioned above to limit matrix effects when performing LC-MS/MS analysis, for example the use of an improved sample preparation procedure to successfully limit matrix effects (Kanda et al. 2011). This approach was successful, but it resulted in the addition of a time consuming sample pre-purification technique using the QuEChERS technique (quick, easy, cheap, effective, rugged and safe), followed by SPE extraction. The use of roxithromycin as an internal standard (Nozal et al. 2008; Dmitrovic and Durden 2013) was shown to be suitable to overcome the matrix effects related to fumagillin analysis. The choice of roxithromycin as an internal standard for fumagillin is not ideal since its chemical structure is very different from that of fumagillin (**Figure 6**). Roxithromycin also elutes at a different retention time than fumagillin (**Figure 9**), which could make it susceptible to different matrix effects than those fumagillin is subject to. An attempt to use a structurally more closely related analogue of fumagillin as an internal standard, namely the commercially available TNP-470 (T1455, Sigma-Aldrich, St. Louis; MO), proved to be unsuccessful owing to the instability of this compound in extracted honey samples, as observed during this method development. The lack of a commercially available stable isotopically-labelled fumagillin, or of a closely related structural analogue of fumagillin, necessitates the use of roxithromycin. No matrix effects associated with the use of roxithromycin as an internal standard to analyze fumagillin were however reported (Nozal et al. 2008; Dmitrovic and Durden 2013), nor did we observe any detrimental matrix effects relating to fumagillin analysis using roxithromycin as an internal standard. Matrix effects were evaluated by spiking a known quantity of fumagillin and DCH (after samples were extracted and analyzed) into a processed sample of domestic honey, as well as into a Buram honey sample. Both samples were analyzed again, and the analyte recovery for fumagillin was observed to be similar in both Buram and the domestic honey using roxithromycin as internal standard, while a recovery of 20-30% lower than expected was observed for DCH in the domestic honey, but not in the Buram honey, when using roxithromycin as the internal standard. Roxithromycin did therefore not compensate for the difference between the two honey matrices when analyzing for DCH, and a more suitable internal standard was required to perform DCH analysis.

The lack of a commercially available stable isotopically labelled analogue of DCH, combined with the fact that roxithromycin was not able to compensate for the observed matrix effects (20-

30% signal suppression), necessitated the in house development of a labelled analogue of DCH. The preferred internal standard for use in LC-MS/MS methodology is a ^{13}C analogue of that compound, having a different molecular mass, but behaving in all other aspects in same manner as the analyte with respect to chromatography and sample extraction. This makes the internal standard subject to the same matrix effects as the analyte. No readily available potential ^{13}C starting materials for the synthesis of labelled DCH could be commercially obtained at a reasonable cost within the time frame of our method development, but several deuterium labelled precursors to DCH were readily available.

A d_{21}-DCH deuterium labelled analogue of DCH was first synthesized (**Figure 7**) in a one pot reaction from readily available starting materials, based on established methodology (Pelter et al. 1984). However when this synthesized d_{21}-DCH was used as an internal standard to analyze DCH in honey samples, a slow decrease in the area counts with time was observed for the d_{21}-DCH both in standards and in samples. After two days no d_{21}-DCH could be detected in the extracts of any samples or standards, while fumagillin, DCH and roxithromycin could still be detected at levels comparable to the originally observed values. This change in signal response with time for d_{21}-DCH was not acceptable, since it could lead to inaccurate results being generated during an extended analysis sequence. Examination of the chemical structure of d_{21}-DCH indicated the presence of a labile deuterium atom on the carbon atom adjacent to the nitrogen atom. This deuterium atom readily undergoes exchange with protons from the acidic sample matrix, leading to a net reduction in molecular mass by one atomic mass unit, translating into a decrease in signal response when using LC-MS/MS in the MRM mode. A d_{11}-DCH analogue, which was not synthesized, would also be subject to the same proton-deuterium exchange, making this potential compound unsuitable for use as a "stable" internal standard as well.

Another analogue, d_{10}-DCH, was therefore synthesized, employing the same methodology as described for the initial d_{21}-DCH synthesis (**Figure 7**). The d_{10}-DCH was observed to be stable in the acidic sample matrix, since it is not subject to deuterium-proton exchange, owing to it having no labile deuterium atom adjacent to the nitrogen atom. In addition to its stability to deuterium-proton exchange, it was also found to be free from any observable residues of unlabeled DCH (**Figure 8**) that may result from the small amount of non-deuterated cyclohexanone residue being

present in the d_{10}-cyclohexanone (99.3% atom %D). This residue of cyclohexanone is also subsequently converted to unlabeled DCH during the synthesis of the labelled DCH. Using this synthesized d_{10}-DCH as an internal standard, we were able to successfully counteract the observed matrix effects associated with the DCH analysis in honey when honeys of different floral origins were employed as matrix matched calibration standards as compared to the samples being analyzed.

Fumagillin (and the associated DCH) is registered for, and extensively used in Canada (Fumagilin-B®; Medivet Pharmaceuticals Ltd) and in the United States. Even though there are reports that fumagillin is allowed for emergency use under veterinary supervision in several European countries including the United Kingdom, Spain, Belgium, Greece, Hungary and Romania (Higes et al. 2011), there is currently no maximum residue limit (MRL) listed for fumagillin in any animal species in Europe, and its use is therefore not allowed. Other countries like Argentina, Australia, Korea, Libya and Uruguay do permit the use of fumagillin (WHO 2010).

In a previous study, no fumagillin or related biologically active degradation products could be detected in any commercial honey samples at levels above 100 ng g^{-1} using a HPLC-UV method (Assil and Sporns 1991). During a more recent analysis of 20 commercial honey samples from Japan, no fumagillin was detected above the LOQ of 0.1 ng g^{-1} (Kanda et al. 2011). In the most recent report, 30 honey samples imported into Canada were analyzed for fumagillin by the Canadian Food Inspection Agency, using a LC-MS/MS method with a LOD of 0.368 ng g^{-1}. Only 2 samples contained fumagillin, but at levels below 1.12 ng g^{-1} (Dmitrovic and Durden 2013). Other methods describing the analysis of fumagillin in honey focused on either the stability of fumagillin under laboratory and field conditions, where fumagillin was applied at different concentrations using different application techniques, and did not report the analysis of incurred honey samples destined for human consumption (Kochansky and Nasr 2004; Nozal et al. 2008). No detectable fumagillin residues below a method detection limit of 1 ng g^{-1} were observed in a study where samples were analyzed one year after treatment at 120 mg fumagillin per bee colony (Higes et al. 2011).

In the time frame at our disposal we could not find any domestically produced raw honey samples on hand in our laboratory that were completely free from traces of both fumagillin and DCH. Our search for a suitable honey to be used for preparation of calibration standards was expanded to include off the shelf retail honey, and was stopped as soon as the honey from the Buram honey company in Turkey was sourced and tested to be free from both fumagillin and DCH. A few other commercially available honeys were tested and found to contain trace levels of DCH, making them unsuitable for preparation of matrix matched calibration standards. The Buram honey was also available for purchase in the quantity we required for method validation, and was therefore used for all of the method development and validation. The detection of DCH in quantifiable amounts in all but one of the incurred domestic samples at our disposal, as well as the difficulty in finding honey that was completely free from both fumagillin and DCH, highlight the significance of including DCH as an analyte of concern in honey destined for human consumption. DCH in honey could be of real concern, owing to its reported tumorigenic and genotoxic and properties (Pliss 1958; Stoltz et al. 1970; Purchase et al. 1978; Pegg et al. 1983; Mortelmans et al. 1986; Heil et al. 1996; Greim et al. 1998; Woldegiorgis et al. 2007). In **Chapter 2** it was concluded that DCH is at least five times more toxic than fumagillin to rats (van den Heever et al. 2014).

A potential environmental source of DCH contamination could result from its usage in aquaculture (El-Matbouli and Hoffmann 1991; Kent and Dawe 1994; Molnar 1994). The use of the commercial formulation of fumagillin is however not the only possible source of DCH residues in honey and other agricultural products. Other potential sources of DCH in honey could be related to environmental contamination associated with industrial activity (vehicle antifreeze, corrosion inhibitor, rubber additive, oil refineries), and needs to be investigated in order to establish whether this is of any real concern for human or bee health (Woldegiorgis et al. 2007; Brorström-Lundén et al. 2011). If environmental contamination with DCH is indeed present in quantities that could be of concern, it could have an impact on apicultural and other agricultural activities located in close proximity to busier major roadways and industrial centers, including oil refineries (Brorström-Lundén et al. 2011). The effect of DCH on honey bees at various stages of bee development also needs to be studied, because the lipophilic nature of DCH, having an estimated log K_{ow} value of between 3.5 to 4.37 and solubility of 800 mg L^{-1} in water at 25 ^{o}C

(Brorström-Lundén et al. 2011), indicates that there is a probability that DCH could accumulate in wax comb. The close proximity of developing bee larvae to the comb wax may then make them more susceptible to the genotoxic effects of DCH.

3.5 Conclusions

The relevance of including DCH as an analyte in honey destined for human consumption is evident from the results obtained for the 16 domestic honey samples that were tested, where 15 samples had quantifiable residues of DCH (**Table 5**), even in the absence of fumagillin. The SPE procedure described in this method is compatible with those used in other published methods (Higes et al. 2011; Kanda et al. 2011; Dmitrovic and Durden 2013), and these existing methods could be easily adapted to include DCH as an analyte. DCH is present in at least a 1:1 molar ratio with fumagillin in the commercial formulations (Fumagilin-B® and Fumidil-B®), and was observed to be significantly more stable than fumagillin in honey during our method development. This relative stability of DCH was reflected by the difficulty in finding honey that was completely free from trace levels of both fumagillin and DCH intended for preparation of matrix matched calibration standards and controls. The long term stability of DCH relative to that of fumagillin in honey however still needs to be determined.

The analysis of honey from different floral origins present a challenge, since different matrices may influence detector response differently, necessitating the use of suitably isotopic labelled internal standards to mitigate these effects. The limited commercial availability of suitably labelled internal standards remains a challenge for LC-MS/MS methodology. We were able to overcome this problem for the DCH analysis, by using the described simple one-pot synthesis to afford d_{10}-DCH in acceptable yield and purity. However, as we have demonstrated care should be taken when using deuterium labelled compounds as internal standards, since the chemical structure of a compound may cause some of the deuterium atoms to be labile and subject to exchange with protons from the matrix, which could lead to errors in quantitation. Although ^{13}C-labelled internal standards are preferred for this reason, it does not exclude the use of suitable stable deuterium labelled compounds.

The stability of fumagillin in solvent or sugar syrup solutions is well studied, but surprisingly limited information is currently available regarding the prevalence of fumagillin in commercially incurred honey samples destined for consumption. The prevalence of DCH in honey is not established at all, even though DCH is clearly a significantly better and more relevant marker residue when evaluating fumagillin usage in beekeeping. DCH is also of greater concern for human and bee health than fumagillin or any of its degradation products.

No maximum residue limit (MRL) is currently set for fumagillin in any jurisdiction that allows its use in apiculture, but a MRL of 25 ng g^{-1} has been proposed for Canada (Fishbein 2013). It is imperative that any future MRL for fumagillin should also take into account the potential presence of DCH when conducting a risk assessment. Comprehensive statistically valid surveys needs to be conducted to determine the frequency and concentration of DCH as well as fumagillin in honey destined for human consumption, especially in jurisdictions that allow the use of fumagillin to control Nosema disease. The environmental occurrence of DCH also needs to be investigated in order to determine if it is indeed of concern for beekeeping. An alternative formulation of fumagillin that does not include DCH would be beneficial to limit unwanted residues in honey destined for human consumption.

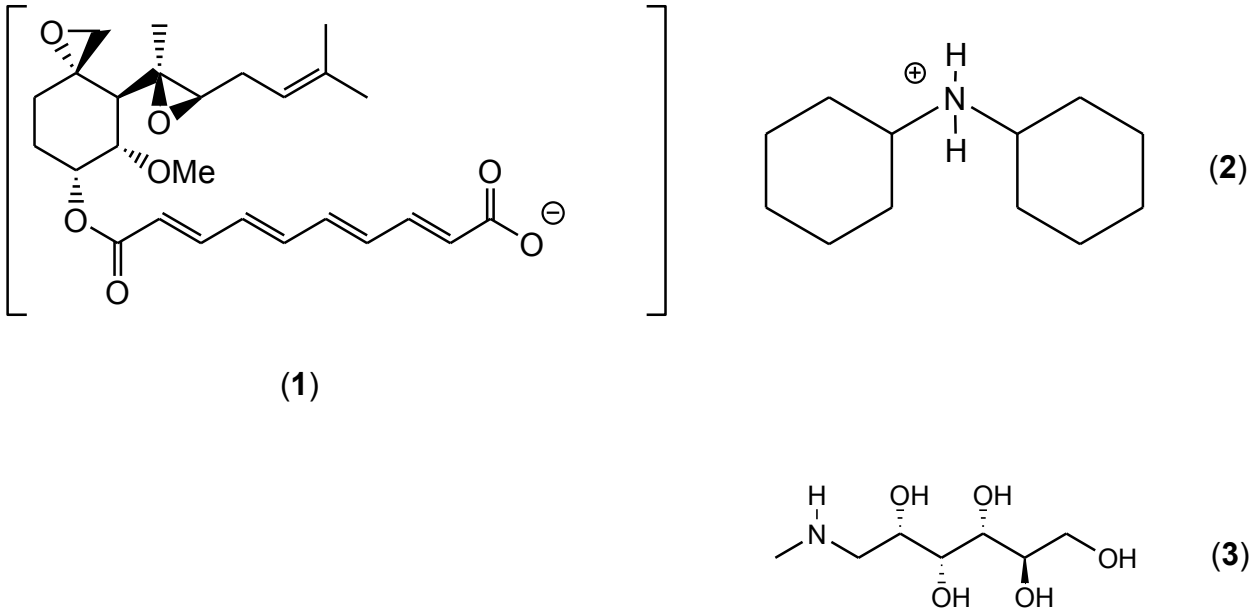
Chapter 4 Stability of dicyclohexylamine and fumagillin in honey

4.1 Introduction

Fumagillin is a potent fungal metabolite that was first isolated from *Aspergillus fumigatus* Fres. (Hanson and Eble 1949) and is extensively employed to control the well-known microsporidian fungal diseases caused by *Nosema apis* Zander (Katznelson and Jamieson 1952; Bailey 1953; Fries 1993) and the more recent *Nosema ceranae* Fries et al. infections plaguing the European honey bee (*Apis mellifera* L.) (Cox-Foster et al. 2007; Martín-Hernández et al. 2007; Higes et al. 2008, 2009a, 2010; vanEngelsdorp et al. 2009; Fries 2010; Botías et al. 2012b; Martínez et al. 2012). The two epidemiologically and etiologically distinct diseases are collectively referred to as Nosema disease. Fumagillin is currently the only registered chemical treatment against Nosema disease (Williams et al. 2008a, 2011), and has been extensively used in apiculture since its discovery in the 1950's. For a more detailed review on the history, usage and properties of fumagillin and DCH, the reader is referred to **Chapter 2** of this thesis (van den Heever et al. 2014).

A problem with compounds such as fumagillin, which have been in use for several decades, is that the initial residue and toxicity investigations during the product's commercial development were performed using analytical methodology that was relatively insensitive compared with currently available techniques. Consequently, higher limits of detection (LOD) and limits of quantitation (LOQ) were established during initial assessments of the drug. Furthermore, the significance and biological activity of metabolites or excipients used in the product's formulation may have been unintentionally overlooked due to the lack of available information at the time of commercialization. It is therefore important that compounds that have been in use for long periods of time be re-evaluated to incorporate all information obtained since the initial commercialization. A critical re-evaluation of fumagillin reveals that the commercial formulation (Fumagilin-B®) contains fumagillin as a salt (**Figure 10**), the counter ion being dicyclohexylamine (DCH). The importance of DCH as a chemical contaminant in hive products with potential human health and food safety implications is extensively described in **Chapter 2** of this thesis (van den Heever et al. 2014). Previous studies report that DCH is five times more

toxic than fumagillin in rats, and that it also exhibits genotoxic properties, making it an important possible contaminant of honey and other hive products.



