Potential of insect proteins for food and feed

Effect of endogenous enzymes and iron-phenolic complexation

Renske H. Janssen

Propositions

1. Crude protein content overestimates the potential of insects as protein source.

   (this thesis)

2. During grinding of insects, iron can cause off-colour formation besides enzymatic browning.

   (this thesis)

3. Playing is the most underestimated course for development in the school system. *(McNamara L. (2013), CJAR, 14:2, 3-21)*

4. Insects will be one of the allergies of the future.

5. A graph does not represent the effort to produce it.

6. The maximum speed of an e-bike for adults should be inversely correlated to the age of the adult.

Propositions belonging to the thesis entitled;

'Potential of insect proteins for food and feed

Effect of endogenous enzymes and iron-phenolic complexation'

Renske H. Janssen
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Renske H. Janssen

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ABSTRACT

Insects have been identified as excellent alternative source of proteins due to their high protein content. The acceptance of insects increases when used as ingredient in an invisible manner and hence grinding is necessary. Off-colour formation occurs upon grinding larvae, which can hamper their potential use as ingredient for food and feed. The aim of this thesis was to investigate potential of insect larvae as protein source, the mechanisms responsible for the browning or blackening of larvae during grinding, and its impact on protein functionality. This was investigated for the larvae of three species: *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*.

The specific Kp factor of 4.76±0.09 was determined for the three species to determine protein content based on nitrogen, and 5.60±0.39 after protein extraction and purification. Thus, the general Kp factor of 6.25, used until now, overestimated the protein content in insects. Off-colour formation upon grinding was caused by both enzymatic and non-enzymatic browning. Phenoloxidase was found to be mainly responsible for browning in *T. molitor* and likely in *A. diaperinus*, whereas iron-phenolic complexation likely contributed to the black colour in *H. illucens*. A model system of L-DOPA and iron was used to elucidate the structures of the iron-L-DOPA complexes by mass spectrometry.

Enzymatic browning did not influence the solubility of the proteins of all three species. Upon *in-vitro* hydrolysis by pepsin and trypsin, soluble proteins from *H. illucens* were more digestible compared to those of *T. molitor* and *A. diaperinus*. Phenoloxidase activity during processing negatively affected *in-vitro* pepsin hydrolysis. Besides phenoloxidase activity, also endogenous proteases remained active at pH 8 in extracts of insect larvae. Summarizing, endogenous enzyme activities and iron complexation should be taken into account for future application, as well as the specific Kp factor to prevent overestimation of the protein content of insects.

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1

GENERAL INTRODUCTION

INTRODUCTION

The demand for sustainable protein sources to feed the increasing world population is growing at a fast pace, as the current world population is expected to reach 9.7 billion in 2050, according to the UN DESA report (United Nations 2015). This means that the present food, and concomitantly its protein, production will not be sufficient to support this growth. The Food and Agricultural Organization of the United Nations (FAO) states that the world food production will need to rise by 70% to feed the world in 2050 (Bruinsma 2009). The demand for proteins is expected to increase even more with the change in diets towards more animal proteins due to increasing welfare in developing countries that adopt Western dietary habits (Belluco et al. 2013). The meat production is expected to increase from 258 million tonnes in 2007 to 455 million tonnes in 2050 (Alexandratos & Bruinsma 2012). In the world, on average 40% of the human protein intake is derived from animal proteins (Boland et al. 2013). There is high interest for alternative protein sources like seaweed, microalgae, duckweed and insects (van der Spiegel & Noordam 2013). Proteins from insects are the focus of this thesis. Insects are not regularly eaten in the Western world and seen as culturally inappropriate (Rozin & Fallon 1987). Using insects as ingredient in an invisible form can increase its acceptability. To make insects invisible, grinding is necessary. Previous research has shown that enzymatic browning occurs during this process (Yi et al. 2013). The aim of this research is to investigate the potential of insects as protein source, to study the mechanisms of browning, and to determine how browning affects protein functionality.

1.1. Need for novel proteins

In general, animal proteins are considered of higher value than plant proteins (Belluco et al. 2013). Nowadays, around 80% of the agricultural land is used to produce livestock (van Huis & Tomberlin 2017), and further increase in agricultural land for this purpose cannot be done in a sustainable way. Thus, alternative protein sources should be explored, such as insects. This is necessary for both food and feed (Boland et al. 2013). Also scientists of the European Academies Science Advisory Council (EASAC) recently stated that the food habits across Europe need to change drastically for food security, by for example using insects (EASAC 2017).

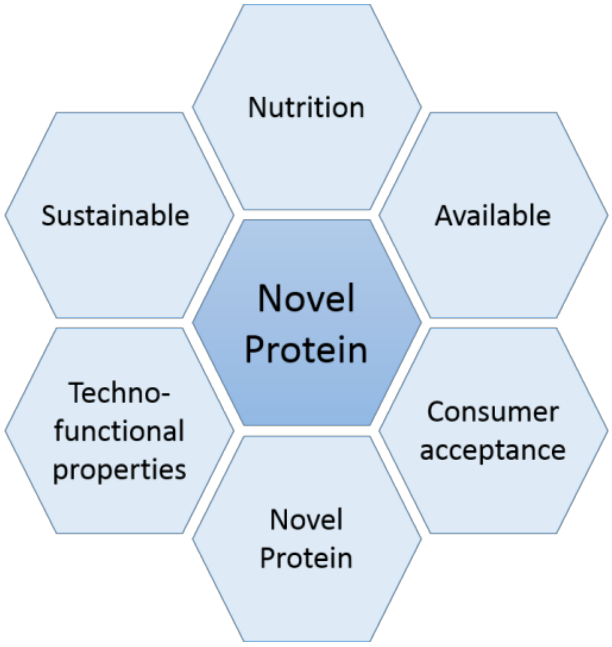

Figure 1.1. Overview of relevant parameters of novel protein sources.

Besides protein supply for food, also alternative protein sources for feed are necessary (Manceron et al. 2014). Nowadays, the three main sources for quality feed protein in Europe are oilseed (316 million tonnes), animal by-product (14 million tonnes) and fishmeal (7 million tonnes) (FAO 2014) and other protein sources are necessary to meet the protein demand.

Novel protein sources should comply with different parameters, as indicated in **Figure 1.1**. Those novel proteins should have a good nutritional profile, having all essential amino acids, and limited anti-nutritional factors. It should be mass-produced to decrease the cost price to compete with other protein sources. Replacing only 5% of the meat in 2050 with novel proteins would mean 23 million tonnes of novel proteins. In addition, the novel source should be accepted by consumers, safe, allowed to use, have good techno-functional properties and be more sustainable than conventional protein sources.

Of novel protein sources, insects have received much attention in the last decade. The first authorized insect production for food in The Netherlands was by Kreca Ento-Food BV and multiple companies followed in the last years. Over 200 start-ups and organisations worldwide are working on edible insect food products or promotion of insects, and this number is still increasing (Bug Burger 2018).

1.2. Insects as novel protein source

The habit of eating insects is called entomophagy. Entomophagy has been part of the traditional human diet in many countries in Africa, Asia, Australia, Middle-East and Middle-America (DeFoliart 1999). Estimates of 2 billion people already consuming insects have been often mentioned to convince Western consumers that entomophagy is not uncommon. This number is likely overestimated as not everyone in a country consumes insects. Over 2000 insect species have been listed as edible (Jongema 2017). Consuming insects only gained attention Western countries in the last decade as sustainable novel protein source.

1.2.1. Sustainability

The habit of entomophagy gained a lot of interest in recent years in Europe. This focussed mainly on the lower environmental impact of insect production compared to that of conventional livestock for example cattle or chicken. This is because insects are poikilothermic (cold-blooded) and need no energy to maintain their body temperature, as they use the temperature from the environment, whereas conventional animals are homeothermic (warm-blooded) and need energy to keep their body temperature constant. In addition, the feed conversion efficiency, which is the amount of feed necessary to obtain one kg of edible animal, is more efficient. The feed conversion efficiency of commercially produced *T. molitor* is 2.2, similar to that of chicken (2.3), but significant lower compared to those of pigs (4.0) and cattle (8.8) (Oonincx & de Boer 2012; Oonincx et al. 2015). Besides, the growth of *T. molitor* requires less water and land use. Furthermore, rearing of *T. molitor* emits fewer greenhouse gasses, decreasing its global warming potential compared to milk, pork, chicken and beef production (Oonincx & de Boer 2012; Oonincx et al. 2010). Only a higher amount of energy is required to produce insects compared to other animal sources. This is partly due to maintaining a constant ambient environment temperature, which is necessary for growth of poikilothermic animals. Most evidence for a low environmental impact exists for *T. molitor* and can be likely extrapolated to other insect species. However, this should be investigated.

Insects have been also indicated as potential alternative ingredient for animal diet, particularly for aquaculture and poultry (Verbeke et al. 2015). Low value organic waste can be used by insects, to convert into high value insect proteins for animal feed, which are comparable as protein source to the widely used soybean meal (Rumpold & Schlüter 2013a; Boland et al. 2013). The feed requirement for insects is less specific in comparison to conventional livestock and they can grow on waste streams, like brewer's spent grains, catering and vegetable waste and thus decreasing the environmental impact. To make

insect a viable option and decrease the environmental impact even more, upscaling of insect production is necessary for the use as novel protein source in industry and to decrease the production cost to compete with other protein sources (Ghaly & Alkoaik 2009).