(**1**) (**2**) (**3**)

Figure 10 Schematic representation of fumagillin (**1**) as its dicyclohexylamine (DCH) salt (**2**) in the commercial formulation Fumagilin-B®. Meglumine (**3**), an alternative compound that could potentially be utilized as a replacement for DCH is also shown.

Current analytical methods to detect fumagillin in honey only recognize fumagillin and its UV, thermal and hydrolysis degradation products (**Figure 11**), without recognizing the importance of DCH (Gochnauer and Furgala 1962; Brackett et al. 1988; Assil and Sporns 1991; Guyonnet et al. 1995; Kochansky and Nasr 2004; Lopez et al. 2008; Nozal et al. 2008; Higes et al. 2011; Kanda et al. 2011; Dmitrovic and Durden 2013). DCH is, however, briefly mentioned as a component of the commercial formulation Fumgilin-B®, that is detectable by LC-MS/MS methodology, but without recognizing its importance as a potential chemical contaminant in honey (Dmitrovic and Durden 2013). Several *cis-trans* as well as *E-Z* diastereoisomeric UV-degradation products exist (Nozal et al. 2008), as indicated (**Figure 11**). These diastereoisomers have the same molecular mass, and are observed to elute as one peak under the chromatographic conditions used in this analysis, using a recently described LC-MS/MS method that allows accurate identification and

quantification of both fumagillin and DCH in honey destined for human consumption (van den Heever et al. 2015a).

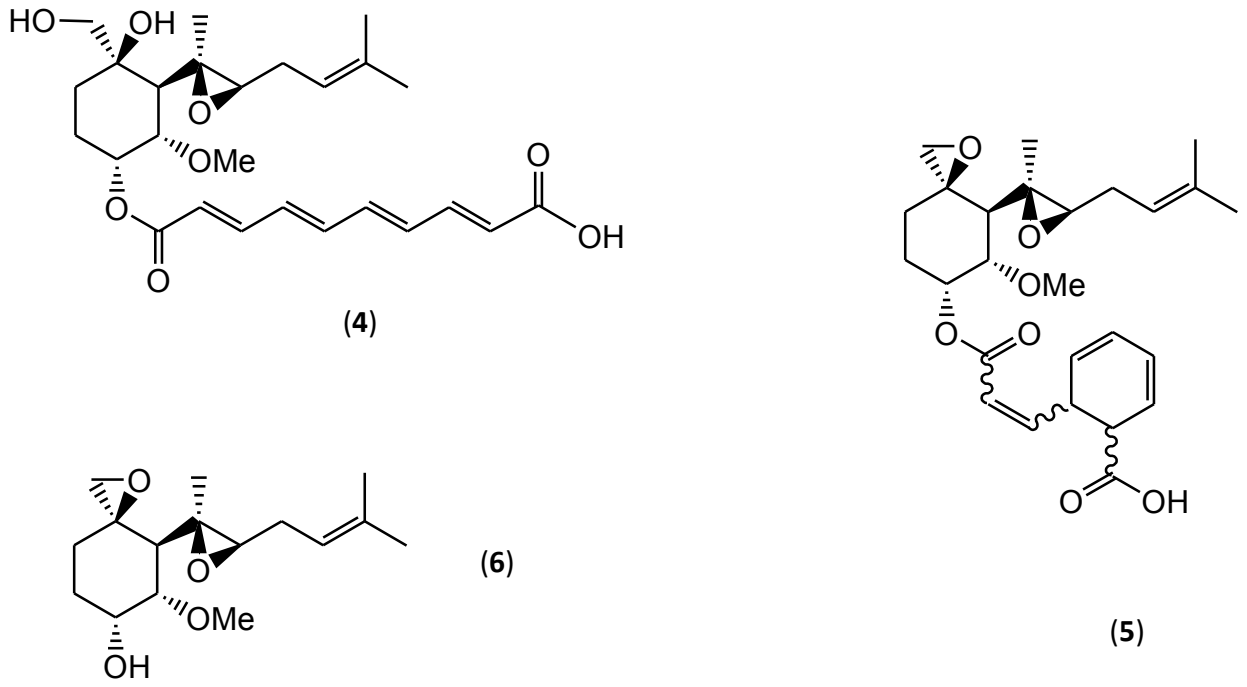


Figure 11 Schematic representation of the biologically inactive thermally decomposed fumagillin (**4**) and it's two other biologically active breakdown products, namely the UV decomposition products (**5**), and the semi-synthetic potential hydrolysis product, fumagillol (**6**).

The stability of fumagillin under the influence of light has been extensively examined in solutions such as sugar syrup (50% w/w) and 50% aqueous ethanol, with the half-life determined to be in the order of seconds to minutes (Kochansky and Nasr 2004). Earlier studies also focused on the stability of fumagillin to light in ethanol/water solutions (Eble and Garrett 1954; Garrett and Eble 1954) as well as the thermal degradation of the molecule (Garrett 1954).

Recently, a study examining the best method for applying fumagillin to honey bee colonies to ensure that the maximum amount of active fumagillin reached infected adult bees (with a minimum of decomposition) was reported (Higes et al. 2011). The investigators examined the

stability of fumagillin in 50% sugar syrup spiked with fumagillin at three concentrations of 1, 1.5 and 2.5 g Fumidil-B® in 250 mL of sugar syrup. The stability of fumagillin was determined at each concentration in the presence and absence of light at 4, 22, 30 and 40 ºC. Fumagillin was observed to rapidly decompose upon exposure to light, and this decomposition was accelerated by elevated temperatures, with 30% decomposition being observed at 4ºC, 60% decomposition at 22 ºC, and 65% decomposition at 30 ºC after 70 days for all samples. At 40 ºC, when exposed to light, no fumagillin could be detected past 20 days. In the absence of light (amber vials) fumagillin was observed to be more stable at lower temperatures, with only 12% decomposition at 4 ºC being observed after 70 days. Exposure to UV irradiation, even when amber vials were used, resulted in complete decomposition of fumagillin after 40 days, with the UV-decomposed fumagillin products only being observed for up to 60 days. Similar observations were made when evaluating the stability of fumagillin in sucrose/honey patties consisting of 1 part sucrose and one part honey, spiked with fumagillin. Fumagillin was however observed to be more stable in the sucrose/honey patties than in the 50% sugar syrup solution. The presence of fumagillin in honey after treatment of a hive was reported as ranging from 66 ng g^{-1} to below their method limit of detection (LOD) of 1 ng g^{-1}, but no other details are given regarding fumagillin degradation products in these incurred samples (Higes et al. 2011). The susceptibility of fumagillin to photolytic degradation was also suggested as the reason for the observed absence of fumagillin residues in commercial honey samples originating from hives that have been treated with Fumidil-B® (Nozal et al. 2008).

The stability of fumagillin under the influence of light and temperature variations in sugar syrup and in organic solvents is therefore well documented. However, the stability of fumagillin in honey under hive (34 ºC in darkness) and typical storage conditions (21 ºC in either darkness or when exposed to light) has not been reported, and neither has the stability and presence of DCH in honey. This study was conducted in order to establish the relative stabilities of both fumagillin as well as DCH in honey under simulated hive and storage conditions, both in the presence and in the absence of light. The observed half-lives will provide apiculturists with information that could be used with caution to estimate the levels of fumagillin and DCH in honey destined for human consumption, based on the amounts of Fumagilin-B® being applied. This estimate could be used as a general guideline, as the actual fate of DCH in honey destined for human

consumption should be further examined by field dosage experiments and subsequent analysis of the harvested honeys.

4.2 Materials and Methods

4.2.1 Reagents and Materials

Fumagillin as the free acid (F6771), roxithromycin (R4393), dicyclohexylamine nitrite (317837, 97% purity) and formic acid (≥98 %; 33015) were all obtained from Sigma-Aldrich (St. Louis; MO). Acetonitrile, methanol and ammonium formate were sourced from Caledon Laboratory Chemicals (Ontario, Canada). Purified water was obtained using a Barnstead NANOPURE Diamond system. Amber coloured 50mL SuperClear^{TM} centrifuge tubes (525-0437) for use with light sensitive samples were bought from VWR International (Edmonton, AB). Strata X (33 µm, 200 mg, 6 mL, 8B-S100-FCH) polymeric reversed phase extraction cartridges were from Phenomenex (Torrence, CA). Honey from the Buram honey company in Turkey was used to prepare all matrix matched standards, controls and samples for this stability study. The d_{10}-DCH internal standard used for DCH analysis (to limit the observed matrix effects) was prepared according to published methodology (Pelter et al. 1984; van den Heever et al. 2015a).

4.2.2 LC-MS/MS Equipment

The liquid chromatography system used was a Waters 2695 separations module in tandem with a Waters Quattro Ultima Pt mass spectrometer. The column used for this analysis was a Waters Xterra MS-C18 with internal column dimensions of 4.6 x 100 mm with 3.5 µm packing particle size, protected by a matching 4 x 2.0 mm guard column (Phenomenex, Torrence, CA). The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode using positive electrospray ionisation (ES+). The two mobile phases consisted of acetonitrile/methanol (9:1, v:v) and water, both buffered with ammonium formate/formic acid. For a complete description of all the instrumental and other parameters see **Chapter 3** (van den Heever et al. 2015a).

4.2.3 Preparation of standards and stability samples

A combined internal standard working solution consisting of roxithromycin (20 μg mL^{-1}) and d_{10}-DCH (300 μg mL^{-1}) was prepared in acetonitrile. Honey matrix matched calibration standards, free from both fumagillin and DCH, were prepared by weighing honey (5 g) into amber coloured 50 mL centrifuge tubes, followed by addition of 50 μL of the internal standard working solution. Positive control samples were fortified at 200 ng g^{-1} with both DCH and fumagillin as the free acid, while the negative controls were only spiked with the internal standard working solution. Calibration standards were fortified at 10, 20, 50, 100 and 500 ng g^{-1} respectively for each analyte.

Samples for the stability study were also weighed (5 g) into either amber coloured or clear 50 mL centrifuge tubes. The samples were fortified at 500 ng g^{-1} with both fumagillin (free acid form) and with DCH, followed by shaking for 30 min on a mechanical shaker to homogenize the samples as well as possible. Simulated shelf condition stability samples (n=93) were weighed into clear 50 mL centrifuge tubes to enable exposure of the honey to ambient fluorescent light. Amber-coloured centrifuge tubes were divided into two sets consisting of 93 tubes per set, with one set being stored in an incubator (Thelco Precision Scientific, Cat. # 31485, Chicago, IL) at 34°C in darkness, to simulate hive conditions, and the other set kept at 21°C in the dark, simulating bulk storage conditions in drums. The temperatures for all storage conditions were monitored and recorded using HOBO H8 Pro Series temperature data loggers (Onset Computer Corporation, Bourne, MA).

All standards, control samples and stored honey samples were then processed in the manner previously described in **Chapter 3** (van den Heever et al. 2015a), by dilution with water, shaking and brief centrifugation, followed by percolation through preconditioned Strata X polymeric (Phenomenex Part 8B-S100-FCH; 33μm; 200 mg; 6 mL; Torrence; CA) solid phase extraction (SPE) cartridges. Analytes were eluted off the cartridges using acetonitrile acidified with 5% formic acid. Sampling proceeded with 3 random samples from each of the three sets of samples being analyzed at 3 to 4 day intervals after fortification (up to day 48). The sampling period was then extended to once a week up to day 112, and samples were then subsequently analyzed once every three weeks.

Linear regression analysis was conducted using Excel 2010 (Microsoft Corporation, Redmond, WA, USA), and analyte half-lives were calculated by substituting 250 ng g^{-1} (half of the formulated analyte concentration) into the formulas obtained for the respective regression analyses, and solving the equation to obtain the value of x (the time needed for the respective analytes to decompose to 50% of their original formulated values).

4.3 Results

Temperature was recorded every hour over the entire duration of the study for both the incubator (hive temperature study) and the room temperature study using data loggers. The average incubator temperature was 33.9 ± 4.1(SD) ^{o}C, while the room temperature averaged 21.2 ± 1.5(SD) ^{o}C. The data obtained during this stability study is summarized in **Table 6**, and graphically portrayed in **Figure 12, 13** and **14**.

From **Figure 12** it is evident that fumagillin and its UV degradation products are highly unstable upon exposure to light. This rapid degradation of fumagillin and its UV decomposition products in light is contrasted by the relative stability observed for DCH under the same conditions. In the absence of UV decomposed fumagillin standards, the calibration curve of fumagillin was used to quantitate the UV decomposed fumagillin. DCH was also observed to be significantly more stable than fumagillin at hive and room temperatures, in the absence of light.

Linear regression analysis of the observed data was used to estimate the half-lives for both DCH and fumagillin under all the conditions examined (**Table 7**). Due to the rapid degradation of both fumagillin and its biologically active UV degradation products when exposed to fluorescent light at 21^{o}C, the half-life of fumagillin and UV decomposed fumagillin was estimated to be approximately 3 days for both, because there were insufficient data points to enable accurate regression analysis to be conducted for the short time interval it took to reach 50% decomposition for these compounds. No fumagillin or its UV decomposition products could be detected in the honey 30 days when exposed to light. No UV-decomposition products of fumagillin were detected in honey stored in darkness at either 21 ^{o}C or 34 ^{o}C. The observed half-lives for DCH ranged from a minimum of 368 days (34 ^{o}C in darkness) to a maximum of 852 days (21 ^{o}C in darkness), compared to that of fumagillin ranging from 141 days (34 ^{o}C in

darkness) to 246 days (21 ^{o}C in darkness) when determined over a 6 month time period. At room temperature upon exposure to light, fumagillin has an estimated half-life of 3 days, compared to that of 829 days for DCH. Exposure to light therefore appears to have little or no effect on the rate of degradation of DCH in honey. All of the observations indicate that DCH is more stable than fumagillin in honey under all the conditions studied.

Table 6 Results for the study simulating bulk storage (21 ºC in darkness), shelf (21 ºC exposed to light) and hive conditions (34 ºC in darkness) for both fumagillin and DCH in fortified honey samples.

Day	Concentration (ng g^{-1})±SD						
	Bulk Storage		Shelf			Hive	
	Fumagillin	DCH	Fumagillin	UV-Fumagillin	DCH	Fumagillin	DCH
0	500*	500*	500*	0*	500*	500*	500*
3	479 ± 23	431 ± 17	154 ± 18	181 ± 67	418 ± 11	269 ± 30	413 ± 19
7	432 ± 56	342 ± 37	55 ± 20	74 ± 26	409 ± 11	405 ± 39	361 ± 16
10	286 ± 26	411 ± 7	13 ± 8	16 ± 4	413 ± 27	162 ± 15	431 ± 9
14	458 ± 10	451 ± 23	23 ± 7	20 ± 6	450 ± 21	290 ± 62	441 ± 24
17	458 ± 28	348 ± 7	9 ± 1	12 ± 2	359 ± 17	315 ± 32	406 ± 20
21	438 ± 43	391 ± 7	17 ± 5	13 ± 8	392 ± 12	343 ± 34	360 ± 17
24	459 ± 27	440 ± 9	15 ± 2	17 ± 1	412 ± 5	379 ± 76	400 ± 2
28	402 ± 34	461 ± 23	<LOQ	<LOQ	420 ± 9	340 ± 9	432 ± 6
31	402 ± 34	408 ± 7	ND	ND	400 ± 29	281 ± 26	389 ± 36
35	442 ± 40	395 ± 22	ND	ND	443 ± 35	304 ± 34	410 ± 13
38	220 ± 18	404 ± 31	ND	ND	342 ± 38	145 ± 21	385 ± 18
42	342 ± 42	415 ± 12	ND	ND	393 ± 20	268 ± 20	363 ± 8
45	321 ± 18	373 ± 7	ND	ND	352 ± 13	180 ± 8	357 ± 5
48	410 ± 46	405 ± 12	ND	ND	396 ± 5	234 ± 69	374 ± 5
55	395 ± 37	372 ± 11	ND	ND	367 ± 3	254 ± 71	340 ± 26
63	231 ± 51	379 ± 8	ND	ND	370 ± 14	143 ± 22	386 ± 3
71	382 ± 20	387 ± 22	ND	ND	370 ± 13	143 ± 31	362 ± 11
77	387 ± 22	379 ± 7	ND	ND	373 ± 17	329 ± 60	353 ± 7
84	376 ± 30	400 ± 4	ND	ND	399 ± 5	188 ± 23	362 ± 7
91	309 ± 5	389 ± 11	ND	ND	378 ± 27	200 ± 87	344 ± 11
98	337 ± 58	371 ± 20	ND	ND	341 ± 17	168 ± 56	343 ± 9
112	234 ± 54	435 ± 36	ND	ND	406 ± 10	218 ± 20	383 ± 8
126	325 ± 28	411 ± 6	ND	ND	416 ± 17	223 ± 21	372 ± 5
155	293 ± 49	401 ± 6	ND	ND	409 ± 9	153 ± 45	349 ± 2
175	304 ± 55	379 ± 9	ND	ND	387 ± 21	135 ± 10	340 ± 9
196	306 ± 20	343 ± 9	ND	ND	343 ± 6	135 ± 31	280 ± 4

Three replicate samples were analyzed for both DCH and Fumagillin on each day at each simulated condition. Limit of quantitation (LOQ) is 10 ng g^{-1} for all analytes, while limit of detection (LOD) is 1.2 ng g^{-1} for fumagillin, and 0.24 ng g^{-1} for DCH. Non-Detectable (ND) denotes values less than the LOD, while * indicates fortified values.

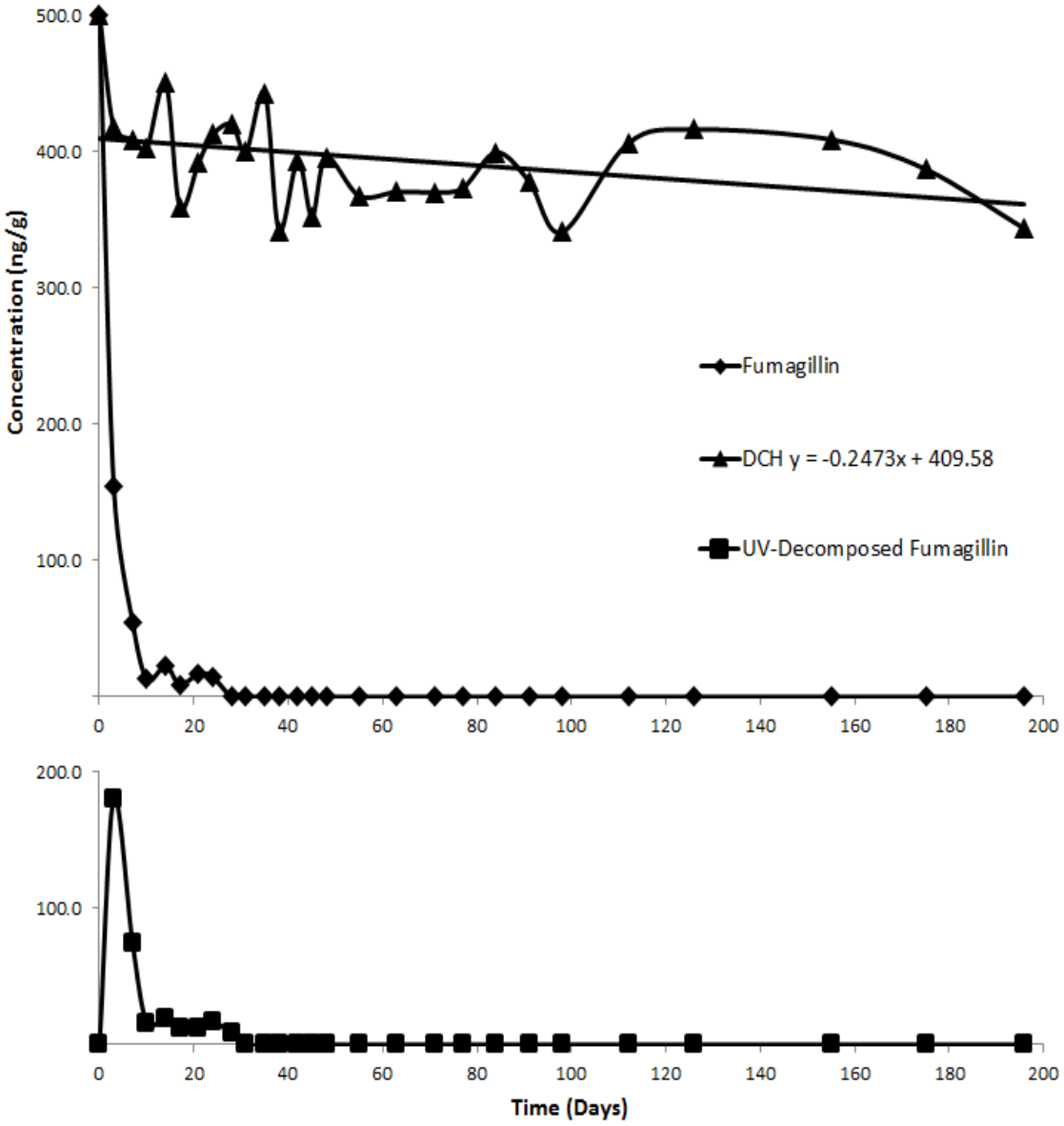


Figure 12 Graphical representation of data recorded for fumagillin and DCH under simulated shelf conditions (21 ºC exposed to light) indicating that no fumagillin or UV decomposed fumagillin is present after 30 days.

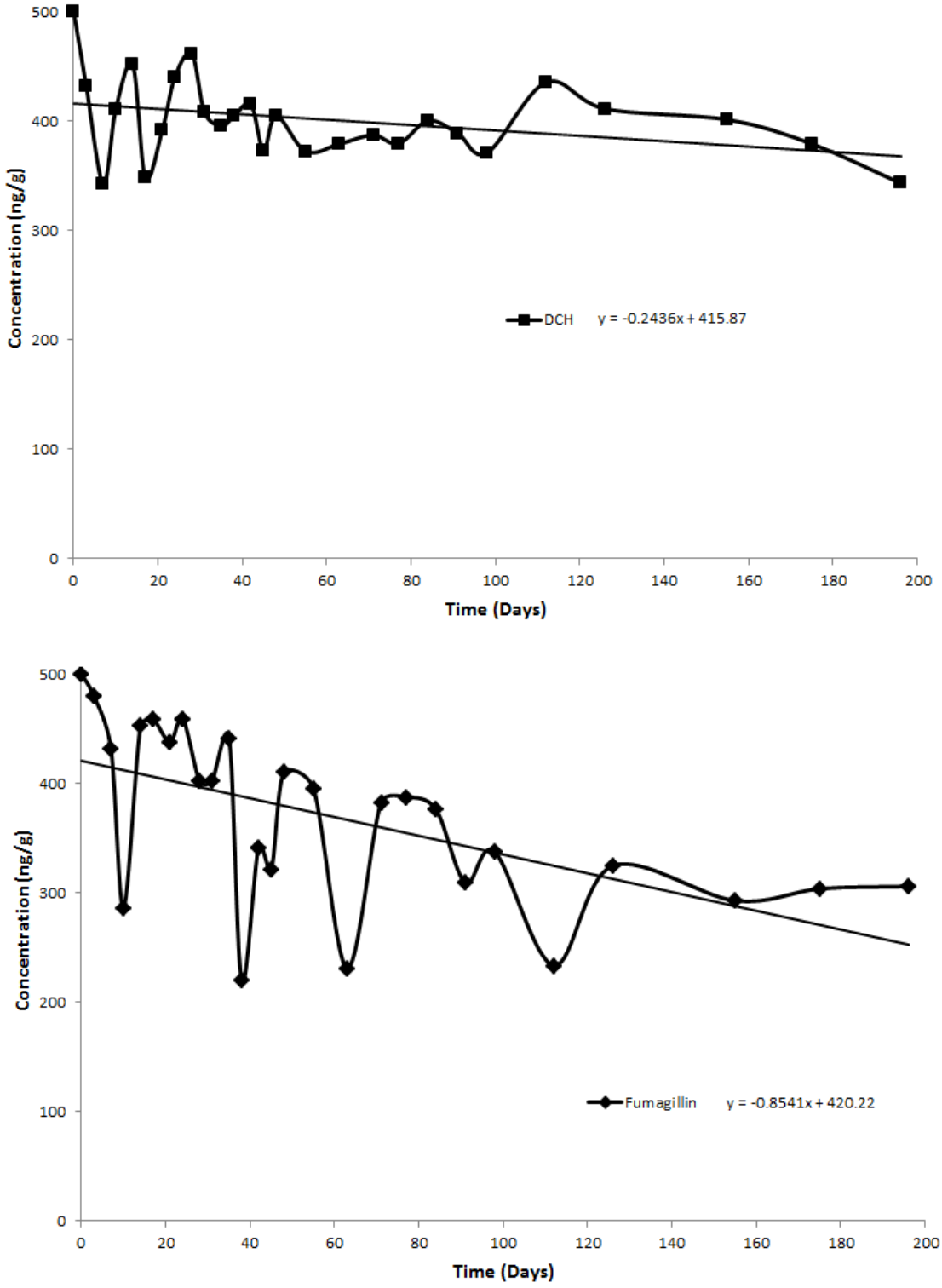


Figure 13 Graphical representation of data recorded for fumagillin and DCH under simulated bulk storage conditions (21 ºC in darkness).

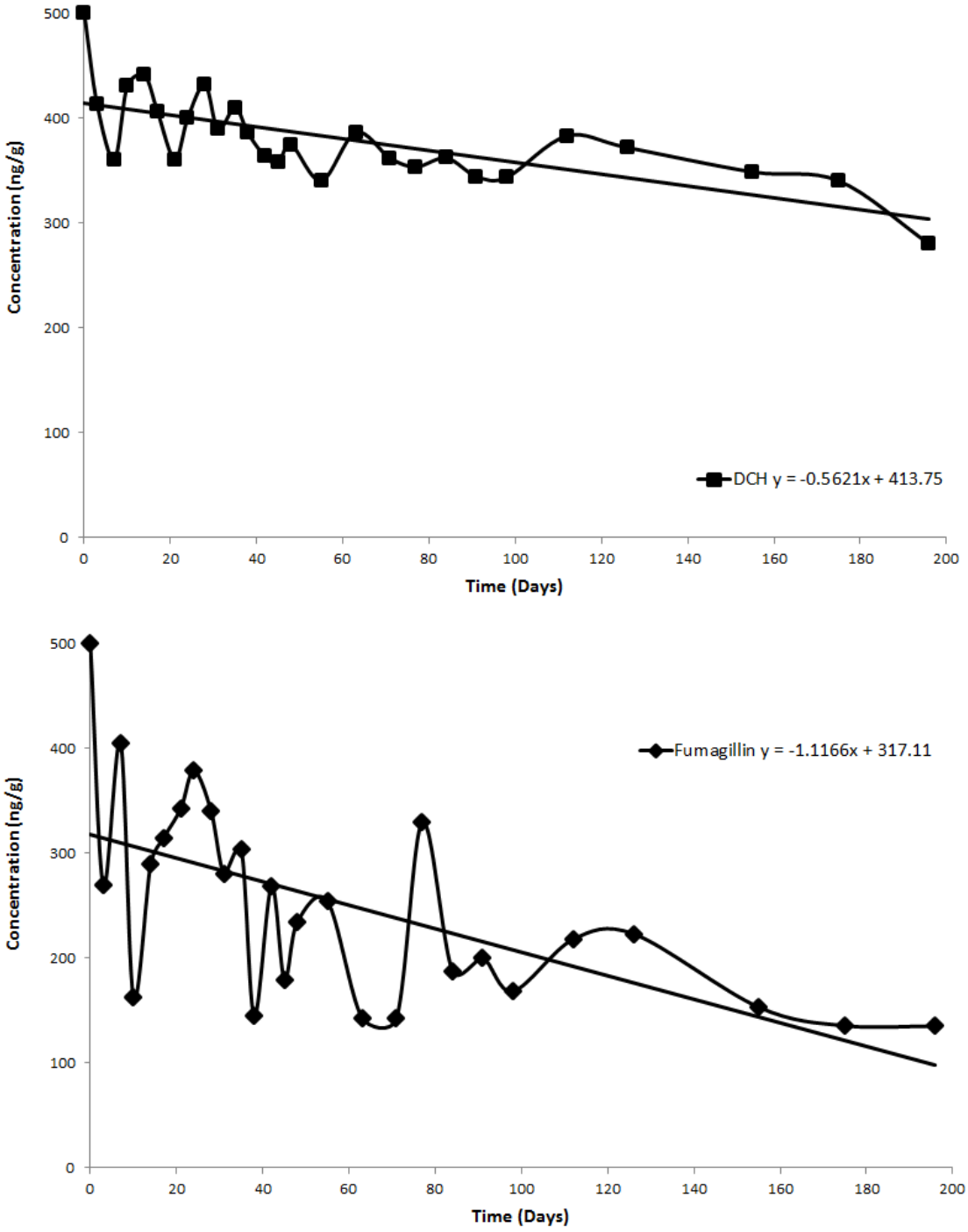


Figure 14 Graphical representation of data recorded for fumagillin and DCH under simulated hive conditions (34°C in darkness)

Table 7 Results for the linear regression analysis of fumagillin and DCH for data recorded under simulated shelf (21 ^{o}C exposed to light), bulk storage (21 ^{o}C in darkness) and hive (34 ^{o}C in darkness) conditions.

Compound	Test Condition	Linear Regression Equation	r^2	Calculated half-life (days)*
DCH	21^{o}C Light	y = - 0.25 x + 409.6	0.071	829
	21^{o}C Dark	y = -0.24 x + 415.9	0.072	852
	34^{o}C Dark	y = - 0.56 x + 413.8	0.376	368
Fumagillin	21^{o}C Light	estimated		3
	21^{o}C Dark	y = - 0.85 x + 420.2	0.358	246
	34^{o}C Dark	y = -1.12 x + 317.1	0.303	141
UV-Fumagillin	21^{o}C Light	estimated		3

*The calculated half-life for each compound under each of these conditions is shown. The half-life of fumagillin and its UV decomposition products under shelf conditions is estimated to be approximately 3 days (**Figure 12**), while no fumagillin, or its metabolically active UV decomposition products, could be observed after one month at shelf conditions

4.4 Discussion

Previous research by Higes et al. (2011) reported 30% decomposition of fumagillin in 50% sugar syrup solution after 70 days in clear vials at 4 ^{o}C, and 12% decomposition at 4 ^{o}C after 70 days in amber vials. Increased temperatures of 22 and 30 ^{o}C for the 50% sugar syrup solution caused 60% and 65% decomposition respectively after 70 days, with total decomposition of fumagillin being observed after 20 days at 40 ^{o}C. When exposed to UV light, 80% decomposition was observed within 24 hours, and no detectable residues of fumagillin could be observed after 10 days (Higes et al. 2011). Higes et al. (2011) also examined the stability of fumagillin in 50% honey and sugar patties, and found that fumagillin was observed to be more stable in this matrix than in 50% sugar syrup solutions, with only 10% decomposition of fumagillin being observed after 4 weeks at 4, 22, 30 and 40 ^{o}C respectively when stored in amber vials. In our study 20% decomposition of fumagillin was observed in honey stored at 21 ^{o}C and 32% decomposition in honey stored at 34 ^{o}C, both in darkness. In field trials, Higes et al., observed levels of fumagillin ranging from less than the limit of detection (1 ng g^{-1}) to 66 ng g^{-1} in honey samples. Nevertheless, the field trial data is incomplete as no surplus honey was available to be harvested for some of the colonies.