1.2.2. Acceptance as food and feed

The book 'why not eat insects?' was already written in 1886 by Vincent Holt. Human consumption of insects is still uncommon in the Western world and can evoke 'disgust', as they are mainly seen as pest. This resistance of consumers toward eating insects is an important determinant for its use in food. Often insects are considered a culturally inappropriate food, which are not considered as edible (Rozin & Fallon 1987). Sensory appeal and appropriateness of developed food products might help to overcome disgust (Shan et al. 2015). On the other hand, insects for feed, particularly for poultry and fish, is widely accepted, as insects belong to the diet of these animals in their natural habitat (Rumpold & Schlüter 2013b).

Most people are already eating insect constituents, but they are not aware of this. For instance, the red colourant carmine (E120) is used in many products, like cakes, yoghurts but also in cosmetics. This is derived from the female cochineal insects *(Dactylopius coccus Costa* / *Coccus cacti L*) (Belluco et al. 2017). Another food additive is shellac (E904), which is used to make apples and candies shiny. Shellac is derived from *Kerria lacca* excretions.

Reduction of their visibility can help Western consumers to overcome initial barriers for trying insects and improve consumers acceptance. (Shan et al. 2015; Tan et al. 2016; Schösler et al. 2012). Thus, insect powders are necessary to prepare products in which insects are invisible and grinding is necessary. However, when insects are ground, enzymatic browning occurs directly. This will give an off-colour to the insect powder and products made thereof. Dark colour will decrease consumer acceptance and should be avoided. This research therefore focusses on the browning and its effect for future application of insect proteins.

1.2.3. Legislation

Legislation is besides consumer acceptance a barrier for insects as food. Edible insects were not specifically addressed in the European legislation. The old novel food regulation (258/1997), which was in place until end of 2017, stated that food ingredients were considered novel when not consumed by humans to a significant degree in one of the EU member states before 15 May 1997 and have a safe history of use. This is not the case for insects and their traditional consumption outside the EU was not enough. The new food regulation 2013/2283, which is applicable from this year onwards, aims to accelerate the

authorisation process of products already consumed outside EU, for example insects, with a centralized procedure by European Food Safety Authority (EFSA). Seven insect species i.e. *Hermetia illucens*, *Musca domestica*, *Tenebrio molitor*, *Alphitobius diaperinus*, *Acheta domesticus*, *Gryllodes sigillatus* and *Gryllus assimilis* are already allowed as aquaculture animal feed when fed with feed grade substrates according to the EU regulation 2017/893.

For the new novel food legislation, also more information about the risk, such as allergies, biological or chemical hazards is necessary (EFSA 2015). The long history of entomophagy in non-EU countries suggests safety upon consumption of insects, with little evidence of negative health effects (DeFoliart 1992). Yet, this should be confirmed in order to enter the EU market. Insects have been shown to accumulate certain heavy metals. *T. molitor* larvae were shown to accumulate arsenic, whereas *H. illucens* larvae accumulated lead and cadmium (van der Fels-Klerx et al. 2016). The limit in feed should therefore be determined per species. The microbial load of insects is high and should be taken into account, but this is similar to that in animal farming (Belluco et al. 2013). Most of the micro-organisms are present in the gut of the insects and regarded as harmless to animals and humans. Microbial hazards will originate from rearing, handling, processing and preservation (EFSA 2015). Blanching, chilling and drying are mostly used to reduce this microbial load (EFSA 2015). Belluco et al. (2013) stated that the safety of some insects was comparable to conventional animal products and simple hygienic measures, like cooking and freezing, should be applied. To increase the sustainability of insects as protein source, insects have a high potential to valorise low value side streams (van der Spiegel & Noordam 2013) into higher value edible insect biomass. However, according to legislation, it is currently not allowed to feed manure, catering waste or former foodstuff containing meat and fish as insects are considered as 'farmed animals' and can only be fed with safe feed (EFSA 2015).

1.2.4. Food allergy

A food allergy towards insects might develop and will follow the same regulation as other food allergens. All novel protein sources can potentially cause an allergic reaction. Symptoms can range from oral allergy to anaphylactic shock. Besides direct sensitisation to a specific protein, also cross-reactivity can occur. Multiple allergens are known to give a cross-reaction between crustaceans, chelicerates and between some insect species, including tropomyosin, α-amylase, hexamerin 1B precursor, myosin, arginine kinase and glutathione S-transferase (Verhoeckx et al. 2014; Broekhoven et al. 2016). For crustacean allergic people, 87% had a probable cross-reactivity towards mealworm (Broekman et al. 2016). Thermal processing and *in-vitro* digestion diminished, but did not prevent a cross-reactivity with crustacean and house dust mite allergic people (Broekhoven et al. 2016), whereas other research indicated that thermal processing only

decreased the solubility and not the allergenicity (Broekman et al. 2015). Future tests should confirm the allergenicity *in-vivo*. Besides cross-reactivity, specific allergy towards *T. molitor* indicated mealworm larval cuticle proteins as allergen (Broekman et al. 2017). Anaphylaxis has been reported for *T. molitor* before (Freye 1996). As anaphylaxis can occur and there is a high chance for cross-reactivity, it is important to use appropriate labelling when insects are used in food products.

1.3. Insect species

Tenebrio molitor (yellow mealworm) and *Alphitobius diaperinus* (lesser mealworm) larvae are reared on larger scale. They are used in multiple food applications within Europe and are a promising alternative to conventional food protein sources (Ghaly & Alkoaik 2009). *Hermetia illucens* (black soldier fly) is promising for industrial feed application (van Huis et al. 2013; Oonincx et al. 2015). Therefore, all three species are studied in this thesis (**Figure 1.2**). Larvae are often used for food and feed, as metamorphosis will use energy and thus decrease the food conversion efficiency.

Figure 1.2. Larvae of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*.

T. molitor and *A. diaperinus* belong to the order of Coleoptera and to the family of Tenebrionidae. Coleoptera species are the most commonly consumed insects globally, as they are easy to rear and maintain (Ghaly & Alkoaik 2009). A female *T. molitor* deposits 400-500 eggs (Ghaly & Alkoaik 2009) on average, which hatch into white-coloured mealworm larvae after about 10-12 days. Larvae turn yellowish brown upon feeding. The transition of larvae to the pupal stage is 83-227 days, depending on the feed (Oonincx et al. 2015). The development from pupa to adult is 7 days, independent of the feed (van Broekhoven et al. 2015). The adult beetle is shiny black, about 10 mm in length and has a life span of 37-96 days (Ghaly & Alkoaik 2009). The lifecycle of *A. diaperinus* is similar to that of *T. molitor* as shown in **Figure 1.3A**. Larvae are white at the beginning and obtain a yellowish-brown appearance upon feeding.

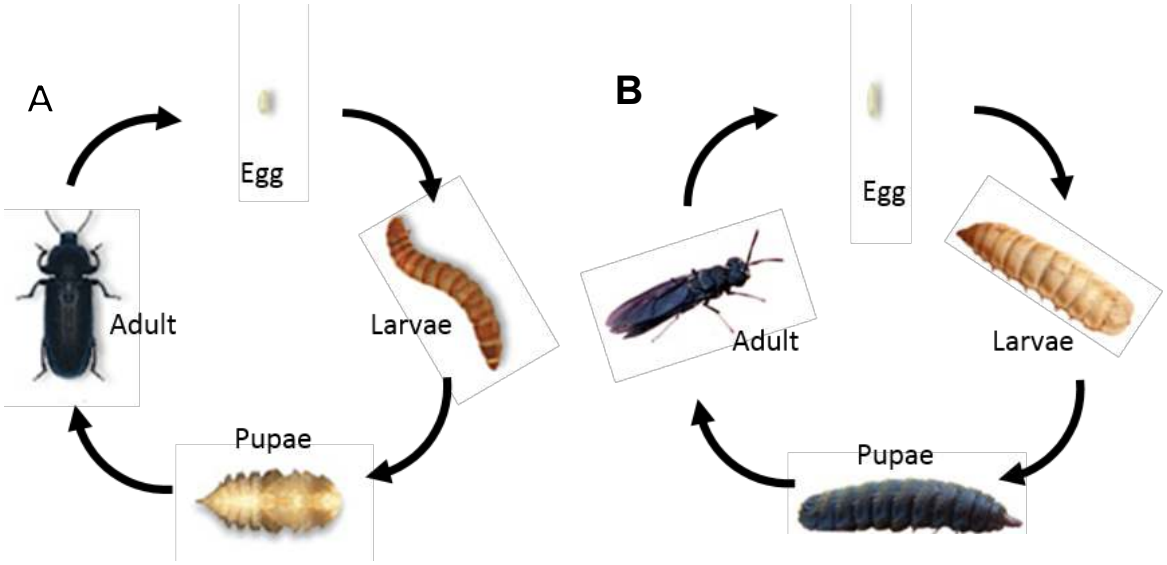

Figure 1.3. Life cycle of (A) *Tenebrio molitor* and *Alphitobius diaperinus*, (B) *Hermetia illucens*.