Nozal et al. (2008) found that fumagillin was stable for one month at mild temperature conditions (40ºC) in either sugar syrup or water when not exposed to light. The same study also confirmed the instability of fumagillin upon exposure to light. In field studies, no fumagillin could be observed at concentrations above their LOD (ranging from 1-24 ng g^{-1} for clear rosemary honey and 4-45 ng g^{-1} for darker heather honey respectively) for hives treated with either 120 mg or 240 mg (twice the recommended dosage in Spain). The fumagillin treatment was applied for six weekly periods in a 50% honey and water mixture (250 mL) in the late fall to early winter (December to January), and surplus honey was sampled 6 months later, in June.

The instability of fumagillin upon exposure to light observed during this study is similar to that reported previously using fluorescent and UV light sources (Kochansky and Nasr 2004; Higes et al. 2011), even though the stability of fumagillin was examined in ethanol or sugar syrup solutions in those studies, while the stability of fumagillin was evaluated in a honey matrix in our study. Fumagillin was however observed to be more stable in honey when it is not exposed to light at elevated temperatures (34 ºC), simulating hive conditions. This indicates that residual fumagillin in the stored honey of overwintered hives should remain active against Nosema disease. Because of the instability of fumagillin, greater variability in the measured concentrations of residual fumagillin was observed compared to that seen for residual DCH.

In addition to DCH residues originating from fumagillin treatment, the possible environmental origins of DCH related to industrial activity (rubber, steel corrosion inhibitors, vehicle antifreeze formulations) need to be examined in order to establish if this is of any concern for human and bee health, especially for apiaries located close to major roadways and industrial centers (Woldegiorgis et al. 2007). The effect of DCH on bees at various stages of development also needs to be evaluated, since the lipophilic nature and stability of DCH could lead to accumulation in the comb wax, where the developing larvae may be more susceptible to the effects of DCH.

An alternative less harmful formulation of fumagillin that does not contain DCH as the counter ion would be desirable. A potential candidate as an alternative counter ion of formulated fumagillin could be for example a compound such as meglumine (CAS Registry No. 6284-40-8) shown in **Figure 10**, which is a basic amino sugar derived from sorbitol, and which is acceptable

for use in human medicine as a pharmaceutical excipient to obtain crystalline precipitates from organic acids (Blood et al. 2007). An example of this is the meglumine salt of the organic acid, flunixen (Sigma-Aldrich product F0429, CAS Registry No.42461-84-7). The reported MSDS LD_{50} value for the meglumine is 5000 mg kg^{-1} orally for both rat and rabbit (Sigma-Aldrich product M9179 v3.5 07/02/2014), as compared to the reported oral LD_{50} for rat of 373 mg kg^{-1} for DCH (Sigma-Aldrich product 185841 v5.0 07/24/2012). Similar values of 200-373 mg kg^{-1} was also reported elsewhere for DCH (Greim et al. 1998). The use of meglumine instead of DCH as the counterion of fumagillin formulation could therefore reduce the toxicity of the formulated fumagillin significantly. The presence of multiple hydroxyl groups on meglumine might also increase the water solubility of the formulated fumagillin. The suitability of the suggested meglumine or other alternative counter ions to replace DCH in the formulated fumagillin needs to be investigated further.

4.5 Conclusions

The previously reported accelerated decomposition of fumagillin at higher temperatures and upon exposure to light was confirmed by the results obtained for our study. Our observations also support the conclusions reached in other studies, namely that it is unlikely that fumagillin or its UV degradation products will be detected in any appreciable amounts in honey destined for human consumption, when the commercial formulation is used as prescribed by the label instructions (Kochansky and Nasr 2004; Lopez et al. 2008; Nozal et al. 2008; Higes et al. 2011; Kanda et al. 2011; Dmitrovic and Durden 2013). It should however be noted that the presence of DCH, resulting from the application of the commercial formulations of fumagillin (Fumidil-B® and Fumagillin-B®), is not recognized by any of these studies.

The long half-lives observed for DCH in this stability study (ranging from 1 to almost 3 years) indicate that DCH will likely be detectable in honey at higher concentrations than fumagillin for extended periods of time. It is important to remember that DCH and fumagillin are applied in theoretically equimolar amounts when using the current commercial formulations. The expected higher prevalence of DCH in honey, compared to that of fumagillin, was demonstrated in our recent study, where incurred DCH was detected at levels above the analytical method LOD of 10 ng g^{-1} for all but one of 16 random honey samples analyzed, with DCH levels ranging from

20.0 - 234.6 ng g^{-1} (van den Heever et al. 2015a). In contrast, the same study found fumagillin above the LOD of 10 ng g^{-1}, in only two of the samples, at values of 11.9 and 11.6 ng g^{-1} respectively. The extended half-life of DCH, in combination with its reported toxicity and genotoxic properties, clearly makes it a contaminant of concern in honey (Greim et al. 1998). DCH is clearly a much better marker residue than fumagillin for monitoring fumagillin usage in apiculture, provided that other industrial exposures to DCH are not occurring. A thorough risk assessment needs to be conducted in order to establish a maximum residue limit (MRL) for both fumagillin, as well as for DCH in honey.

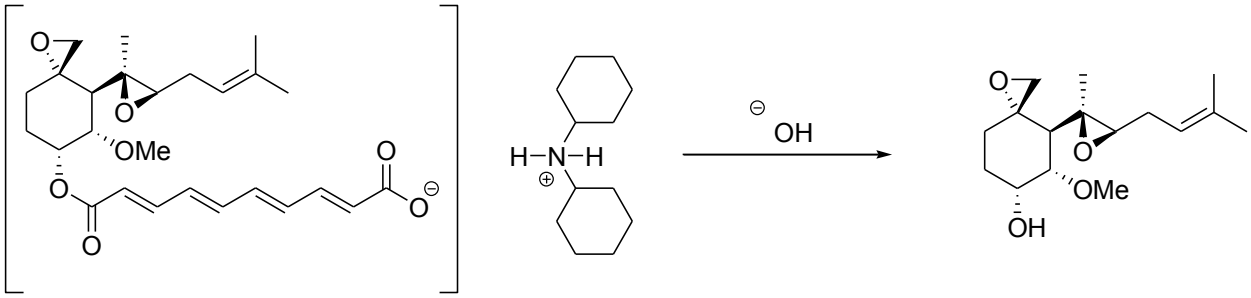
Chapter 5 Evaluation of Fumagilin-B® and other potential alternative chemotherapies against *Nosema ceranae*-infected honey bees (*Apis mellifera*) in cage trial assays

5.1 Introduction

Nosema disease of honey bees, *Apis mellifera* L., is caused by infection from two distinct species of single-cellular microsporidian fungal parasites (Genersch 2010), *Nosema apis* Zander and *Nosema ceranae* Fries et al., respectively. Infections of *N. ceranae* have been shown to cause high levels of colony loss in some regions, while both species have been implicated as part of the pathogen complex associated with colony collapse disorder (CCD) (Cox-Foster et al. 2007; Martín-Hernández et al. 2007; Higes et al. 2008, 2009c; vanEngelsdorp et al. 2009). Fumagilin-B® is the only registered chemotherapy currently available to treat Nosema disease in apiculture in North America (Williams et al. 2008a, 2011), and is reportedly allowed for use in special circumstances in Spain, the Balkan countries, and other parts of Europe (Higes et al. 2011; Stevanovic et al. 2013). Shortly after its discovery (Hanson and Eble 1949; Eble and Hanson 1951), fumagillin (**Figure 15**) was found to be effective in controlling *N. apis* in honey bees (Katznelson and Jamieson 1952; Bailey 1953). Since that time, the commercial formulations of fumagillin, namely Fumagilin-B® and Fumidil-B®, have been continuously employed to treat either *N. apis* or *N. ceranae* in North America. It should be noted that the commercial formulations of fumagillin consist of the dicyclohexylamine (DCH) salt of fumagillin, with fumagillin and DCH being present in a 1:1 stoichiometric ratio (**Figure 15**). For a more comprehensive description of fumagillin and its significance for apiculture, the reader is referred to **Chapter 2** of this thesis (van den Heever et al. 2014).

In human cancer research, fumagillin and its analogues were found to inhibit the formation of new blood vessels around tumors (angiogenesis), thereby limiting the blood supply to tumors and thus impeding their growth (Ingber et al. 1990). The mode of action responsible for these anti-angiogenic properties was elucidated with the aid of a crystal structure showing fumagillin covalently bound to a histidine moiety (His^{231}) within the enzymatic active site of the methionine aminopeptidase type 2 (MetAP-2) enzyme (Griffith et al. 1997; Sin et al. 1997; Liu et al. 1998). This covalent binding of fumagillin to the MetAP-2 enzyme results in the irreversible opening of

the spiro-epoxide group on the core cyclohexane skeleton of fumagillin (Sin et al. 1997; Liu et al. 1998; Zhang et al. 2002). Because the spiro-epoxide is reportedly required for the biological activity of fumagillin, opening of the epoxide by thermal decomposition leads to the loss of biological activity (Griffith et al. 1997, 1998; Kochansky and Nasr 2004; Lu et al. 2006).



(**1**) (**2**) (**3**)

Figure 15 The commercial Fumagilin-B®, consisting of fumagillin (**1**) as the dicyclohexylamine (**2**) salt, which can be hydrolyzed to afford the alcohol, fumagillol (**3**).

MetAP-2 enzymes are ubiquitous to most eukaryotic organisms (Zhang et al. 2002), and are also important in apiculture, as the MetAP-2 enzyme is present in both the host honey bee as well as in the disease-causative *N. apis* and *N. ceranae* (Zhang et al. 2002; Huang et al. 2013). Fumagillin binds only to the MetAP-2 enzyme, and not to the MetAP-1 enzyme (Sin et al. 1997). Comparison of the MetAP-2 enzyme binding site amino acid sequences among humans, *A. mellifera*, *N. apis* and *N. ceranae* revealed that the MetAP-2 enzyme protein sequences were identical in this region for honey bees and humans, while the *Nosema* spp. sequences were only different at two amino acids (Huang et al. 2013).

In the search for new chemical treatments against human disease, numerous research efforts have been aimed at modulating the mode of action of fumagillin by synthesizing analogues that replace the alkene side chain of fumagillin (Han et al. 2000; Baldwin et al. 2002; Drahl et al. 2005; Lu et al. 2006; Lee et al. 2007; Chen et al. 2009b; Balthaser et al. 2011). These analogues were synthesized with the objective of reducing the cytotoxicity of fumagillin, while at the same time retaining the beneficial properties of the parent compound. The cytotoxicity of fumagillin

makes it unsuitable for most human applications. Fumagillin can be hydrolyzed with a suitable base to remove the alkene side chain (**Figure 15**), yielding the alcohol, fumagillol (Tarbell et al. 1961; Gochnauer and Furgala 1962; Assil and Sporns 1991). A new chemical moiety, such as a carboxylic acid, can then be coupled to fumagillol, resulting in a new semisynthetic fumagillin analogue (Han et al. 2000; Baldwin et al. 2002; Lee et al. 2007). An example of such a semisynthetic fumagillin analogue is TNP-470, which is a potential new treatment for malaria (Zhang et al. 2002; Chen et al. 2009b).

I postulated that the known mode of action of fumagillin against the MetAP-2 enzyme in humans is similar to MetAP-2 enzymes found in the host honey bee as well as in *N. apis* and *N. ceranae*. As such, the core cyclohexane skeleton of fumagillin with the intact spiro-epoxide was used as a template for the design and synthesis of two semisynthetic analogues of fumagillin, as well as for the synthesis of several purely synthetic compounds aimed at targeting the MetAP-2 enzyme in *N. ceranae*. These, and several other commercially-available compounds that reportedly exhibited activity against Nosema disease, were evaluated for disease suppression and adult bee survival using cage trial assays. The effects of DCH, the counter ion of fumagillin in Fumagilin-B®, were also evaluated.

5.2 Materials and Methods

5.2.1 Reagents and materials

Pure fumagillin isolated from *Aspergillus fumigatus* Fres. containing no DCH (Cat. # F6771), dicyclohexylamine nitrite (Cat. # 317837), trimethylsulfoxonium iodide (Cat. # T80500), *N,N′*-dicyclohexylcarbodiimide (Cat. # D80002), 4-(dimethylamino) pyridine (Cat. # 107700), acetyl salicylic acid (Cat. # A5376), piperonylic acid (Cat. # P49805), thymol (Cat. # T0501), carbendazim (Cat. # 378674), toltrazuril (Cat. # 34000), thiabendazole (Cat. # T8904) and enilconazole (also known as imazalil or chloramizole, Cat. # Y0000136) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Purified fumagillol was produced in-house by hydrolysis of a quantity of the commercial fumagillin formulation, Fumagilin-B®(Medivet Pharmaceuticals Ltd., High River, Alberta, Canada; DIN 02231180), followed by purification using column chromatography (Assil and Sporns 1991). The commercial formulation of fumagillin,

Fumagilin-B® (fumagillin as the DCH salt), was used as a positive control in the cage trial assays. The Dess-Martin reagent used for oxidation reactions was prepared in-house according to literature procedures (Dess and Martin 1991; Frigerio et al. 1999). Nozevit solution, a natural plant polyphenol extract, was obtained from Complete Bee (AK, USA).

5.2.2 General synthetic methodology

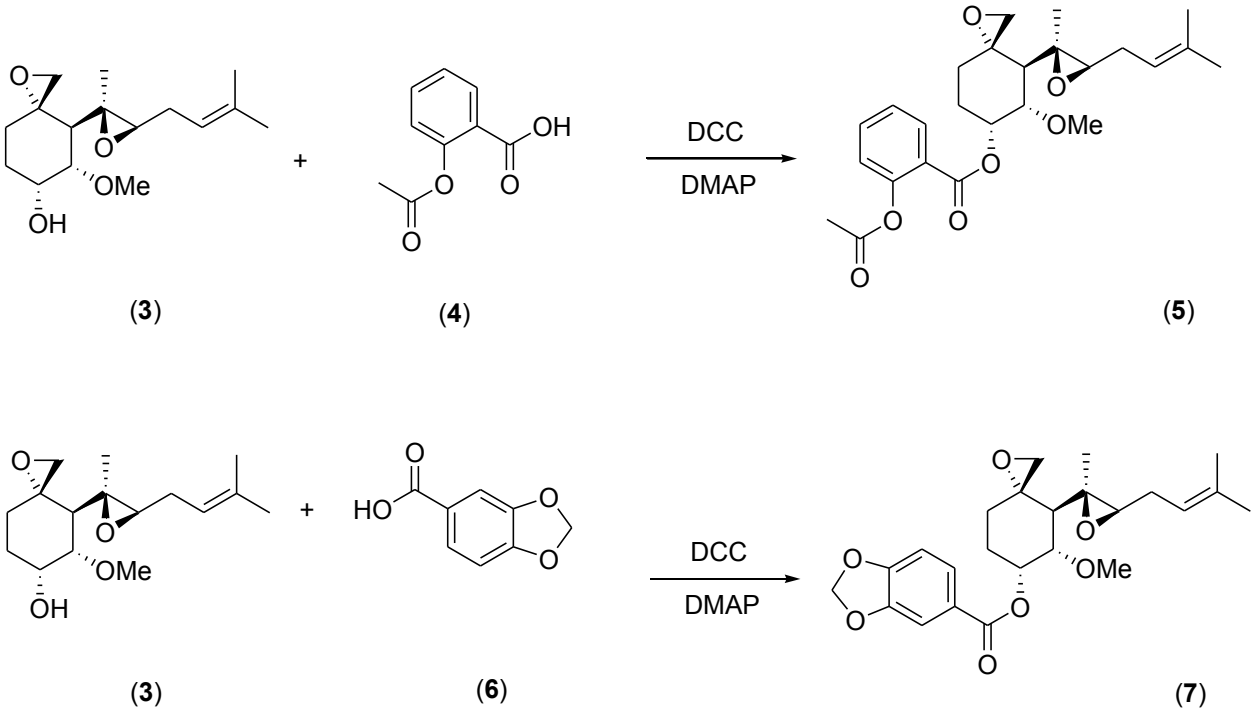


Figure 16 Coupling of fumagillol (**3**) with aspirin (**4**) or piperonylic acid (**6**) respectively, under Steglich reaction conditions using *N,N′*-dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP), to afford the aspirin analogue (**5**) and piperonylic acid analogue (**7**) of fumagillin.

Fumagillol was prepared by basic hydrolysis according to established methodology (Assil and Sporns 1991) from the commercial formulation Fumagilin-B® (**Figure 15**). Oxidation reactions to transform alcohol starting materials to the corresponding ketones were done using the Dess-Martin reaction (Dess and Martin 1991; Frigerio et al. 1999). The reactions used to prepare the semisynthetic and synthetic compounds generally involved the esterification of alcohols with carboxylic acids (**Figure 16**), and the epoxidation of ketones (**Figure 17**). General procedures for performing these reactions are given in following sections. During the chemical synthesis no

attempt was made to synthesize single stereoisomers of compounds, hence presumably racemic mixtures of analogues were prepared for initial screening. The identity of all the synthesized compounds was verified by high resolution mass spectrometry on an Agilent 6224 time of flight (TOF) mass spectrometer, with a summary of the results given in **Table 8**.

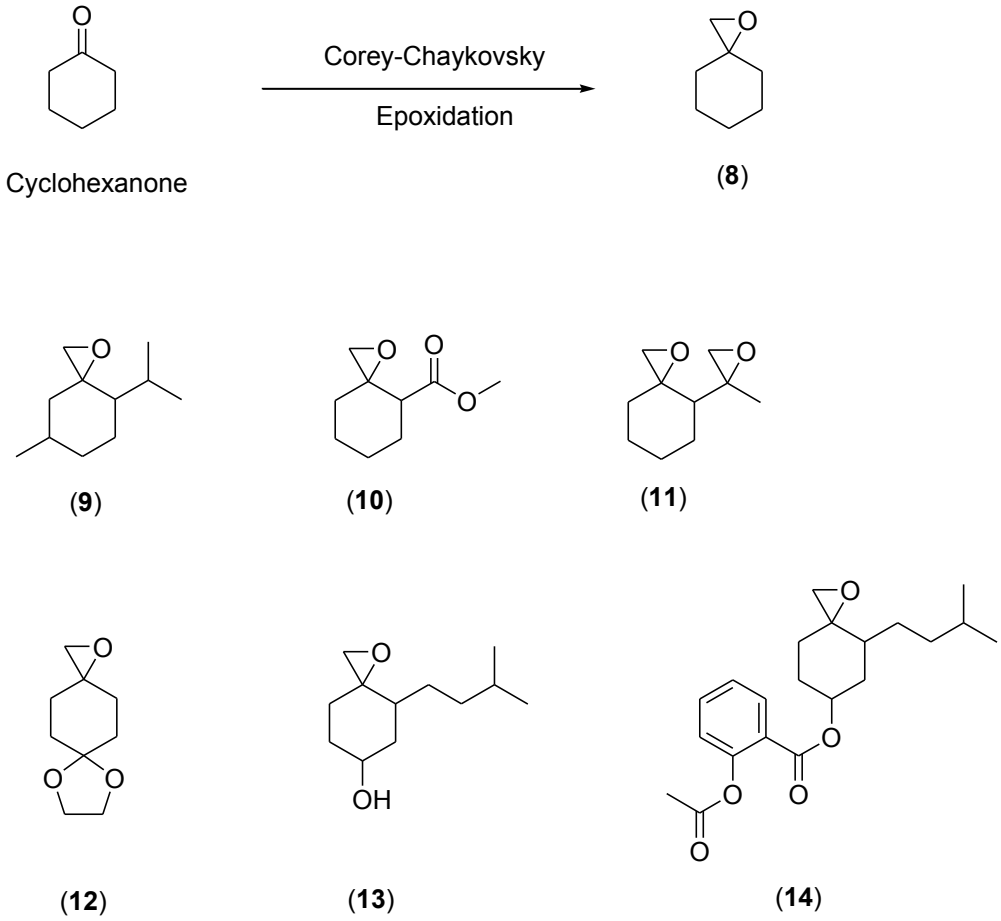


Figure 17 Purely synthetic compounds prepared by using the Corey-Chaykovsky epoxidation reaction to afford the cyclohexanone derivative (**8**), menthol derivative (**9**) as well as other synthetic analogues (**10**, **11**, **12**, **13**, **14**).

Table 8 Confirmation of the identity of semisynthetic and synthetic compounds that were tested against *N. ceranae* by high resolution mass spectrometric analysis.

Compound	Found Formula	Ion	Mass Found	Mass Calculated	Δ ppm
3	$C_{16}H_{24}O_3Na$	$[M+Na\ (-H_2O)]^+$	287.1631	287.1618	4.65
5	$C_{25}H_{32}O_7Na$	$[M+Na]^+$	467.2033	467.2040	-1.49
7	$C_{24}H_{28}O_6K$	$[M+K\ (-H_2O)]^+$	451.1512	451.1517	-1.23
8	$C_7H_{12}ONa$	$[M+Na]^+$	135.0776	135.0780	-3.49
9	$C_{11}H_{21}O$	$[M+H]^+$	169.1592	169.1587	2.72
10	$C_9H_{13}O_2$	$[M+H\ (-H_2O)]^+$	153.0913	153.0910	1.96
11	$C_{10}H_{20}NO$	$[M+NH_4]^+$	170.1531	170.1539	-4.72
12	$C_9H_{15}O_3$	$[M+H]^+$	171.1024	171.1016	4.96
13	$C_{11}H_{20}O_2Na$	$[M+Na]^+$	207.1351	207.1356	-2.02
14	$C_{25}H_{33}O_7$	$[M+H]^+$	445.2234	445.2221	3.00

5.2.3 Ester synthesis

The Steglich esterification (**Figure 16**) was used to prepare esters from the corresponding carboxylic acids and alcohols (Neises and Steglich 1978). The general procedure involved stirring a solution of the carboxylic acid (10 mmol) in anhydrous CH_2Cl_2 (10 mL). The catalyst, dimethylaminopyridine (DMAP) was added (5 mmol) followed by drop-wise addition of the alcohol (11 mmol, 1.1 equivalent). The mixture was then cooled to 0 ^oC, and then dicyclohexylcarbodiimide (DCC; 11 mmol, 1.1 equivalent) was slowly added. The mixture was stirred for 5 min at 0°C, and then for 3 h at room temperature. The precipitated dicyclohexyl urea (DCU) by-product was then filtered off, and the filtrate washed successively with 0.5 N HCl (2 x 10 mL) and saturated $NaHCO_3$ solution (1 x20 mL). The CH_2Cl_2 fraction was dried ($MgSO_4$) and concentrated *in vacuo*. The residue was then purified by flash chromatography on silica (230-400 mesh) with a suitable eluent, usually consisting of a mixture of *n*-hexane and ethyl acetate.

5.2.4 Epoxide formation from ketones

A modified version of the Corey-Chaykovsky reaction (Corey and Chaykovsky 1965) was used to prepare epoxides from ketone compounds (Ng 1990). The procedure involved dissolving trimethylsulfoxonium iodide (10 mmol) into dimethylsulfoxide (15 mL) under a nitrogen

atmosphere at room temperature. After stirring for 5 min the ketone (10 mmol) was added, followed by addition of potassium tert-butoxide (10 mmol), dissolved in dimethylsulfoxide (10 mL). Stirring was continued overnight at room temperature under nitrogen, and the mixture was then diluted with water (30 mL) and extracted with CH_2Cl_2 (3 x 30 mL) and concentrated *in vacuo*. The crude epoxide product was then purified by flash chromatography on silica (230-400 mesh) with a suitable eluent system, usually consisting of a mixture of *n*-hexane and ethyl acetate. An example of this procedure is shown for the conversion of cyclohexanone to the corresponding epoxide (**Figure 17**).

5.2.5 Cage assays – general procedures

Trials were conducted over a four-year period, from 2010 to 2013. To obtain adult honey bees (*A. mellifera* L.) for the assays, frames of sealed brood with newly-eclosing bees were obtained from several colonies at Agriculture and Agri-Food Canada's Research Farm, in Beaverlodge, Alberta, Canada (55° 18' N; 119° 17' W). These colonies were repeatedly tested to be free from both infections of *N. apis* and *N. ceranae*, using both light microscopic and molecular methods, described below. Frames were kept overnight in an incubator (Percival Model 136NLC9, Percival Scientific Inc., Perry, IA, USA) maintained at hive temperature (33 ± 0.5ºC) and a relative humidity of 70 ± 5%. Adult workers were pooled and mixed from all frames with 100 bees added to each wooden screened cage (8.0 x 9.5 x 12.0 cm I.D.). Bees were then fed 4 mL of a 60% (w/v) of aqueous sucrose syrup for 24 h, using gravity feeders fashioned from disposable centrifuge tubes (Cat. #93000-020, VWR International, Radnor, PA, USA).

After the initial 24 h period, each cage was mass inoculated with 5 mL of 60% syrup solution containing 1×10^7 freshly-harvested *N. ceranae* spores, prepared from previously-identified colonies of honey bees with high levels of *N. ceranae* infection. Workers from these colonies were euthanized on dry ice, and then their abdomens were removed and suspended in ultrapure water (1 mL per bee). After maceration, the crude suspension was filtered through a sieve (~0.8 mm) to remove large body parts, and spores were then counted (described below) to prepare solutions with the correct inoculation doses. After consumption of the inoculum for 48 h, cages of bees were fed the test compounds in 60% sucrose syrup, *ad libitum* for 17 days at prescribed dosages.

Five or six replicate cages of bees were evaluated for each concentration of each compound tested. The efficacy of test compounds for suppressing *N. ceranae* spore development was assessed by determining the average number of spores per bee at 17 days post-inoculation. Cumulative bee mortality per cage over the duration of the trials was also recorded. For the trials in which the toxicity of DCH was being assessed (Section 5.2.9), the mortality of bees was recorded each day, up to 17 days post-inoculation.

5.2.6 Determination of spore levels

To determine mean spore numbers per bee from colonies, 60 adult workers were collected from peripheral frames of the brood nest; these were used to confirm a source of inoculum for infections, or a source of *Nosema*-free bees for cage trials. When conducting the cage trials, 30 surviving workers were analyzed, collected from each cage on day 17 post-inoculation.

Worker abdomens were removed and placed in a stomacher bag containing 70% ethanol (1 mL per bee), which was then macerated for 1 min at medium speed (Seward Stomacher® 80 Biomaster, Seward Laboratory Systems Inc., Davie, FL, USA). To determine *Nosema* spore counts per bee, 6 µL of the macerate was withdrawn using a micropipette, loaded onto a Helber Z30000 counting chamber (Hawksley, Lancing, UK) and counted according to the generalized methods of Cantwell (1970), under phase contrast microscopy at 400× magnification. Samples of the remaining crude macerate were portioned into 1.5 mL microcentrifuge tubes and stored at -20°C until required for *Nosema* spp. identification.

5.2.7 *Nosema* spp. Identification

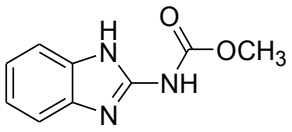
Identification and confirmation of *N. ceranae* as the only microsporidian pathogen infecting the caged bees was conducted, in order to provide evidence that no *N. apis* infection, or co-infection of *N. ceranae* and *N. apis* had occurred in the caged bees used for testing. The crude frozen macerate, described in section 5.2.6, was thawed, vortexed, and then 200-400 µL was centrifuged to remove the ethanol from the sample. DNA extraction was performed using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, CA, USA). The concentration of the extracted DNA was determined spectrophotometrically (NanoDrop 2000C, Thermo Scientific, West Palm

Beach, FL, USA), whereafter 50-100 ng of this DNA extract was amplified using polymerase chain reactions (PCR).

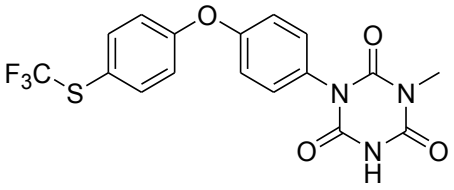
A multiplex system that co-amplified the 16S rRNA gene of *N. apis* and *N. ceranae* (Martín-Hernández et al. 2007) as well as the honey bee ribosomal protein RpS5 gene (Thompson et al. 2007b) was used within the same reaction. A modified version of the PCR protocol was used, owing to the fact that early pre-tests indicated that these modifications increased the sensitivity of simultaneous detection of both *N. apis* and *N. ceranae* within any given sample. All PCR reactions were performed using a Mastercycler® proS thermocycler (Eppendorf, Mississauga, Canada) and utilizing the Illustra™ PuReTaq Ready-To-Go™ PCR beads (GE Healthcare Life Sciences, Baie d'Urfe, Quebec, Canada). PCR beads were reconstituted to a 25 µL final volume by adding sterile H_2O, 0.5 µL of 20 mM forward and reverse primers (a final concentration of 0.4 mM) and the DNA (50-100 ng per reaction). To amplify a 218 bp 16S rRNA PCR product specific for *N. ceranae*, primers Mitoc-For (5'-CGGCGACGATGTGATATGAAAATATTAA-3') and Mitoc-Rev (5'-CCCGGTCATTCTCAAACAAAAAACCG-3') were used and to amplify a 321 bp 16S rRNA PCR product specific for *N. apis* primers Apis-For (5'-GGGGGCATGTCTTTGACGTACTATGTA-3') and Apis-Rev (5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3) were used, according to Martín-Hernández et al. (2007). In addition, the honey bee housekeeping gene, RpS5, was also amplified within the same reaction as a reference, which yielded a PCR product of 115 bp. The primer pairs used were RpS5-For (5'-AATTATTTGGTCGCTGGAATTG-3'), and RpS5-Rev (5'-TAACGTCCAGCAGAATGTGGTA-3') respectively (Thompson et al. 2007). The thermocycler program consisted of an initial DNA denaturation step at 95°C for 5min, followed by 35 cycles of 94°C for 30sec, 61.8°C for 30sec, and 72°C for 30sec. A final extension of 72°C for 7min was followed by holding the reactions at 4°C until stopped. All PCR products were visualized on a 2% agarose gel and stained with SYBR® Safe DNA gel stain (Life Technologies, Carlsbad, CA, USA).

5.2.8 Testing of semisynthetic, synthetic and commercially available compounds

In the 2010 trials, Fumagilin-B® was used to prepare three solutions at concentrations of 4, 40, and 400 μM fumagillin (and DCH) in 60% sucrose solution. This concentration range was chosen to encompass the manufacturer's recommended dose concentration of 41 μM fumagillin in 60% sucrose solution (105 mg fumagillin DCH salt, corresponding to 5 g of the formulation, dissolved into 4 L of syrup). This was achieved by first dissolving the appropriate amount of Fumagilin-B® formulation into 1.0 mL ethanol, before adding it to the 60% sucrose solution, in order to better solubilize the Fumagilin-B® powder. All test compounds were similarly prepared, at the same three concentrations of 4, 40 and 400 μM. A negative control consisting of only 60% sucrose, with 1 mL of added ethanol, was also prepared. The semisynthetic aspirin and piperonyl analogues of fumagillin (**Figure 16**, compounds **5** and **7**), as well as fumagillol (**Figure 15**, compound **3**) and the commercially available carbendazim, thiabendazole, thymol (**Figure 18**, compounds **15**, **17**, and **18**) were tested. Nozevit, sold as a bee supplement consisting of a mixture of unspecified polyphenols and other compounds, was prepared as per the manufacturer's instructions and tested as well. Several synthetic compounds (**Figure 17**, compounds **8**, **9**, **10** and **11**) were also evaluated.