The lifecycle of *H. illucens*, belonging to the order of Diptera and Stratiomyidae family, is shown in **Figure 1.3B**. A female deposits up to ~600 oval shaped eggs, which hatch into larvae (Tomberlin et al. 2002). Larvae become brown with distinct body segments upon growth. It takes between 21-37 days for larvae to develop in pupae, but the actual time depends on feed quality (Oonincx et al. 2015). After pupation, an adult emerges with a wasp-like appearance. This adult is black or dark blue with a length of 13 to 20 mm (Tomberlin et al. 2002). *H. illucens* are found throughout the Western hemisphere, but are most common in North America. Due to their ability to grow on for example manure, they are commercially of interest.

1.4. Insect proteins

Insects are highlighted as a good supplementary protein source for food and feed because of their well-balanced amino acid profile (Rumpold & Schlüter 2013a). They contain all essential amino acids needed for human consumption in sufficient quantities (Yi et al. 2013). The crude protein content of insects is on average 40−75% on dry matter basis (Bukkens 1997). The overall nutritional value of insect proteins is comparable to that of other animal protein sources as crustaceans, fish and meat (Belluco et al. 2017). The average composition of the *T. molitor*, *A. diaperinus* and *H. illucens* in larval stage is shown in **Table 1.1.**

Table 1.1. Nutritional composition (crude protein, fat, ash, and fibre content of *Tenebrio molitor* (n=7) (Rumpold & Schlüter 2013a; Bosch et al. 2014; Zielińska et al. 2015; Barroso et al. 2014), *Alphitobius diaperinus* (n=2) (Despin & Axtell 1994; Bosch et al. 2014) and *Hermetia illucens* (n=6) (Bosch et al. 2014; Barroso et al. 2014; Sánchez-Muros et al. 2014; Makkar et al. 2014) larvae based on dry matter). (mean ±S.D.) Fibre was not determined in part of the references.

	Protein [%]	Fat [%]	Ash [%]	Fibre [%]
Tenebrio molitor	50.9 ± 3.85	34.7 ± 5.95	3.2 ± 0.52	7.2 ± 4.82
Alphitobius diaperinus	66.3 ± 2.16	21.4 ± 1.07	4.6 ± 0.64	7.3
Hermetia illucens	41.9 ± 7.42	23.7 ± 8.54	14.7 ± 3.94	7.0

Insects contain different types of proteins; haemolymph, cuticular and muscle proteins. Research on *T. molitor* has shown that water soluble proteins mainly consist of haemolymph proteins (Yi et al. 2016), whereas insoluble proteins are mainly cuticular and muscle proteins (Holten-Andersen et al. 2011). An overview is shown in **Table 1.2**.

Haemolymph is the fluid that circulates in the interior of arthropods comparable to blood in vertebrates and contains high concentrations of free amino acids. Most abundant haemolymph proteins in larvae are storage proteins or hexamerins, which are used for synthesis of proteins needed in the adult stage (Kanost 2009). Small proteins (< 14 kDa) are suggested to be anti-freeze type of proteins (Graham et al. 1997; Graham et al. 2001).

Table 1.2. Type, size, and solubility of proteins present in *T. molitor* larvae.

Abundant proteins	Size (kDa)	Solubility
Haemolymph		Soluble
Anti-freeze proteins	<14	
Storage proteins or hexamerins	~80	
Cuticular	14-30	Insoluble
Myofibrillar (muscle)		Salt-soluble or insoluble
Myosin	31, ~220	
Actin	42	

The cuticle, which is part of the exoskeleton, is a complex composite material, made of chitin filaments embedded in a protein matrix (Andersen, Hojrup, et al. 1995). Chitin is a polymer of *N*-acetyl-β-D-glucosamine, and represents a major component of the insect cuticle. The cuticle in larvae is soft bodied, pliant and flexible, because of its high water content and equal amounts of chitin and protein (Andersen 2002). The cuticle in adult

insects on the other hand, is solid and sclerotized with little water and contains more chitin than protein (Andersen 2002). Most cuticular regions in insects contain hydrophilic proteins having weak interactions with other components to allow for stretching and expansion during growth. Cuticle proteins in *T. molitor* have molecular weights predominantly between 14 and 30 kDa (Andersen, Rafn, et al. 1995).

T. molitor and *A. diaperinus* larvae have skeletal muscles present, consisting of large size proteins. Muscle proteins (myofibrillar protein) are classified as salt-soluble or insoluble (Andersen, Rafn, et al. 1995). Muscle protein in *T. molitor* includes tropomyosin and actin (42.1 kDa) and sarcoplasmic Ca-binding proteins (Verhoeckx et al. 2014). *T. molitor* tropomyosin can be divided into myosin heavy chain with molecular weight of ~220 kDa and myosin light chain with molecular weight of 31.3 kDa (Verhoeckx et al. 2014; Yi et al. 2016).

Research on the total protein content of insects has focused on the crude protein content determined by multiplying the total nitrogen content with the general nitrogen-to-protein factor (Kp) of 6.25 (Bukkens 1997; Yi et al. 2013; Zhao et al. 2016; Rumpold & Schlüter 2013a; Finke 2013; Finke 2002). Yet, the nitrogen in insects is not only present in proteins but also from nonprotein constituents, such as chitin. Therefore, it is likely that the protein content in insects is often overestimated, which calls for determining specific Kp's for insects to calculate protein content more accurately.

1.5. Enzymatic browning

Acceptability has been shown to increase when insects are used in an invisible manner. However, off-colour formation occurs upon grinding of insect larvae, likely caused by enzymatic browning (Yi et al. 2013). This off-colour formation is different between species ranging from light brown for *A. diaperinus* to brown for *T. molitor*, and black for *H. illucens*. Multiple enzymes can play a role in enzymatic browning: phenoloxidases, laccases, tyrosine hydroxylase, peroxidase and DOPA decarboxylase (**Figure 1.4**) (Kanost & Gorman 2008; Andersen 2012). These enzymes have been studied for roles in sclerotization, wound healing and immunity in insects (Andersen 2012; Sugumaran 2002). Yet, no research has been performed on which enzymes are responsible for browning upon grinding and what causes the difference in off-colour formation between species. This will be investigated in this research.

Enzymatic browning widely occurs in plants, fungi, seafood, animals as well in insects (Yoruk & Marshall 2003; Matheis & Whitaker 1984; Martinez & Whitaker 1995; Dittmer & Kanost 2010). This browning is mostly undesired and causes a loss of quality by changing

the colour, flavour, texture and nutritional value of the product (Yoruk & Marshall 2003). In some cases, however, enzymatic browning is desired in for example tea and cocoa (Yoruk & Marshall 2003). Polyphenol oxidase is the most general name for enzymes causing browning and includes multiple enzymes. Other names are used for polyphenol oxidase from different sources. Tyrosinase is commonly used for microorganisms, humans and animals referring to the substrate L-tyrosine (Sollai et al. 2008). In insects, this enzyme is called phenoloxidase and this notation was used throughout this thesis.

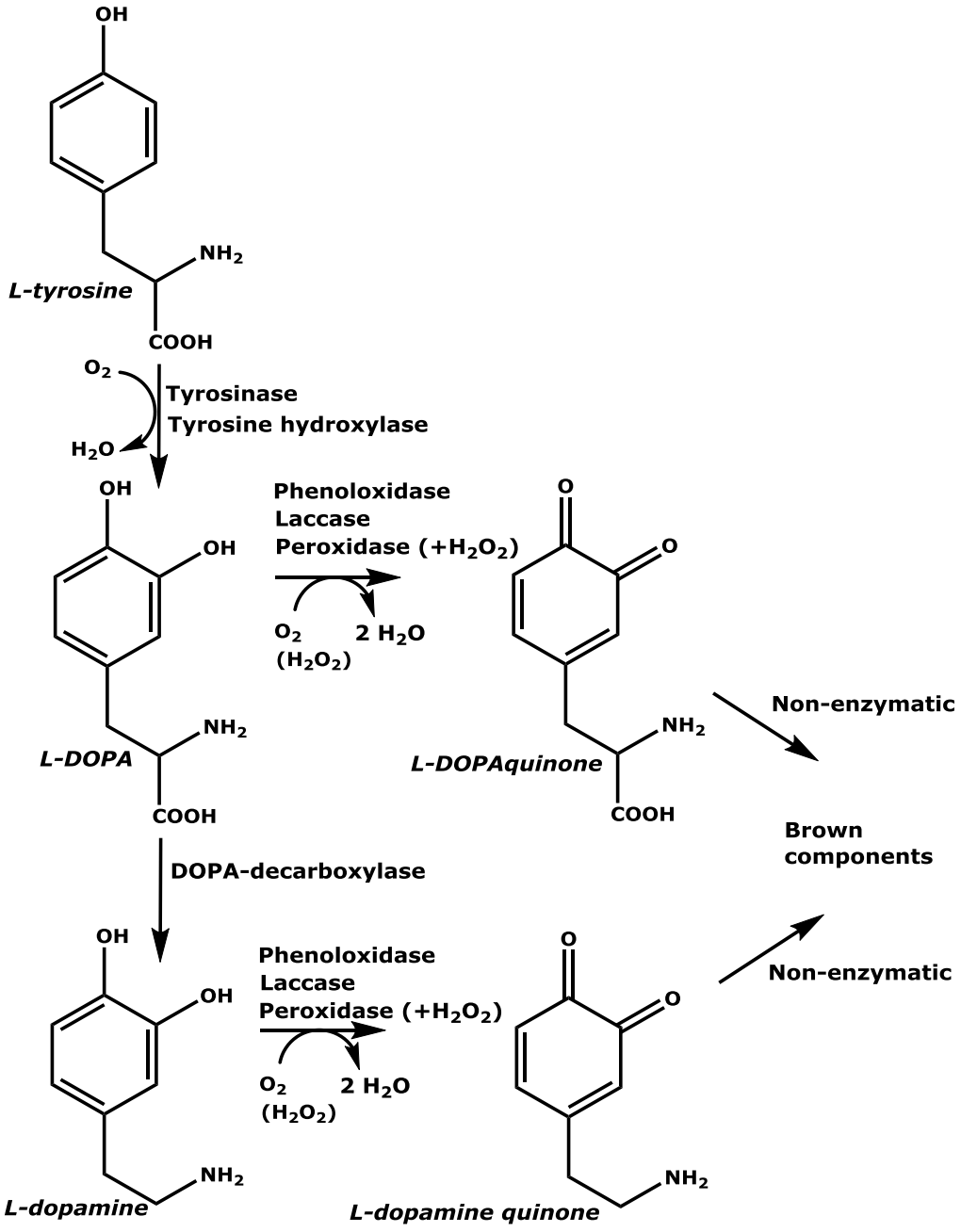

Figure 1.4. General reaction scheme of enzymes involved in enzymatic browning.

Phenoloxidase is a metallo-enzyme, which can catalyse two reactions: the *o*-hydroxylation of a monophenolic compound to a diphenol and the oxidation of an *o*-diphenol to an *o*-quinone. Oxygen is necessary for both reactions. *O*-quinones are highly reactive and react further non-enzymatically into melanins, or they react with proteins (Matheis & Whitaker 1984; Yoruk & Marshall 2003; Bittner 2006). The melanins are deposited on the surface of encapsulated parasites, wound sites and haemocyte nodules (Kanost & Gorman 2008). The melanins produced during synthesis appear to help killing invading parasites and pathogens. This mechanism is responsible for darkening of insect proteins when exposed to air, as O_2 is involved in the reaction (Kanost & Gorman 2008). DOPA decarboxylase converts L-DOPA into L-dopamine which is a better substrate for phenoloxidase. DOPA decarboxylase was active upon bacterial injection into *T. molitor* (Sugumaran 2002; Kim et al. 2000).

Besides the involvement of enzymatic browning in defence mechanism, the browning enzymes also play a role during sclerotization of the cuticular structure (Andersen 2010). Laccase is hypothesized to play a role in cuticle sclerotization by oxidizing catechols in the cuticle into quinone (Dittmer & Kanost 2010). Laccase is able to oxidise both *ortho*-diphenols and *para*-diphenols to their corresponding quinones and cannot hydroxylate monophenols. Oxidation occurs via an intermediate step in which a radical is formed, which is not the case for phenoloxidases (Dittmer & Kanost 2010). Tyrosine hydroxylase is also involved in sclerotization and brown pigmentation as shown for *Tribolium castaneum* (Gorman & Arakane 2010). Tyrosine hydroxylase can only catalyse the hydroxylation of a monophenol into a diphenol as shown in **Figure 1.4**, but not the subsequent oxidation into quinones (Gorman & Arakane 2010). Peroxidase can oxidize diphenols using hydrogen peroxide instead of oxygen and peroxidase activity was also linked to cuticular sclerotization (Matheis & Whitaker 1984; Andersen 2010).

1.6. Properties of insect proteins

Proteins are often used as ingredient to achieve certain characteristics in a food product. In order to replace conventional proteins, the novel proteins should have similar techno-functional properties as the conventional proteins. So far, there are no general functionality-related characteristics to predict whether novel proteins can replace animal-derived proteins (Boland et al. 2013). The following criteria are relevant for novel proteins to replace conventional proteins. These criteria include: (i) extraction and isolation in non-denatured state, (ii) absence of 'co-passengers' in the preparation like anti-nutritional compounds, flavour and colour, (iii) a high solubility, over a wide range of pH and ionic strength and (iv) a comparable amino acid profile with common protein sources (Boland et. al. 2013). The relevance of each criterion individually and feasibility to

replace animal proteins depends on the specific characteristics necessary for its future application.

To extract proteins in a non-denatured state, mild extraction methods are necessary. Denaturation of proteins often decreases their solubility. Solubility is an important aspect of proteins as it is necessary for many techno-functional properties, like foaming, emulsification and gelation and are most versatile in use (Boland et al. 2013; Kinsella & Melachouris 1976).

In order to use insects as novel protein source, it is necessary to understand the properties. Currently, limited information is available on the techno-functional properties of insect proteins (Yi et al. 2013; Bußler et al. 2016; Zhao et al. 2016). Heating is known to decrease the solubility of proteins from *T. molitor* (Broekman et al. 2015). Gelation has been shown for protein extract of multiple insects species at different conditions (Yi et al. 2013). Upon extraction of protein from insects, off-colour formation occurs, likely due to enzymatic browning. The quinones formed upon enzymatic browning can modify proteins by reacting with side chains of amino acids to form crosslinks (Friedman 1997; Bittner 2006), which might lead to altered functional properties and therewith altered suitability for food application (Matheis & Whitaker 1984; Bittner 2006). The proteins might also have undesirable colour that will decrease their use (Matheis & Whitaker 1984). Solubility can be decreased by enzymatic browning as was previously observed for other protein sources (Matheis & Whitaker 1984). The effect of enzymatic browning in insects and differences between species is currently unknown and will be investigated. Understanding chemical and physical properties of insect proteins is necessary to produce products with good sensory properties and can help choosing between species for a specific purpose.

Interchanging conventional proteins with novel proteins is only possible when functional properties are maintained as well as good nutritional characteristics, like good amino acid composition and digestibility. The digestibility of insects has been investigated before in among others fish (Sánchez-Muros et al. 2016), broiler chicken (De Marco et al. 2015), cat and dog feed (Bosch et al. 2014). *In vitro* protein breakdown in *T. molitor* and *A. diaperinus* was comparable to soybean meal and better than fish meal and poultry meat meal (Bosch et al. 2014). Studies reported that digestibility of soluble proteins was higher compared to that of insoluble proteins extracted from *T. molitor* and in presence of chitin (Sánchez-Muros et al. 2016; Bosch et al. 2014; Yi et al. 2016).

When proteins from ground insect larvae are used as ingredient, enzymatic browning can change functional properties of the proteins. Besides, enzymatic browning can also reduce the susceptibility to enzymatic hydrolysis during digestion. However, this effect of

enzymatic browning on hydrolysis, solubility and colour depends on the type of proteins and phenolics (Kroll et al. 2003). For insect proteins, no such studies have been performed so far. Thus, the effect of enzymatic browning on protein solubility and *in vitro* digestibility will be investigated in this thesis.

1.7. Inhibition of enzymatic browning

Multiple inhibition mechanisms of phenoloxidase-like enzymes, from various sources, have been described before. An overview of general browning inhibition strategies is shown in **Table 1.3**. Most research on inhibition of PO was performed on the mushroom tyrosinase from *Agaricus bisporus*, due to its commercial availability. Yet, research by Kuijpers et al. (2014) showed that tyrosinases from different sources, in this case from mushroom and potato, were differently inhibited or activated by similar plant extracts. So, extrapolation of inhibitors to insects should be done with care.

Table 1.3. Inhibition methods for phenoloxidase (PO) including advantages and disadvantages of the methods.

Inhibition	Mechanism	Advantage	Disadvantage
Sulphur containing compounds (e.g. HSO_3^-)	Irreversible inactivation of PO and addition to quinones	Cheap and irreversible inactivation	Chemical component, allergies possible
Heating	Denaturation PO	No addition chemicals	Denaturation other proteins
Low pH	PO inactive at low pH	Effective when low pH desired	Maintaining low pH necessary
Chelating agent (e.g. EDTA/DTT)	Chelating of copper from active site	-	Chemical component, reversible upon addition copper
Reducing agent (e.g. ascorbic acid)	Reduction of quinones to diphenols	Natural and cheap	High concentration necessary and only effective until depleted
Competitive inhibitor (e.g. plant extracts)	Competitive inhibition by binding active site of PO	Natural component	High concentration necessary, expensive
Removal of O_2	O_2 is a co-substrate for reaction	No addition of chemicals	Difficult to keep O_2 free environment

To investigate the effect of enzymatic browning on the proteins from insects, an efficient method is desired which inhibits the enzymes irreversibly in a food grade manner. The type of inhibition desired depends on the application of the insects. Based on the disadvantages in **Table 1.3**, reducing agents, chelating agents, competitive inhibitors and removal of oxygen were not considered during this thesis. Heating can effectively inhibit phenoloxidases by denaturation of the enzymes (Martinez & Whitaker 1995). Yet, other proteins in insects denature as well which might decrease the solubility of proteins. Another option is to lower the pH below 4, as phenoloxidases are not active, which leads to light extracts (Martinez & Whitaker 1995). However, low pH can also change the solubility of insect proteins (Bußler et al. 2016; Azagoh et al. 2016). Therefore, heating and sulphite have been used as inhibitors in this thesis.