(**15**)



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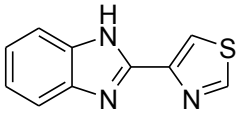

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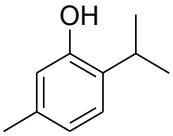
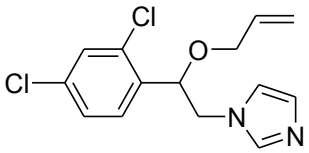


(**18**)



(**19**)

Figure 18 Commercially available compounds evaluated included carbendazim (**15**), toltrazuril (**16**), thiabendzole (**17**), thymol (**18**) and enilconazole (**19**). Nozevit, another commercially available product tested, is a bee supplement consisting of a mixture of unspecified polyphenols and other compounds.

In the 2011 trials, only a single concentration of fumagillin (and DCH) was prepared from Fumagilin-B® at 40 μM, to be used as a positive control, along with the previously described negative control. This was done because Fumagilin-B® was observed to almost completely eliminate *N. ceranae* spores in honey bees at a concentration of 40 μM during the 2010 trials, and due to the fact that we observed a significant increase in bee mortality at higher concentrations of Fumagilin-B®. Hence, only the one positive control concentration of 40 μM Fumagilin-B® was employed for 2011 and subsequent testing. The screening of synthetic compounds at concentrations greater than 40 μM facilitated easier detection of any potential biological activity that they may exhibit, given the possibility that these compounds may not be as biologically active at the same concentrations as Fumagilin-B®. Thus, the test concentrations of compounds in 2011 (and subsequent years) were increased to 40, 400 and 800 μM. Testing at 4000 μM was impractical, due to limitations on the amount of synthetic material that would be needed and dissolved into the 60% sugar solution. The semisynthetic aspirin analogue (**Figure**

16, compound **5**), as well as the commercially available carbendazim and toltrazuril (**Figure 18**, compound **15** and **16**) were tested during the cage trial assays conducted during 2011.

In 2012, testing was conducted using the same 40, 400 and 800 μM concentration regimen as described above for the 2011 tests. Three synthetic compounds (**Figure 17**, compounds **12**, **13** and **14**), as well as the commercially available aspirin (**Figure 16** compound **4**) and enilconazole (**Figure 18**, compound **19**), were tested during this year.

5.2.9 Testing of DCH in the commercial Fumagilin-B®

Our observation that Fumagilin-B® appears to lead to an increase in bee mortality at higher concentrations, along with the fact that the commercial formulations of fumagillin (Fumidil-B®, Fumagilin-B®) contain the reportedly genotoxic and tumorigenic DCH (Greim et al. 1998), caused us to suspect that DCH might be a contributing factor to this higher observed bee mortality (**Table 11**). This led to an experiment conducted in 2013 that was designed to evaluate bee mortality associated with the use of purified fumagillin (no DCH), Fumagilin-B® (fumagillin as the DCH salt) and DCH by itself. Test solutions for each of these compounds or groups of compounds were prepared at a single concentration of 40 μM in 60% sucrose solutions, along with a 60% sucrose negative control, as previously described.

5.2.10 Statistical Analysis

For the cage trials conducted to evaluate alternative chemotherapies, the effectiveness of compounds was determined by using regression models of spore counts that included compound dose as the explanatory variable. Indicators for each dose were included, compared to 0 μM negative controls as the referent, because the relationship between dose and spore count was not linear. Separate negative binomial (NB) or zero-inflated negative binomial (ZINB) regression models were run for each compound. Models were selected using the following criteria (Dohoo et al. 2009): 1) Linear regression models for spore count violated residual assumptions and were not used, despite a log transformation of spore count. 2) NB models were selected as the mean number of spore counts were not equal to the variance of Poisson models for count data (the over-dispersion parameter of NB models was significantly different than 0). 3) ZINB models were selected when the Vuong test was significant ($P \leq 0.05$), indicating that there may be a

separate process involved for those bees with zero spore counts. 4) For the inflated (logistic) portion of ZINB models, Akaike's and Bayesian Information Criteria (AIC and BIC) were used to determine the best fit for compound dose (dose indicators compared to treated versus non-treated, lower AIC and BIC representing a better fitting model).

Likelihood ratio tests (LRT) were used to determine if groups of concentration indicators were significantly associated with changes in spore count ($P \leq 0.05$). If the LRT was significant, Wald tests were used to compare each individual dose to the referent concentration (0 μM). The fit of NB models was assessed using deviance chi-squared tests ($P \leq 0.05$ indicating the model did not fit the data) and residual analysis. The fit of ZINB models was assessed by residual analysis.

Differences in mortality for compounds significantly related to spore count were evaluated descriptively by comparing proportions of mortality for bees in all replicates of a given dose compared to controls. Probit analysis to determine median lethal concentrations (LC_{50}) for each compound was attempted, using Abbott's formula for correction for control mortality.

For the cage trial assays used to assess the influence of DCH on the mortality of *N. ceranae*-infected bees, data was analyzed using semi-parametric, Cox proportional hazards models with a complimentary log-log link for survival data (Corrente et al. 2003; Dohoo et al. 2009). The bee survival for indicator variables for each treatment group was considered against negative controls using LRT ($P \leq 0.05$). To test for proportional hazards across treatment groups, an interaction between treatment group and survival time was considered. A LRT $P \leq 0.05$ indicated non-proportional hazards, which was solved by including the interaction in the model. Linear regression was used to assess the effects of these treatment methods on the natural log of spore counts to normalize the data (zero spore counts were changed to one to allow for the log value to be zero). The effect of an indicator variable treatment group was tested using the extra sum of squares F-test (Dohoo et al. 2009). The model was assessed for fit by evaluating the normality and homoscedasticity of the standardized residuals.

All analysis was conducted in Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and STATA Intercooled 13.1 (StataCorp LP, College Station, TX, USA).

5.3 Results

5.3.1 Potential alternative chemotherapies against *N. ceranae*

Molecular identification of *N. ceranae*, using the described PCR techniques confirmed that bees were only infected with *N. ceranae.* The results of the NB and ZINB models are shown in **Table 9** and **Table 10**, respectively. Fumagilin-B®, as well as fumagillol (**Figure 15**, compound **3**), exhibited statistically significant biological activity against *N. ceranae* under our test conditions (**Table 10**). Both semisynthetic analogues of fumagillin, namely the aspirin analogue (**Figure 16**, compound **5**) and the piperonyl analogue (**Figure 16**, compound **7**) showed statistically significant biological activity against *N. ceranae*-infected bees under the test conditions described.

From **Table 9** it can be seen that the purely synthetic compounds (**Figure 17**, compounds **8**, **10**, **11** and **14**) also exhibited statistically significant biological activity against *N. ceranae* under the described test conditions. The commercially available thymol and enilconazole (**Figure 18**, compound **18** and **19**) were also active against *N. ceranae* (**Table 10**). Model assessment for the NB models indicated that they were a good fit (Deviance chi-squared $P>0.05$ and normal residuals), with the exception of one NB model for carbendazim, where the $P=0.05$; however, carbendazim was not significantly associated with reduced spore counts. Model assessment for the ZINB models indicated that they were a good fit using the tools available (Vuong $P\leq0.05$). Detected outliers were evaluated and kept in all models.

Cumulative bee mortalities by concentrations for those compounds shown to significantly impact spore count are shown in **Table 11**. It is interesting to note the higher bee mortality associated with Fumagilin-B®, when compared to the negative control. The mortality associated with Fumagilin-B® also increases with an increase in concentration. Further, initial attempts to conduct probit analysis to determine LC_{50} values determined that models did not fit the data using Pearson Chi-squared tests (data not shown). For most compounds, testing was only conducted at three concentrations, making it difficult to determine an LC_{50} value, where it is generally accepted that having two to three concentrations above as well as below the LC_{50} is best for this calculation.

5.3.2 Toxicity of DCH in the commercial formulation Fumagilin-B®

The final Cox proportional hazards model found that the treatment group and an interaction between the treatment group and survival time were significant (LRT $P<0.01$ for both). This indicated that the hazards for each treatment group varied over time.

The predicted hazards by treatment group are shown in **Figure 19**. Specific comparisons between two treatments at distinct time points (hazard ratios) are shown in **Table 12**. For the first 10 days of the trial, there were no obvious differences in the hazards (probability of death) for any treatment group. Starting on Day 11, the commercial Fumagilin-B® (fumagillin with DCH) caused significantly higher bee mortality than any other group. By day 13, both Fumagilin-B® and DCH alone had significantly higher mortality than either the control or purified fumagillin, but not from one another. Commercial Fumagilin-B® and purified DCH consistently had higher bee mortality than purified fumagillin after Day 11. There were no significant differences in mortality between the purified fumagillin and the control group.

Table 9 Results of the negative binomial models to assess the relationship between test compound concentration and average *N. ceranae* spore count at 17 days post-infection after feeding each compound *ad libitum* in 60% sugar solution.

Compound	n[1]	Dose (μM)[2]	IRR[3]	LRT *P*-value[4]	Wald *P*-value[5]	95% CI[6]
Cyclohexanone derivative (**8**)	28	0	Referent	0.03	--	--
	6	4	0.35		0.02	0.14-0.87
	6	40	0.41		0.06	0.16-1.03
	6	400	0.32		0.02	0.13-0.81
Compound(**10**)	28	0	Referent	0.03	--	--
	6	4	0.24		0.01	0.08-0.73
	6	40	0.21		0.01	0.07-0.63
	6	400	0.44		0.15	0.15-1.35
Compound (**11**)	28	0	Referent	<0.01	--	--
	6	4	0.55		0.22	0.21-1.43
	6	40	0.08		<0.01	0.03-0.21
	6	400	0.43		0.09	0.17-1.13
Compound (**14**)	28	0	Referent	<0.01	--	--
	5	40	2.56		0.07	0.93-7.04
	5	400	3.49		0.02	1.27-9.60
	4	800	5.64		<0.01	1.85-17.20
Aspirin (**4**)	43*	--	Dose	0.52	--	--
Compound (**9**)	46*	--	Dose	0.07	--	--
Compound (**12**)	46*	--	Dose	0.44	--	--
Compound (**13**)	46*	--	Dose	0.85	--	--
Carbendazim (**15**)	63*	--	Dose	0.67	--	--
Toltrazuril (**16**)	47*	--	Dose	0.09	--	--
Thiabendazole (**17**)	46*	--	Dose	0.12	--	--
Nozevit	46*	--	Dose	0.32	--	--

[1]Total number of cages of 100 bees for each dose. Spore counts for each cage represent the average number of spores per bee.
[2]The dose of each compound tested compared to negative controls (0 μM).
[3]Incident rate ratio (ratio of spore counts per bee of the dose in question compared to the referent negative control dose). IRRs are not shown for compounds where the dose variable was not significantly associated with changes in spore counts (see 5).
[4]Likelihood ratio test of whether or not the group of indicators for dose is significant to the model for spore count.
[5]Wald tests of whether or not IRR's for each individual dose compared to controls is significantly different than 1.0.
[6]The 95% confidence interval for the IRR.
*These numbers also include 28 cages of controls

Table 10 Results of the zero-inflated negative binomial models to assess the relationship between test compound concentration and average *N. ceranae* spore count at 17 days post-infection after feeding each compound *ad libitum* in 60% sugar solution.

Compound (model portion)[1]	n[2]	Dose (μM)[3]	IRR/OR[4]	LRT/Wald P-value[5]	Wald P-value[6]	95% CI[7]
Fumagilin-B® (**1**)	28	0	Referent	<0.01	--	--
NegBin	11	4	0.36		<0.01	0.19-0.68
	23	40	0.04		<0.01	0.02-0.07
	11	400	1.1e-7		1.00	0-∞
Inflated (treated)	28	No	Referent	N/A	--	--
	45	Yes	31.82		<0.01	3.71-273.14
Fumagillol (**3**)	28	0	Referent	<0.01	--	--
NegBin	6	4	0.84		0.62	0.41-1.70
	6	40	0.27		<0.01	0.12-0.59
	5	400	0.03		<0.01	0.01-0.15
Inflated (dose)	28	0	Referent	0.02	--	--
	6	4	0.00		1.00	0-∞
	6	40	6.75		0.21	0.35-130.32
	5	400	107.77		<0.01	5.58-2100.65
Aspirin Analogue (**5**)	28	0	Referent	<0.01	--	--
NegBin	6	4	0.55		0.11	0.27-1.14
	12	40	0.38		<0.01	0.22-0.64
	13	400	0.10		<0.01	0.06-0.28
	5	800	0.13		<0.01	0.06-0.28
Inflated (treated)	28	No	Referent	N/A	--	--
	36	Yes	6.51		0.09	0.75-56.49
Piperonyl Analogue (**7**)	28	0	Referent	<0.01	--	--
NegBin	6	4	0.26		<0.01	0.13-0.52
	6	40	0.28		<0.01	0.14-0.56
	6	400	0.43		0.03	0.20-0.92
Inflated (treated)	18	No	Referent	N/A	--	--
	28	Yes	7.69		0.08	0.79-75.94
Thymol (**18**)	28	0	Referent	<0.01	--	--
NegBin	6	4	0.31		<0.01	0.16-0.62
	6	40	0.30		<0.01	0.15-0.59
	6	400	0.54		0.08	0.27-1.07
Inflated (treated)	28	No	Referent	N/A	--	--
	18	Yes	5.42		0.16	0.52-56.83
Enilconazole (**19**)	28	0	Referent	<0.01	--	--
NegBin	5	40	0.47		0.051	0.22-1.005
	5	400	0.16		<0.01	0.07-0.34
	3	800	0.12		<0.01	0.04-0.37
Inflated (treated)	28	No	Referent	N/A	--	--
	13	Yes	11.25		0.04	1.19-106.70

[1]Model portion refers to the Negative Binomial (NegBin) portion or the zero-inflated logistic (inflated) portion.
[2]Total number of cages of 100 bees for each dose. Spore counts for each cage represent the average number of spores per bee.
[3]The dose of each compound tested compared to negative controls (0 μM).
[4]Incident rate ratio (ratio of spore counts per bee of the dose in question compared to the referent negative control dose) for the NegBin portion. Odds ratio for the Inflated portion (either indicators for each dose or for a variable that is treated – yes or no).
[5]Likelihood ratio test of whether or not the group of indicators for dose is significant to the model for spore count for the NegBin model. Wald test of the group of indicators for dose for the Inflated model. If treatment – yes or no – was used, refer to the Wald test in 6.
[6]Wald tests of whether or not IRR's or OR's for each individual dose compared to controls is significantly different than 1.0.
[7]The 95% confidence interval for the IRR or the OR.

Table 11 Cumulative mortalities for bees infected with *N. ceranae* that were fed varying concentrations of test compounds *ad libitum* in 60% sugar solution. Only compounds that had a statistically significant impact on spore counts are included.