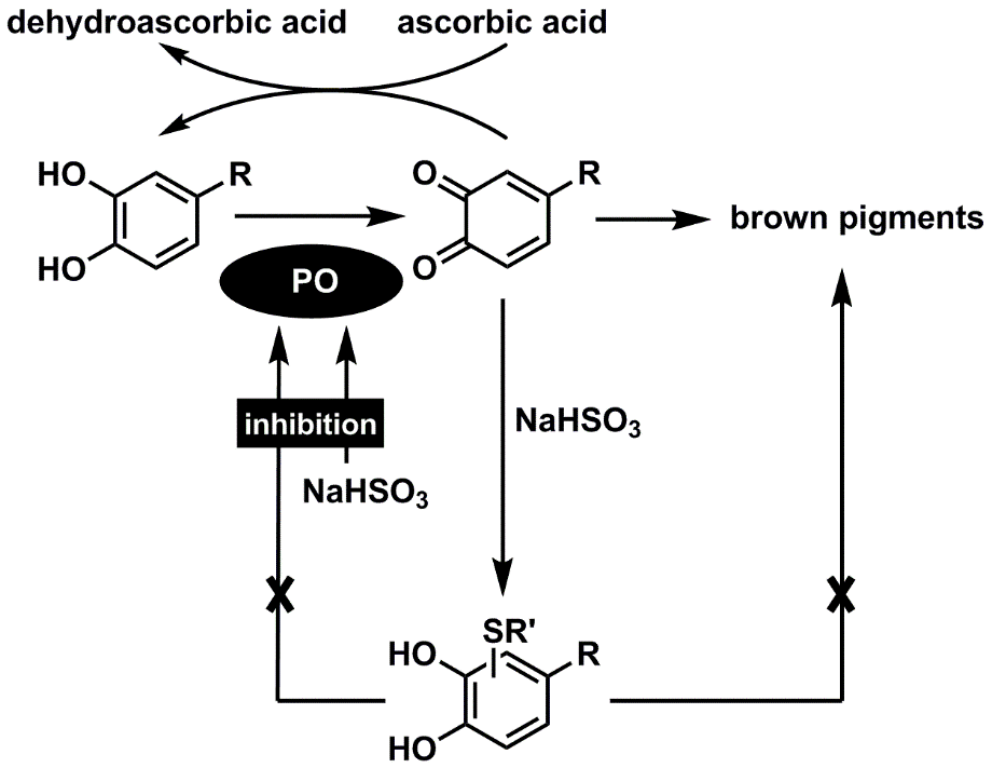

Figure 1.5. PO inhibition mechanism of sulphur-containing components on oxidation of diphenolic structures. Adjusted from Kuijpers et al. (2012).

Sulphur containing compounds are widely used as food additive. Sodium bisulphite has been used as inhibitor as it is an effective cheap way of inhibiting the enzyme irreversibly (Kuijpers et al. 2012; Kuijpers et al. 2013). The inhibition mechanism of sulphite has been elucidated before as shown in **Figure 1.5**. Enzymatic browning is prevented in a dual manner, by the formation of colourless addition products (sulpho-phenolics) with the oxidized phenolics, while at the same time tyrosinase activity was inhibited in a time-dependent way by binding of sulphite to a histidine residue in the active site of

tyrosinase (Kuijpers et al. 2012; Kuijpers et al. 2013). In recent years, there is a demand for alternatives for sulphites due to health concerns for allergic people (Lester 1995).

1.8. Aim and outline

Most research on insects as novel protein source has focused on the crude protein content determined by the general nitrogen-to-protein factor (Kp) of 6.25 (Bukkens 1997; Yi et al. 2013; Zhao et al. 2016; Rumpold & Schlüter 2013a; Finke 2013; Finke 2002). A specific factor for insect proteins for quantification of insect proteins was not determined before. Moreover, browning occurs upon grinding certain insect species, which can hamper its potential as a food or feed ingredient, by altering techno-functional and nutritional properties. Yet, the dark colour formation in insects during processing has not been investigated and little is understood about how it can be mitigated.

The insects investigated in this thesis are *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. The specific aims were to: (i) Determine the specific nitrogen-to-protein factor for insect proteins, as the general nitrogen-to-protein factor of 6.25 was expected to overestimate the protein content in insects. (ii) Understand the mechanisms causing off-colour formation upon grinding of insects were investigated. The brown or black off-colour formation differs between species. Involvement of enzymatic browning is expected yet specific mechanisms and differences between the species are unclear. (iii) Establish the effect of browning on protein solubility and digestibility of insect proteins. The hypothesis is that solubility and digestibility of proteins will be negatively affected by browning. An overview is shown in **Figure 1.6**.

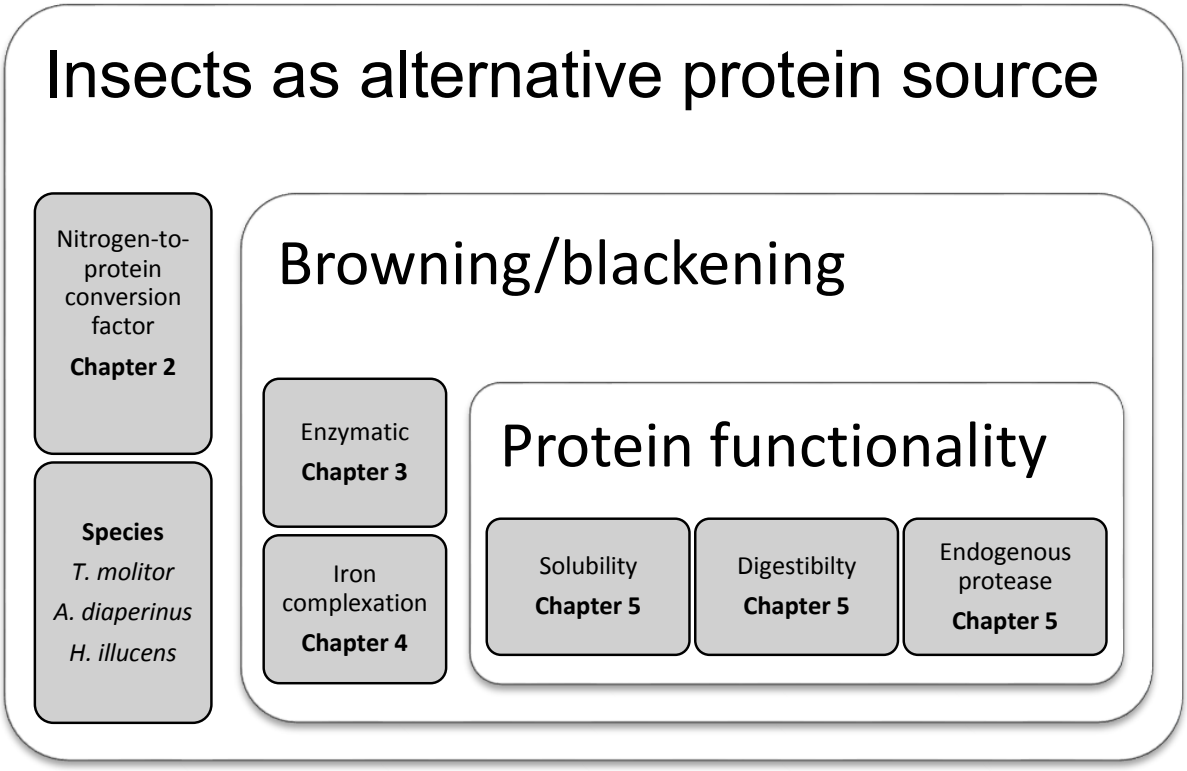

Figure 1.6. Schematic overview of this thesis.

In **Chapter 2,** the specific nitrogen-to-protein conversion factor Kp for the three insect species and their respective soluble protein extracts was determined to quantify the protein content more accurately from the nitrogen content than with the Kp of 6.25.

As browning was observed during grinding, **Chapter 3** focussed on characterization of the enzymes responsible for this browning. The oxidative activity was compared at different pH values for the three species. Enzymes were separated based on activity by anion exchange chromatography and subsequent using polyacrylamide gel electrophoresis under non-denaturing conditions (native PAGE). The native PAGE was stained with the substrate L-DOPA showed the different isoforms and the bands of *T. molitor* were sequenced to confirm the presence of the enzyme active upon grinding insect larvae.

The PO activity could not fully explain the off-colour formation in *H. illucens*. **Chapter 4** therefore investigated the effect of iron and phenolic compounds on dark colour formation. To investigate the complexation between phenolic compounds and iron, a model system was used to elucidate the mechanism.

Chapter 5 focussed on the effect of browning on the properties of soluble insect proteins. Three protein extracts were compared with respect to solubility and digestibility: (i) without

PO inhibition, (ii) PO inhibition using sulphite and (iii) PO inhibition using blanching. Furthermore, the activity of endogenous proteases was investigated in the protein extracts from insects.

The general discussion in **Chapter 6** focusses on the potential of insects as alternative protein source. The specific Kp factor obtained for the larvae of the three insect species in **Chapter 2** is compared to newly calculated Kp factors for other insects, for example *Acheta domesticus* (house cricket), and commercial insect flours and protein powder. Moreover, the effect of endogenous enzymes and pH on the solubility and gelation of proteins from insects is elaborated. Finally, the potential of insect larvae as protein source in future application is discussed in relation to processing methods and differences between species.

2

Nitrogen-to-protein conversion factors for three edible insects: *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*

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ABSTRACT

Insects are considered a nutritionally valuable source of alternative proteins and their efficient protein extraction is a prerequisite for large-scale use. The protein content is usually calculated from total nitrogen using the nitrogen-to-protein conversion factor (Kp) of 6.25. This factor overestimates the protein content, due to the presence of nonprotein nitrogen in insects. In this paper, a specific Kp of 4.76±0.09 was calculated for larvae from *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*, using amino acid analysis. After protein extraction and purification, a Kp factor of 5.60±0.39 was found for the larvae of three insect species studied. We propose to adopt these Kp values for determining protein content of insects to avoid overestimation of the protein content.

2.1. INTRODUCTION

There is increasing interest in alternative protein sources to feed the increasing world population (van Huis et al. 2013). Insects represent one of the potential sources to exploit. The high protein content, 40-75% on dry matter basis, makes insects a promising protein alternative for both food and feed (Bukkens 1997). Their nutritional composition and ease of rearing makes insects especially interesting for food and feed production when they are in the larval stage (Ghaly & Alkoaik 2009). To use insects as an alternative food protein source, efficient protein extraction is a prerequisite, as potential consumers do not like to recognize the insects as such.

The protein content of different insect species in the literature is mainly based on nitrogen content using the nitrogen-to-protein conversion factor (Kp) of 6.25 generally used for proteins (Bukkens 1997; Yi et al. 2013; Zhao et al. 2016; Rumpold & Schlüter 2013a; Finke 2002; Finke 2013). The presence of nonprotein nitrogen (NPN) in insects, for example chitin, nucleic acids, phospholipids, and excretion products (e.g., ammonia) in the intestinal tract, could lead to an overestimation of the protein content (Mariotti et al. 2008; Weihrauch et al. 2012). Finke (2007) estimated that the amount of nitrogen present from chitin would not significantly increase the total amount of nitrogen.

The aim of this research was to determine the specific nitrogen-to-protein conversion factor (Kp) for larvae of three insect species and their protein extracts using amino acid composition data. In this way an accurate protein content can be determined from the analysis of the nitrogen content. Larvae of *Tenebrio molitor* (yellow mealworm), *Alphitobius diaperinus* (lesser mealworm) and *Hermetia illucens* (black soldier fly) were used.

2.2. MATERIALS AND METHODS

2.2.1. Materials

T. molitor and *A. diaperinus* larvae were purchased from Kreca Ento-Feed BV (Ermelo, The Netherlands). *H. illucens* larvae were kindly provided by the Laboratory of Entomology (Wageningen University, The Netherlands). Larvae were frozen with liquid nitrogen and stored at -22 °C. The larvae from the three species were freeze-dried before chitin, nitrogen, and amino acid analysis.

2.2.1. Compositional analysis

The dry matter content and ash content were determined gravimetrically by drying and incinerating the samples at, respectively, 105 and 525 °C overnight in triplicate.

For carbohydrate analysis, larvae were frozen and ground in liquid nitrogen. The ground larvae were freeze-dried and subsequently hydrolysed and analysed for carbohydrates according to the method of Gilbert-López et al. 2015) with some modifications. An ICS-3000 Ion Chromatography HPLC system equipped with a Dionex™ CarboPac PA-1 column (2×250 mm) in combination with a Dionex™ CarboPac PA guard column (2×25 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode was used (ThermoFisher Scientific, Breda, The Netherlands). A flow rate of 0.25 mL min^{-1} was used and the column was equilibrated with H_2O. Elution was performed as follows: 0-35 min H_2O; 35-50 min, 0-40% 1 M sodium acetate in 100 mM NaOH; 50-55 min, 1 M sodium acetate in 100 mM NaOH; 55-60 min, 150 mM NaOH; 70-85 min, H_2O. Detection of the monosaccharides was possible after post column addition of 0.5 M sodium hydroxide (0.15 mL min^{-1}). Elution was performed at 20 °C, and to discriminate between glucose and glucosamine an additional run was performed at 28 °C using the same settings.

Fat content was determined gravimetrically after petroleum ether extraction using Soxhlet in duplicate (Tzompa-Sosa et al. 2014).

2.2.2. Protein extraction, amino acid composition and total nitrogen content

For protein extraction, frozen larvae were blended at 4 °C in 0.1 M citric acid - 0.2 M disodium phosphate buffer at pH 6 in a ratio of 1:4 (w/v) using a kitchen blender (Philips, Eindhoven, The Netherlands). The obtained solutions were centrifuged for 20 min. at 25800 *g* and 15 °C using a high-speed centrifuge (Beckman Coulter, Woerden, The Netherlands). The supernatant was filtered twice through cellulose filter paper (grade: 424, VWR, USA) and dialyzed at 4 °C at a cutoff of 12–14 kDa (Medicell Membranes Ltd., London, UK). Dialyzed protein extracts were considered as soluble protein extract and stored at -20 °C after freeze-drying. Extraction was performed in duplicate.

Amino acid composition was determined in duplicate by using the ISO 13903:2005 method, adjusted for microscale. The amide nitrogen from Asn/Gln was measured together with Asp/Glu. The amount of tryptophan was determined on the basis of AOAC 988.15. Total protein content was calculated from the total amino acid content.

Nitrogen content (Nt) was determined in triplicate according to the Dumas method using a Flash EA 1112 NC analyser (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's protocol. Average Kp values were calculated from the ratio of the sum of amino acid residue weights to Nt. Kp values were statistically evaluated by analysis of variance (ANOVA) with the SPSS 23 program. The percentage protein nitrogen from total

nitrogen was determined by total amino acid nitrogen (Naa)/Nt. The lower limit of this percentage was calculated on the basis of the theoretical value with 100% Asp/Glu and the upper level with 100% Asn/Gln (Schwenzfeier et al. 2011).

2.3. RESULTS AND DISCUSSION

2.3.1. Nutritional composition of whole insects

The amino acid profile from both whole larvae and their protein extract contains high amounts of all essential amino acids (**Table 2.1**). Overall, amino acid profiles were comparable as observed before for *T. molitor*, *A. diaperinus* (Yi et al. 2013), and *H. illucens* (Finke 2013; Bosch et al. 2014). From the amino acid profiles, the total nitrogen from amino acids and the accurate protein content were determined (**Table 2.2**).

General composition data are summarized in **Figure 2.1**. The protein values based on amino acid content for *T. molitor* and *A. diaperinus* were lower compared to those of Yi et al. (2013). *A. diaperinus* showed the highest protein content based on total amino acid content within the tested species. The total carbohydrate content within the three species ranged from 15 to 21%. The fat content for the three species ranged from 21 to 24% based on dry matter. In the literature, fat contents between 27 and 49% for *T. molitor* (Yi et al. 2013; Rumpold & Schlüter 2013a; Tzompa-Sosa et al. 2014), between 20 and 22% for *A. diaperinus* (Yi et al. 2013; Bosch et al. 2014) and between 13 and 36% for *H. illucens* (Bosch et al. 2014; Finke 2013) have been reported. Differences in chemical composition were probably caused by different diets (Rumpold & Schlüter 2013a; Oonincx & Dierenfeld 2012). Our results show that proteins, fats, and carbohydrates accounted for around 90% of the total dry matter; the remainder might come from other organic components, that is, phenols and nucleic acids.