Treatment	Dose (µM)	dead	total	mortality	Mean Spore Count
Control	0	1587	2810	56.5%	7,214,286
Fumagilin-B®	4	634	1100	57.6%	4,068,182
	40	1634	2300	71.0%	423,913
	400	939	1100	85.4%	22,727
Fumagillol (**3**)	4	226	600	37.7%	2,916,667
	40	413	600	68.8%	1,000,000
	400	541	600	90.2%	350,000
Aspirin analogue (**5**)	4	304	600	50.7%	3,548,333
	40	267	600	44.5%	2,166,667
	400	1084	1900	57.1%	1,315,789
	800	441	600	73.5%	750,000
Piperonyl analogue (**7**)	4	288	600	48.0%	1,625,000
	40	276	600	46.0%	1,750,000
	400	416	600	69.3%	2,166,667
Compound (**8**)	4	280	600	46.7%	2,500,000
	40	267	600	44.5%	2,958,333
	400	332	600	55.3%	2,333,333
Compound (**10**)	4	302	600	50.3%	1,750,000
	40	351	600	58.5%	1,500,000
	400	217	600	36.2%	3,208,333
Compound (**11**)	4	264	600	44.0%	3,958,333
	40	332	600	55.3%	583,333
	400	320	600	53.3%	3,125,000
Compound (**14**)	40	182	500	36.4%	18,500,000
	400	427	500	85.4%	25,200,000
	800	494	500	98.8%	40,700,000
Thymol (**18**)	4	268	600	44.7%	1,583,333
	40	276	600	46.0%	5,375,000
	400	308	600	51.3%	2,541,667
Eniconazole (**19**)	40	355	500	71.0%	4,950,000
	400	340	500	68.0%	10,800,000
	800	493	500	98.6%	2,416,667

*One cage contained 110, rather than 100 bees

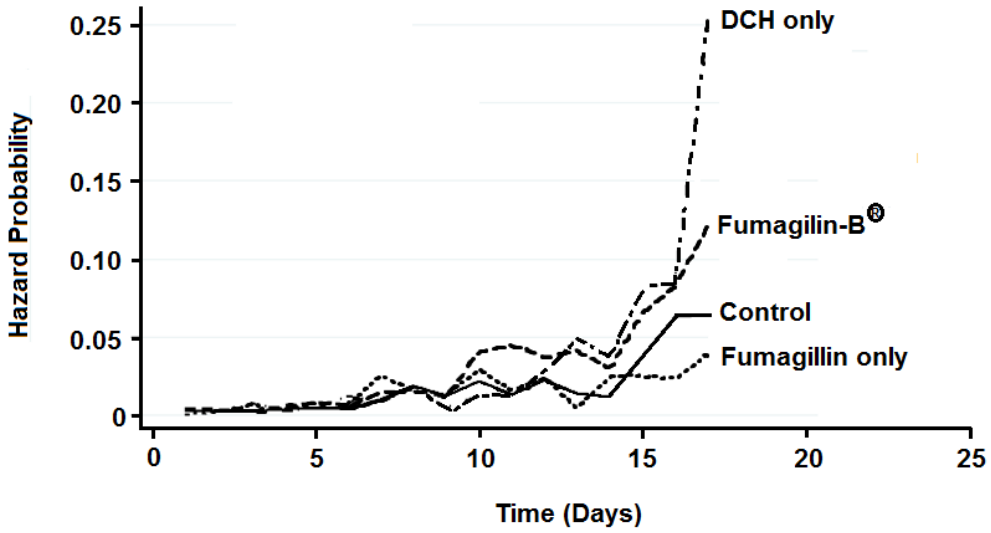


Figure 19 The predicted hazards from the complimentary log-log, Cox proportional hazards survival model for the cage trial to assess toxicity of dicyclohexylamine (DCH), fed *ad libitum* at a concentration of 40 μM in 60% sugar solution, to bees infected with *N. ceranae* over 17 days of treatment.

The results of the linear regression model for spore counts in the comparative cage trials to assess the effects of DCH are shown in **Table 13**. The treatment was significantly associated with altered spore counts ($P \leq 0.01$). The commercial Fumagilin-B® reduced spore counts to zero in all replicates, which was a significant reduction (in the order of millions), compared to all other groups, including the purified fumagillin group ($P \leq 0.01$ for all). Purified fumagillin (no DCH) significantly reduced the spore count compared to the control and pure DCH groups ($P \leq 0.01$ for both); however, the spore reduction was in the order of 20 times less for both, which has questionable clinical significance. Residuals from the model were normally distributed and homoscedastic (data not shown).

Table 12 Results of the complimentary log-log, Cox proportional hazards survival model for the cage trial to assess toxicity of dicyclohexylamine (DCH), fed *ad libitum* at a concentration of 40 μM in 60% sucrose solution, to bees infected with *N. ceranae* over 17 days of treatment.

Treatment	HR[1]	*P*-value	95% CI[2]
DCH only *vs*. Negative Control			
Day 11	0.97	0.96	0.31-3.02
Day 13	3.66	0.01	1.48-9.02
Day 15	2.20	0.01	1.21-4.00
Day 16	1.36	0.24	0.81-2.28
Day 17	4.34	<0.01	2.78-6.77
Fumagilin-B® *vs*. Negative Control			
Day 11	3.44	0.01	1.38-8.57
Day 13	3.06	0.02	1.21-7.76
Day 15	1.77	0.07	0.95-3.32
Day 16	1.34	0.27	0.79-2.27
Day 17	1.94	0.01	1.18-3.20
Fumagillin only *vs*. Negative Control			
Day 11	1.23	0.71	0.41-3.67
Day 13	0.35	0.20	0.07-1.74
Day 15	0.66	0.31	0.30-1.46
Day 16	0.36	0.01	0.17-0.76
Day 17	0.60	0.11	0.31-1.13
DCH only *vs*. Fumagillin only			
Day 11	0.79	0.67	0.27-2.35
Day 13	10.40	<0.01	2.45-44.22
Day 15	3.33	<0.01	1.64-6.76
Day 16	3.82	<0.01	1.82-8.00
Day 17	7.27	<0.01	4.20-12.57
Fumagilin-B® *vs*. Fumagillin only			
Day 11	2.79	0.02	1.18-6.60
Day 13	8.70	<0.01	2.01-37.66
Day 15	2.68	0.01	1.29-5.58
Day 16	3.77	<0.01	1.79-7.93
Day 17	3.25	<0.01	1.80-5.89
Fumagilin-B® *vs*. DCH only			
Day 11	3.53	0.01	1.42-8.79
Day 13	0.84	0.58	0.44-1.58
Day 15	0.80	0.41	0.48-1.35
Day 16	0.99	0.96	0.60-1.62
Day 17	0.45	<0.01	0.31-0.65

[1]HR – hazard ratio
[2]CI – confidence interval

Table 13 Results of linear regression model for the cage trial to assess the effects of various treatment preparations with or without dicyclohexylamine (DCH), fed *ad libitum* at a concentration of 40 µM in 60% sugar solution, on spore counts in bees infected with *N. ceranae* over 17 days of treatment.

Compound	Median Spores (10^6/bee)	SD[1] (10^6)	Coefficient (spores/bee)	*P*-value	95% CI[2]	Mean Spore Count
Negative Control	12.0	3.72	Referent	--		12,900,000
DCH only	13.0	4.51	1	0.47	-1 to 2	15,100,000
Fumagilin-B®	0	0	$-12.38e^{+6}$	<0.01	$(-19.64 \text{ to } -7.91)\ e^{+6}$	0
Fumagillin only	0.75	0.29	-21	<0.01	-33 to -13	650,000
Fumagilin-B® *vs*. Fumagillin only	--	--	$-0.59e^{+6}$	<0.01	$(-0.93 \text{ to } -0.37)\ e^{+6}$	N/A
Fumagilin-B® *vs*. DCH only	--	--	$-14.53e^{+6}$	<0.01	$(-23.05 \text{ to } -9.16)\ e^{+6}$	N/A
Fumagillin only *vs*. DCH only	--	--	-25	<0.01	-39 to -16	N/A

[1]standard deviation.
[2]confidence interval.
e – exponent ($-12.38e^{+6} = -12{,}380{,}000$).

5.4 Discussion

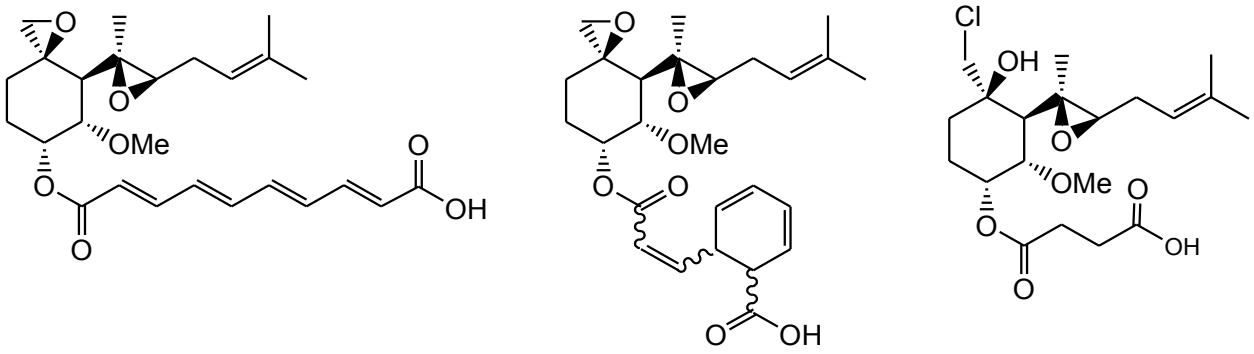
All of the semisynthetic and pure synthetic compounds tested were chosen to be easily preparable from readily available starting materials, which were preferentially selected to be naturally occurring compounds (piperonylic acid, menthol, aspirin – the acetylated derivative of salicylic acid, etc.) possessing antifungal properties. All starting materials were also selected to contain either an alcohol or ketone functional group located on a cyclohexane moiety that mimics the core structure of fumagillin. The alcohols and ketones could then be easily converted into epoxides, using the described synthetic methodologies. Both semisynthetic derivatives of fumagillol (**Figure 15**, compound **3**), namely the aspirin and piperonylic acid analogues (**Figure 16**, compounds **5** and **7**) were observed to exhibit statistically significant, although lower, activity against *N. ceranae*-infected bees in cage trial assays compared with Fumagilin-B® (**Table 10**). Fumagillol itself also exhibited statistically significant, but lower biological activity than Fumagilin-B® (**Table 10**). This is consistent with reports citing lower biological activity of the alcohol (Gochnauer and Furgala 1962). The diastereomeric UV-decomposition products of fumagillin (**Figure 19**, compounds **20**) have been reported to retain some of the biological activity of the parent fumagillin (Kochansky and Nasr 2004). The observed biological activity of the aspirin analogue (**Figure 16**, compound **5**), which is structurally very similar to the UV-decomposition products of fumagillin, is consistent with their activity. Aspirin, alone, did not

exhibit any statistically significant activity against *N. ceranae*-infected bees in our study (**Table 9**), even though it does possess antifungal properties.

Four of the purely synthetic compounds (**Figure 17**, compounds **8**, **10**, **11** and **14**) were also observed to exhibit statistically significant, activity against *N. ceranae*, though inferior to that of Fumagilin-B® (**Tables 9** and **10**). One of these compounds was the simple cyclohexanone derivative (**Figure 17**, compound **8**). This simple spiro-epoxide compound was previously synthesised and tested for activity against the MetAP-2 enzyme, where it was observed to have only trace amounts of biological activity (Arico-Muendel et al. 2009).

Of the six commercially available compounds tested (**Figure 18**, compounds **15**, **16**, **17**, **18** and **19**), including Nozevit, only thymol and enilconazole (**Figure 18**, compound **18** and **19**), were observed to be biologically active against *N. ceranae* (**Table 10**). In our experiments thymol lowered the *N. ceranae* spore count by an average of 40% over all three test concentrations after 17 d, when compared to the control group. In a previously reported cage trial study, a similar beneficial effect was observed for thymol, where *N. ceranae*-infected bees treated with 0.12 mg g^{-1} thymol in candy (85% icing sugar, 10% honey, 5% water and 3.2 µL g^{-1} ethanol) were reported to have only 8.8% of the amount of spores after 25 d, when compared to the control group (Maistrello et al. 2008). In a subsequent study the same researchers reported that thymol, when fed to *N. ceranae*-infected bees in a 0.1 mg g^{-1} concentration in either a 50% (w/v) sugar syrup or in a candy form (85% icing sugar, 10% honey, 5% water and 3.2 µL g^{-1} ethanol), reduced the *N. ceranae* spore counts by 50% after 25d when compared to the control group (Costa et al. 2010). The lifespan of the bees treated with thymol was also increased by three days, when compared to the control group. Resveratrol was also observed to have a similar effect to that of thymol in both studies (Maistrello et al. 2008; Costa et al. 2010). Thymol is registered in Canada under the trade name of Thymovar™ (Reg. #. 29747), and is sold as 15 g thymol impregnated cellulose wafers for use against *Varroa destructor* (Anderson and Trueman 2000) infestations. Thymol is sold worldwide under different trade names and formulations, and is applied as a fumigant. For example, in the EU and the USA it is sold as a gel (Apiguard®), or in combination with menthol, camphor and eucalyptol, as an impregnated wafer (Apilife Var®). In our study, as well as others, thymol was applied orally (Maistrello et al. 2008; Costa et al. 2010). It would be of interest to learn if treatments of varroa mite infestations with products that contain

thymol as the main active ingredient also have the added benefit of providing some secondary protection against Nosema disease.



(**1**) (**20**) (**21**)

Figure 20 Comparison of the chemical structures of fumagillin (**1**), UV-decomposed fumagillin diastereomeric compounds (**20**) and the naturally occurring chlorohydrin, ligerin (**21**), which is reportedly equally or more biologically active against osteosarcoma cell lines than fumagillin, in spite of the fact that the intact spiro-epoxide (which is reportedly responsible for biological activity of fumagillin analogues) is not present in this compound.

The intact epoxide on the core cyclohexane ring of fumagillin is reportedly crucial for its observed activity (Griffith et al. 1997, 1998; Kochansky and Nasr 2004; Lu et al. 2006; Arico-Muendel et al. 2009), but the rest of the fumagillin molecule may also be important for enzymatic recognition by the MetAP-2 enzyme. A recent study on the naturally occurring ligerin (**Figure 20**, compound **21)**, which is structurally very similar to fumagillin, except for the shortened side chain and the spiro-epoxide on the core cyclohexane structure being opened up and replaced by a chlorohydrin moiety. It was reported that ligerin was equally or more active against osteosarcoma cell lines than fumagillin, even though the spiro-epoxide on the core cyclohexane skeleton of ligerin is opened (Blanchet et al. 2014). It was further reported that spiro-epoxides could be easily converted into the corresponding halohydrin compounds by reaction with halogen salts such as lithium chloride or lithium bromide. This study raises the interesting possibility that chlorohydrin compounds could be effective against *N. ceranae*. The synthetic compounds in our study which exhibited an effect against *N. ceranae* could also be easily converted into the corresponding chlorohydrin compounds. However, a major

complication in developing new fumagillin analogues for apicultural usage based on their mode of action against the MetAP-2 enzyme is still the presumably low selectivity of fumagillin and other analogues between the host honey bee and the pathogen (*N. ceranae*) MetAP-2 enzyme (Huang et al. 2013). Further research into the identification of other enzymes that may be present in *N. ceranae* is desirable. This might lead to the discovery of different enzymes that may be crucial for the proliferation of the *Nosema* spp., while ideally not affecting the host honey bee, which could afford different biological targets other than the MetAP-2 enzymes, which could be pursued in future chemotherapy research.

It is important to note that there are limitations to using spore counts from cage trials to evaluate the efficacy of alternative compounds against *N. ceranae*, as well as against other bee diseases. For example, in cage trial studies bees cannot fly, and therefore do not defecate or conduct housekeeping operations such as the removal of dead bees from the hive. This could lead to different results being obtained when applying antibiotics in field trials to whole bee colonies. In our study, which was designed as a preliminary screen to evaluate alternative compounds for use against *N. ceranae*, only those bees that survived to day 17 had their spores counted. It is possible that bees dying earlier than day 17 could have greater spore counts than that of those that survived. Further studies that track daily mortality, using potentially effective compounds are certainly warranted. Testing of these compounds under field conditions would also give a better indication of their effectiveness.

A recent study reported that very low concentrations of Fumagilin-B® (1000 to 20 000 times lower than the manufacturer's recommended dosage) are estimated to be present in beehives at approximately 2 to 5.5 months post Fumagilin-B® treatment, and that these residual concentrations of fumagillin negatively impacted the health of the host honey bee, while not supressing *N. ceranae* spore formation (Huang et al. 2013). This study also provided evidence that these low residual concentrations of Fumagilin-B® leads to a hyperproliferation of *N. ceranae* spore production. These conditions could be conducive to the development of resistance by *N. ceranae* against Fumagilin-B®. Although no resistance to Fumagilin-B® has been reported in apiculture for either *N. apis* or *N. ceranae*, there has been a report of resistance being observed to the usage of Fumidil-B® for an unidentified *Nosema* spp. infecting the diamondback moth, *Pluetella xylostella* L. (Idris et al. 2000, 2001). Based on this report, it could be speculated that

resistance to fumagillin may develop in apiculture in future, owing to the prolonged usage of fumagillin against Nosema disease, dating back almost to its discovery in the early 1950's. Research into the development of new chemical treatments against Nosema disease in apiculture is therefore of utmost importance, given that Fumagilin-B® is currently the only effective chemical treatment available.

It is important to remember that Fumagilin-B® is commercially sold as the dicyclohexylamine (DCH) salt, containing both fumagillin and DCH in a 1:1 stoichiometric ratio (**Figure 15**, compound **1** and **2**). Using Fumagilin-B® or Fumidil-B® therefore introduces not only one, but two potentially biologically active compounds to the beehive (van den Heever et al. 2014). From our study to determine the toxicity of DCH by examining bee mortality, it was observed that the risk of bee mortality of *N. ceranae*-infected caged bees treated with DCH only was not statistically different than commercial Fumagilin-B® for most of the study period, with the exception of days 11 and 17 (**Table 12**). Bees treated with DCH or commercial Fumagilin-B® had significantly higher risk of mortality compared to controls or those treated with purified fumagillin for most days after day 11, with a few exceptions (**Table 13**). The observed high bee mortality associated with the use of the commercial Fumagilin-B® needs to be investigated further, as Fumagilin-B® contains not only fumagillin, but also DCH. DCH has also been shown to be significantly more stable in honey under a variety of conditions (van den Heever et al. 2015b), and is reportedly also genotoxic and tumorigenic. In our study the beneficial properties of purified fumagillin was also observed to be almost twenty times less than that of the commercial Fumagilin-B®. This is presumably due to the fact that the purified fumagillin is not chemically stabilized as a salt and therefore prone to decomposition during handling and application.

5.5 Conclusions

From the results obtained during the cage trial evaluation of alternative chemotherapies against *N. ceranae*, four purely synthetic compounds were found to exhibit statistically significant biological activity against *N. ceranae*, although none are as effective as Fumagilin-B®. Fumagillol, as well as the semisynthetic aspirin and piperonyl analogues also exhibited statistically significant activity against *N. ceranae*, but were also not as effective as

Fumagilin-B®. Despite our knowledge of fumagillin's activity against the MetAP-2 enzyme, no commercially viable alternative to fumagillin was discovered in this study - nevertheless, it is hoped that the information described here will stimulate other research efforts aimed at finding new alternative chemotherapies against *N. ceranae*. Amongst the commercially available compounds evaluated, only thymol and enilconazole were found to have some activity against *N. ceranae*. The high bee mortality related to enilconazole usage makes this compound less attractive for commercial purposes, however thymol is a commercially available product that might be used to combat Nosema disease.

Evaluation of the toxicity of the DCH, which is present in a 1:1 stoichiometric ratio to fumagillin in the commercial formulations of fumagillin, showed that DCH appears to have a negative effect on bee survival. The genotoxic and mutagenic properties of DCH, combined with its lipophilicity, could lead to the accumulation of DCH in comb wax, where it could potentially impact the development of the bee larvae and pupa. DCH is also a contaminant of concern in hive products, with regard to food safety and human health. The frequent detection of DCH residues in honey by LC-MS/MS, even in the absence of detectable fumagillin residues, was recently reported (van den Heever et al. 2015a). The risk to the consumer associated with DCH residues in honey at the levels detected (20-235 ng g^{-1}), should be evaluated. DCH was also reported to be significantly more stable than fumagillin in honey (van den Heever et al. 2015b). A different formulation of fumagillin that uses a less toxic counter ion to form the salt should be further investigated. This would eliminate DCH from the commercial fumagillin formulation, and would clearly benefit bee health, and improve the quality of honey destined for human consumption.

Chapter 6 General discussion and conclusions

6.1 Significance of this research

Several key observations and findings made during the course of this research enhance the body of knowledge available to the apicultural industry. The significance of dicyclohexylamine (DCH), used in the formulation of the salt form of fumagillin in the commercial products (Fumagilin-B® and Fumidil-B®) was recognized (**Chapter 2**) and further investigated with the development of an analytical method to quantitate not only fumagillin and its degradation products, but also DCH in honey.

A LC-MS/MS method and extraction procedure were developed to quantitate and confirm DCH, fumagillin, as well as its UV decomposition products in honey (**Chapter 3**). The sample extraction procedure used in this method is compatible with current published methods which do not yet include DCH as an analyte. This should facilitate the adoption of this analytical method. A stable deuterium labelled analogue of DCH was synthesized to counteract the matrix effects observed for this analyte during the method development. This internal standard is easily prepared in a one-pot reaction form commercially available starting materials. When honey samples were analyzed it was found that DCH was present at significant concentrations in honey, even when no fumagillin was detected. This indicates that DCH is a much better marker residue than fumagillin itself, when analyzing honey to determine Fumagilin-B® or Fumidil-B® usage. The apparent higher stability of DCH in honey, as compared to that of fumagillin, was therefore further examined.

The relative stability of fumagillin and DCH in honey was investigated under a variety of conditions (**Chapter 4**) simulating bulk storage, hive and shelf conditions. It was observed that DCH was significantly more stable than fumagillin in honey under all conditions tested. This information could be used with caution by honey producers and regulators trying to estimate the amount of DCH in honey resulting from fumagillin usage, but further field trial research needs to be performed in order to verify the results obtained from the simulated stability study. In my research, the half-life of DCH was observed to be approximately 1-3 years in honey under the various simulated conditions examined. DCH can therefore be expected to accumulate in beeswax, and to have a long half-life in this matrix, but this remains to be confirmed. The

relative stability of DCH, when compared to that of fumagillin confirms that DCH is indeed a better marker residue than fumagillin, when examining Fumagilin-B® or Fumidil-B® usage in apiculture.

The mode of action of fumagillin against the MetAP-2 enzyme in humans is well established (Ingber et al. 1990). It has also been determined that the MetAp-2 enzyme in humans, honey bees, *N. ceranae* and *N. apis* is almost identical (Huang et al. 2007). I hypothesised that the mode of action of fumagillin is the same in humans, honey bees, *N. ceranae* and *N. apis*, and this hypothesis was tested in the design, synthesis and evaluation of alternative chemical compounds other than fumagillin to treat Nosema disease (**Chapter 5**). Several commercially available, as well as some novel synthetic and semisynthetic compounds, were evaluated *in vivo* against *N. ceranae*-infected bees in cage trial experiments. The effectiveness of the test compounds were evaluated using spore count as the measure of efficacy. Four purely synthetic compounds were found to exhibit statistically significant biological activity against *N. ceranae*, although none were as effective as Fumagilin-B®. Fumagillol (the hydrolysis product of fumagillin) as well as the semisynthetic aspirin and piperonyl fumagillin analogues also exhibited statistically significant activity against *N. ceranae*, but again none were as effective as Fumagilin-B® in suppressing *N. ceranae* spore production from infected caged bees. Amongst the commercially available compounds evaluated, only thymol and enilconazole were found to be active against *N. ceranae*. The high bee mortality related to enilconazole usage makes this compound less attractive for commercial purposes, however thymol might be used as an alternative treatment for Nosema disease. Interestingly, high cumulative bee mortality was observed in the control groups of *N. ceranae*-infected bees being treated with Fumagilin-B®.

The observed high bee mortality related to Fumagilin-B® usage was further examined in an experiment designed to evaluate the effects of fumagillin, DCH and Fumagilin-B® (fumagillin and DCH) on *N. ceranae*-infected caged bees (**Chapter 5**). It was observed that that oral exposure of *N. ceranae*-infected caged honey bees to DCH and to Fumagilin-B® leads to a statistically significant risk of increased bee mortality, when compared to pure fumagillin and the control group. This indicates that DCH appears to have a negative effect on bee health.

6.2 Conclusions and Future Prospects

In this research the prevalence and fate of Fumagilin-B® was examined in honey only. Other hive products such as wax need to be examined for DCH and fumagillin residues. The current analytical method sample extraction (**Chapter 3**) is unsuitable for analyzing wax samples. The labelled d_{10}-DCH internal standard that I developed for the LC-MS/MS method used to analyze honey would however be very useful for the analysis of DCH in matrices such as wax or pollen. Determining the prevalence of DCH in wax would be very useful for apiculturists as well as for the cosmetic industry applications of beeswax. The prevalence of DCH in pollen and runoff water next to busy roads and industries should be examined, since this might indicate whether an environmental source of DCH is indeed of concern. The fact that fumagillin decomposes fairly rapidly when exposed to light could be considered as a benefit as far as residues in honey is concerned, but remains a problem for commercial beekeepers when treating large numbers of colonies, due to the exposure of fumagillin in the prepared sugar solution to light during transit, or when barrel feeding. The results of this research suggest that in order to ensure the least amount of chemical residues in honey destined for human consumption, Fumagilin-B® should only be applied well before the bees start to collect nectar for honey production. A fall application of Fumagilin-B® should concentrate chemical residues in the brood chambers, and minimize their presence in the "supers" used for honey production. Fumagillin should also be more beneficial during the winter in controlling Nosema disease, as the bees cannot leave the hive easily for cleansing flights, and are closely confined with longer lifespans, which leads to conditions conducive to the spread of disease. Spring application should likely be avoided.

The observed relative stability of DCH (**Chapter 4**) in honey indicates that it is likely that DCH will accumulate in wax, and that it will be present at elevated concentrations. The general stability of chemical contaminants in wax (Bogdanov 2004) supports this assumption, but it remains to be investigated. Accumulation of DCH in the comb wax could negatively impact the development of young bee larvae, which are in close proximity to the wax at a stage of their lifecycle that make them potentially more susceptible to the influence of chemical contaminants such as the reportedly genotoxic DCH. The observed increased bee mortality associated with DCH and Fumagilin-B® in *N. ceranae*-infected caged bees (**Chapter 5**) supports this conclusion, but further research is needed.

The development of an alternative salt of fumagillin that does not contain DCH would be desirable, as this would eliminate DCH residues from the hive and hive products destined for human consumption. Fumagillin is not chemically synthesized, but is produced through a fungal fermentation process. The DCH salt of fumagillin is presumably used to crystallize fumagillin from the fermentation broth, before further formulation occurs. The DCH-fumagillin salt could potentially be purified by using for example an ion exchange resin column that would "trap" the DCH, while allowing the fumagillin to exit the column bed along with the eluent. Fumagillin could then be re-crystallized with another suitable less toxic counter ion. This will add another purification step to the process, which would result in an increase in the cost of production, but would ultimately result in improved bee health and food safety.

The potential synergistic effects resulting from the combination DCH and fumagillin with other known chemical contaminants in the hive needs to be examined. Previous research has shown that combined exposure to Fumagilin-B® and to *tau*-fluvalinate leads to an increase in *tau*-fluvalinate toxicity to honey bees, resulting in increased mortality (Johnson et al. 2013). *Tau*-fluvalinate was reportedly the most abundant pyrethroid chemical found North American apiaries (Mullin et al. 2010) in wax (98.1% detection at a median concentration of 3595 ng g^{-1}), pollen (88.3% detection at a median concentration of 40.2 ng g^{-1}) and bees (83.6% detection at a median concentration of 53 ng g^{-1}). The concentration of Fumagilin-B® used for the synergism study (designed to evaluate subtherapeutic concentration effects) was however only 0.78 μM (Johnson et al. 2013), which is 50 times less than the manufacturer's prescribed minimum therapeutic concentration of 40 μM for Fumagilin-B®. A significant increase in toxic synergism with *tau*-fluvalinate can therefore be expected at the therapeutic concentration of Fumidil-B® (or Fumagilin-B®). It would be interesting to establish whether it is fumagillin or whether it is DCH that was responsible for this observed synergistic effect, as the commercially available Fumidil-B® was used in this study (Johnson et al. 2013). Pyrethroids, including *tau*-fluvalinate, are reportedly very stable in wax, where they accumulate, with estimated half-lives of approximately 5 years (Bogdanov 2004). The synergistic effects of both DCH and fumagillin with *tau*-fluvalinate (as well as other frequently found chemical residues in hive products) warrants further research.

Fumagilin-B® (and Fumidil-B®) has been used extensively since almost 1950 (van den Heever et al. 2014), combined with a recent report that resistance to Fumidil-B® was observed in an unidentified *Nosema* sp. infecting the diamondback moth (Idris et al. 2000, 2001), makes finding alternative chemical treatment against Nosema disease important, should antibiotic resistance develop to fumagillin. Our observation that orally administered thymol has a statistically significant effect on *N. ceranae* (**Chapter 5**) is promising, and it would be interesting to determine if fumigation with thymol, which is used to combat mite infections, also acts against *N. ceranae* at the same time. Evaluation of the chlorohydrin analogues of the four synthetic analogues of fumagillin that we tested could also result in increased activity against *N. ceranae*. Further research is however needed in order to determine whether there are enzymes other than MetAP-2 that are crucial for the survival and functioning of *N. ceranae*. In the best case scenario these potential enzymes would be unique to *N. ceranae* (and to *N. apis*) and would not be present in the host honey bee. Such an enzyme, if found, would be a much better target for the design of alternative chemotherapies, since fumagillin (and mimics of fumagillin) target the MetAP-2 enzyme that is also crucial to the survival of the host honey bee. Alternative chemical treatments that target the MetAP-2 enzyme will most likely also become ineffective if resistance to fumagillin should develop, since they are designed to mimic the same mode of action as that of fumagillin.

Cage trial *in vivo* testing used for this research is however very labor and time intensive, and also seasonally bound, as newly-eclosed bees free from infection are required. *In vitro* testing of alternative treatments would be a much better option, but this is dependent on the availability of bee cell lines which can be used to propagate the obligatory intracellular *N. ceranae* microsporidian fungal pathogen. *N. ceranae* needs host cells to propagate, because it can only exist as metabolically inactive spores outside of the host cell (Bigliardi and Sacchi 2001). The susceptibility of the heterologous cell line IPL-LD-65Y obtained from the ovaries of the gypsy moth, *Lymantria dispar* (Goodwin et al. 1978) was however recently used to successfully propagate both *N. apis* and *N. ceranae* in the laboratory *in vitro* (Gisder et al. 2011). This development, along with another cell line derived from honey bee (*A. mellifera*) embryonic tissues (Goblirsch et al. 2013b) could enable the *in vitro* screening of potential new chemical

treatments against Nosema disease, which would improve the efficiency of screening potential alternative chemotherapies.

My research should also lead to more informed decisions with regards to setting a MRL for DCH and fumagillin, since the significance of DCH in Fumagilin-B®, Fumidil-B®, honey and other hive products has been overlooked since 1950. The harmonization of MRL standards worldwide would be the ideal situation that we should aspire to, but it is a moving target at best. The lack of harmonization can impede fair trade, by excluding honey from one country while it is acceptable in another. A recent example of this is the change in the MRL for tylosin in honey from 10 ng g^{-1} to a zero tolerance limit in Japan (The Japan Food Chemical Research Foundation 2014), while there is no requirement for a tylosin MRL in the USA. Disputes can take a long time to resolve through organizations such as the World Trade Organization (WTO). At best it is probably up to the producer to be fully aware of the requirements of the market where he or she hopes to sell his products. A thorough risk assessment of fumagillin and DCH needs to be conducted. The proposed MRL of 25 ng g^{-1} for fumagillin by Health Canada (Fishbein 2013) needs to be re-examined to include a MRL for DCH as well. This research may eventually lead to policy changes regarding the MRL status of fumagillin and DCH in honey destined for human consumption, and also perhaps to a change in the formulated product to exclude the use of DCH as the counter ion of the salt form in the commercial product.

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