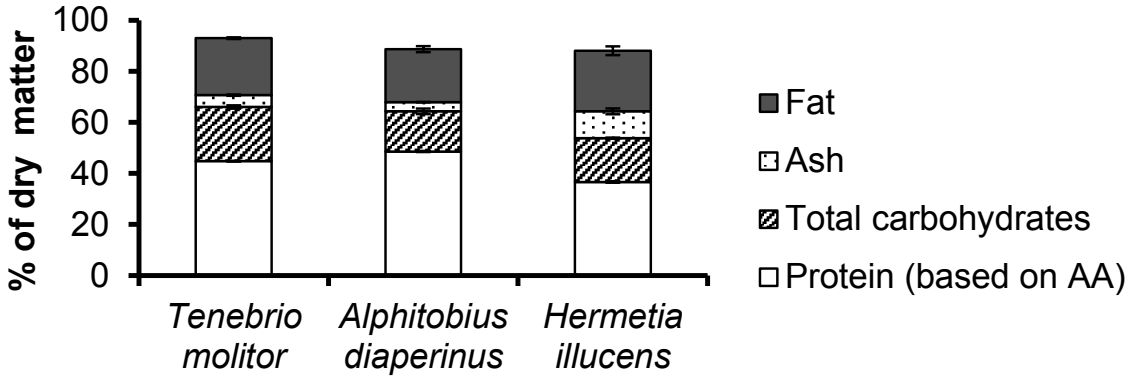


Figure 2.1. General composition (% dm) of whole insect larvae from *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. The protein content was based on amino acid (AA) composition.

Table 2.1. Amino acid composition (g/100 g protein) and total amino acid (AA) content (w/w % dw) for whole *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* and their protein extracts Asx: no separate analysis of Asp/Asn, Glx: no separate analysis of Glu/Gln (mean ±S.D., n=2).

	T. molitor	*A. diaperinus*	*H. illucens*	*T. molitor* extract	*A. diaperinus* extract	*H. illucens* extract
His	3.56 (±0.05)	3.97 (±0.01)	3.85 (±0.02)	2.61 (±0.00)	2.98 (±0.00)	3.64 (±0.01)
Ile	4.99 (±0.02)	4.61 (±0.00)	4.59 (±0.01)	5.54 (±0.01)	5.04 (±0.01)	5.18 (±0.00)
Leu	8.33 (±0.02)	7.32 (±0.01)	7.45 (±0.07)	9.36 (±0.02)	8.03 (±0.00)	7.99 (±0.00)
Lys	6.14 (±0.08)	7.05 (±0.01)	6.91 (±0.02)	6.12 (±0.05)	8.42 (±0.02)	9.16 (±0.00)
Met	1.52 (±0.04)	1.59 (±0.01)	2.00 (±0.01)	1.44 (±0.01)	1.68 (±0.02)	2.53 (±0.02)
Cys	1.13 (±0.01)	0.96 (±0.00)	0.97 (±0.02)	1.92 (±0.01)	1.29 (±0.00)	1.32 (±0.01)
Tyr	5.80 (±0.01)	8.49 (±0.01)	6.54 (±0.02)	5.01 (±0.03)	6.91 (±0.02)	6.28 (±0.00)
Phe	3.64 (±0.00)	5.17 (±0.05)	4.49 (±0.05)	5.1 (±0.00)	5.76 (±0.01)	7.18 (±0.01)
Val	6.42 (±0.04)	5.76 (±0.01)	6.1 (±0.06)	6.16 (±0.00)	5.50 (±0.03)	5.61 (±0.00)
Trp	1.50 (±0.01)	1.47 (±0.04)	1.87 (±0.01)	n.d.	n.d.	n.d.
Thr	4.52 (±0.03)	4.31 (±0.00)	4.34 (±0.01)	5.85 (±0.02)	5.09 (±0.00)	4.95 (±0.00)
Ser	5.03(±0.01)	4.41 (±0.00)	4.54 (±0.01)	5.06 (±0.02)	4.54 (±0.00)	3.99 (±0.01)
Asx	9.21 (±0.09)	9.38 (±0.05)	10.62 (±0.18)	14.29 (±0.13)	12.78 (±0.01)	12.56 (±0.06)
Glx	12.3 (±0.18)	13.01 (±0.04)	13.68 (±0.01)	13.53 (±0.14)	14.85 (±0.06)	12.13 (±0.03)
Gly	4.98 (±0.03)	4.2 (±0.00)	4.92 (±0.05)	4.25 (±0.00)	3.79 (±0.00)	3.88 (±0.01)
Ala	7.40 (±0.16)	6.58 (±0.03)	6.23 (±0.08)	4.89 (±0.01)	4.43 (±0.00)	4.66 (±0.02)
Pro	7.96 (±0.18)	6.36 (±0.08)	5.85 (±0.12)	4.80 (±0.06)	4.58 (±0.11)	4.38 (±0.01)
Arg	5.57 (±0.02)	5.35 (±0.00)	5.06 (±0.05)	4.07 (±0.03)	4.25 (±0.01)	4.57 (±0.01)
Sum AA	44.74 (±0.11)	49.58 (±0.52)	36.00 (±0.31)	67.91 (±1.31)	72.74 (±0.82)	67.77 (±0.60)

n.d. is not determined.

Table 2.2. Total nitrogen (Nt), protein nitrogen (Naa), chitin nitrogen (NGlcN), Naa/ Nt ratio and nitrogen-to-protein conversion factors (Kp) of different whole larvae and protein extracts from *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* (% dw). Protein extraction yield and content based on estimated Kp and Kp 6.25 (mean ±S.D, n=2).

	Nt	Naa Asp-Glu	Naa Asn-Gln	NGlcN	Naa/Nt (%)	Kp	Protein content Kp new	Protein content Kp 6.25	Protein yield(%) Kp new	Protein yield (%) Kp 6.25
T. molitor	9.41 (±0.03)	7.20 (±0.02)	8.31 (±0.04)	0.624 (±0.041)	77 < x < 88	4.75	44.8 (±0.1)	58.8 (±0.2)	-	-
A. diaperinus	10.21 (±0.05)	7.84 (±0.11)	9.13 (±0.12)	0.304 (±0.018)	77 < x < 89	4.86	48.6 (±0.2)	63.8 (±0.3)	-	-
H. illucens	7.70 (±0.06)	5.71 (±0.05)	6.72 (±0.07)	0.529 (±0.000)	74 < x < 87	4.67	36.7 (±0.3)	48.1 (±0.4)	-	-
***T. molitor* extract**	12.15 (±0.53)	10.31 (±0.26)	12.52 (±0.29)	n.d.[a]	85 < x < 103	5.59	68.1 (±3.0)	76.0 (±3.3)	23.5 (±0.4)	17.6 (±1.3)
***A. diaperinus* extract**	13.00 (±1.09)	11.09 (±0.15)	13.43 (±0.18)	n.d.	85 < x < 103	5.59	72.8 (±6.1)	81.3 (±7.0)	19.1 (±0.7)	16.9 (±2.1)
***H. illucens* extract**	12.06 (±0.13)	10.52 (±0.11)	12.48 (±0.13)	n.d.	87 < x < 103	5.62	67.6 (±0.7)	75.4 (±0.8)	17.1 (±1.9)	14.4 (±1.4)
Average Larvae					76 < x < 88	4.76 (±0.09)				
Average insect protein extract					86 < x < 103	5.60 (±0.02)				

[a] n.d. not determined

2.3.2. Nitrogen-to-protein conversion factors

To determine the protein content from total nitrogen content, the Kp and ratio Naa/Nt were calculated (**Table 2.2**). Interestingly, comparable Kp values were found among larvae of the three species with an average Kp value of 4.76±0.09, despite the fact that *H. illucens* belongs to a different order (Diptera) from *T. molitor* and *A. diaperinus* (which are Tenebriodinae family members within the Coleoptera order). This Kp value was significantly lower (P<0.001) than the general nitrogen factor of 6.25, which has been used up to now to calculate the protein content of insects (Yi et al. 2013; Zhao et al. 2016; Rumpold & Schlüter 2013a; Finke 2013; Bosch et al. 2014).

The Kp values found for insects are similar to those calculated for different tropical plants (Kp range 3.7-5.0)(Milton & Dintzis 1981), and microalgae (Kp range 2.53-5.77) (Gilbert-López et al. 2015; Schwenzfeier et al. 2011; Lourenço et al. 2004), as well as different grains and legumes (Kp range 5.09-5.38) (Mosse 1990). Higher values between 5.14 and 6.26 were found for meat, fish, and egg (Sosulski & Imafidon 1990).

This new Kp value gives a more accurate estimation of protein content by taking the presence of NPN into account. This leads to > 20% lower values for protein content compared to literature values, which are based on Kp of 6.25. Therefore, the protein content of *T. molitor* calculated in this study was 45%, which falls in the low range (45-65%) found in the literature based on Kp of 6.25 (Rumpold & Schlüter 2013a; van Broekhoven et al. 2015). The protein content falls out of the range for the larvae of *A. diaperinus*, for which a value of 49% was found compared to the literature values of 58-65% protein (Yi et al. 2013; Bosch et al. 2014; van Broekhoven et al. 2015). Also for *H. illucens* a lower value of 36% was found compared to the range of 37-56% from the literature (Bosch et al. 2014; Sánchez-Muros et al. 2014). When protein content is calculated from our data using a Kp of 6.25, the results do fall again into the ranges reported in the literature.

The average Kp value of 5.60 ± 0.24 obtained for soluble protein extracted from insects was significantly (P<0.001) higher compared to that for whole larvae, due to the removal of NPN. Again, comparable Kp values among the three species were found.

2.3.3. Nonprotein nitrogen

The calculated Naa/Nt ratio showed the presence of 11-26% NPN in whole larvae of all three insect species (**Table 2.2**). *T. molitor* contained 12-23% NPN, which is in line with Finke (2002). The NPN of 16-26% present in *H. illucens* is higher compared to the 2% found in literature, whereas the amino acid composition and content were similar (Finke 2013).

Besides the analytical procedures, differences in composition and recovery might be also caused by different diets fed to the insects (Oonincx & Dierenfeld 2012).

Carbohydrates, such as chitin and chitosan, have glucosamine or *N*-acetylglucosamine with nitrogen as building blocks. During the hydrolysis conditions used, *N*-acetylglucosamine was converted into *N*-glucosamine. The total amount of (*N*-acetyl) glucosamine within polymers for the three insect species was 4.4-9.1% (w/w), corresponding to approximately one-third of the carbohydrates present, similar to results based on acid detergent fibre fraction for *T. molitor* (Barker et al. 1998).

The chitin content comprised 3.0-6.8% nitrogen of the total nitrogen. Apart from chitin, NPN might originate from nucleic acids (Mariotti et al. 2008). Part of the NPN can also come from inorganic nitrogen. Examples of inorganic nitrogen are excretion products in the intestinal tract of the larvae, such as uric acid, urea and ammonia (Weihrauch et al. 2012). This is in agreement with the removal of most NPN during dialysis of the protein extracts.

2.3.4. Protein extraction yields

The average Kp values for the whole larvae and extracts were used to determine the protein content and extraction yield based on nitrogen (**Table 2.2**). Protein extraction yields between 17.1 and 23.5% were calculated using the insect-specific Kp factors, and these were higher compared to those obtained with the general Kp of 6.25 (14.4-17.6%). This is due to a larger overestimation of the protein content within the whole larvae when the factor of 6.25 was used caused by NPN.

When insect larvae are considered as an alternative protein source, overestimation of the protein content, due to the presence of NPN, should be avoided. To avoid overestimation of protein content in insects, we propose the use of a Kp value of 4.76 for the quantification of protein content in whole larvae and a Kp of 5.60 for the protein extracts derived from insects.

3

Involvement of phenoloxidase in browning during grinding of *Tenebrio molitor* larvae

This chapter has been published as Janssen RH, Lakemond CMM, Fogliano V, Renzone G, Scaloni A, Vincken J-P. Involvement of phenoloxidase in browning during grinding of *Tenebrio molitor* larvae. *PloS ONE*. 2017, 12 (12).

ABSTRACT

Insects are investigated as alternative protein source to meet the increasing demand for proteins in the future. Enzymatic browning occurring during grinding of insect and subsequent extraction of proteins can influence the proteins' properties, but it is unclear which enzymes are responsible for this phenomenon. This study was performed on larvae of three commonly used insect species, namely *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. Oxygen consumption measurements on protein extracts showed activity on L-tyrosine, L-3,4-di-hydroxy-phenylalanine (L-DOPA) and L-dopamine, indicating phenoloxidase as a key player in browning. Furthermore, no reaction on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was observed, ruling out an important contribution of laccase to browning. The browning reaction was most prominent at pH 6 for *T. molitor* and *A. diaperinus*, and 7 for *H. illucens*. As the enzyme activity of *H. illucens* was the lowest with the darkest colour formation, this was likely caused by another factor. The activity of phenoloxidase was confirmed for *T. molitor* and *A. diaperinus* by activity measurements after fractionation by anion-exchange chromatography. Colour measurements showed the presence of activity on both L-DOPA and L-tyrosine in the same fractions. Both substrates were converted into dopachrome after incubation with enzyme-enriched fractions. No DOPA-decarboxylase, tyrosine hydroxylase and peroxidase activities were observed. By using native PAGE with L-DOPA as staining-solution, active *T. molitor* protein bands were resolved and characterized, identifying a tyrosinase/ phenoloxidase as the active enzyme species. All together, these data confirmed that tyrosinase is an important enzyme in causing enzymatic browning in *T. molitor* and likely in *A. diaperinus.*

3.1. INTRODUCTION

The United Nations has predicted that the world population will reach 9.7 billion people by 2050 (Melorose et al. 2015). To feed this growing population, new and sustainable protein sources are needed. Insects might be a potential sustainable source of novel proteins, as they contain between 30% and 70% proteins on dry matter basis, and have high quality proteins in terms of amino acid composition (Bukkens 1997). Although insects are consumed in some parts of the world, Westerners dislike them as protein sources. This might change when insects are added as ingredient so that they are not recognizable as such (Schösler et al. 2012). To use insects as ingredient, often they need to be ground and substantial enzymatic browning occurs (Yi et al. 2013). During enzymatic browning phenolic compounds are oxidized, and can further react non-enzymatically into brown pigments or react with proteins and amino acids (Le Bourvellec & Renard 2012; Prigent et al. 2007; McEvily et al. 1992). Browning can influence insect applicability as food ingredient in four different ways.

(i) The visual appearance of browning is often linked to deterioration of foods and will decrease the economic value of food products as is often the case with fruits, vegetables or shrimps (Lee & Whitaker 1995). (ii) Browning can also deteriorate the flavour (Friedman 1997). (iii) Quinones can react with proteins and might decrease their digestibility and its corresponding nutritional quality (Friedman 1997; Rohn et al. 2006; Kroll et al. 2003). The presence of melanisation-engaging proteins, which can react with quinones were already shown in *T. molitor* before (Lee et al. 2000). (iv) Browning of proteins can affect their solubility and techno-functional properties, like foaming and emulsifications characteristics (Kroll et al. 2003). Also protein-chitosan crosslinking might occur and alter techno-functional properties (Yang et al. 2009).

As shown in **Figure 3.1**, five different enzymes can play a role in enzymatic browning in insects, namely phenoloxidase, laccase, tyrosine hydroxylase, DOPA decarboxylase and peroxidase (Andersen 2012; Kanost, Michael R; Gorman 2008). In insects, these enzymes are related to defence mechanisms and can quickly react upon biotic/abiotic stresses, such as bacterial challenges or animal wounding. Besides, these enzymes are known to participate in exoskeletal sclerotization (Andersen 2012; Sugumaran 2002).

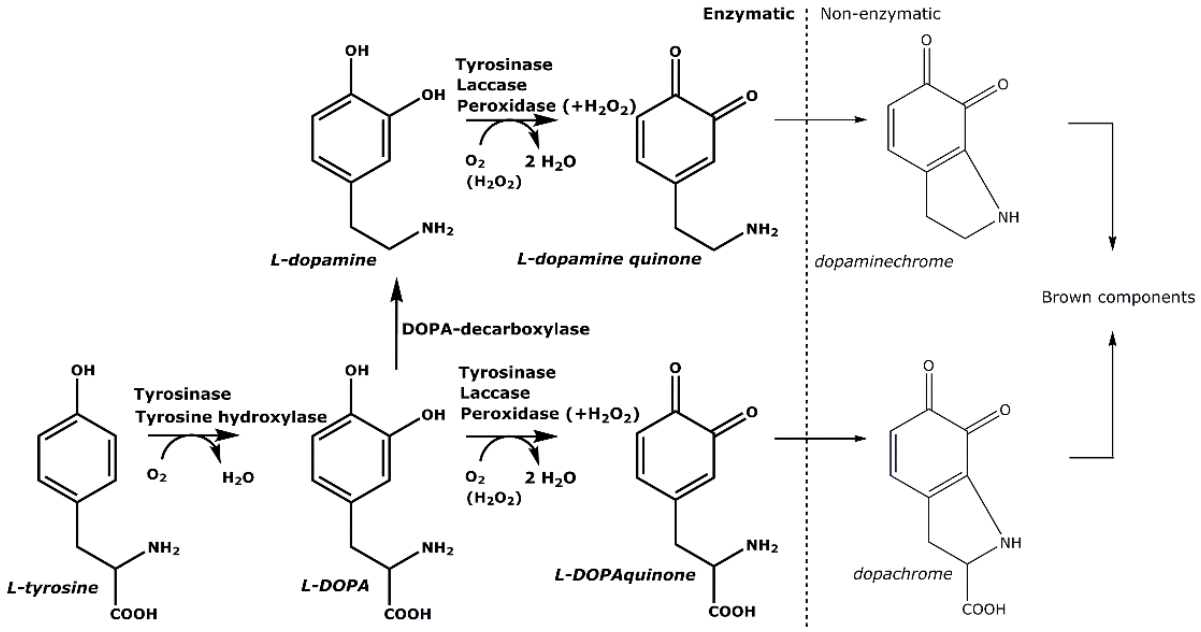


Figure 3.1. Tyrosine-related substrates, reactions and enzymes involved in enzymatic browning in insects, together with subsequent non-enzymatic reactions.

Phenoloxidase, in insects called phenol oxidase, hydroxylates monophenols (e.g., L-tyrosine) to generate corresponding *ortho*-diphenols, and subsequently oxidizes them into *ortho*-quinones (Andersen 2012). These quinones can further react non-enzymatically with proteins and/or form melanins. The hydroxylation involves a lag-period, which can be shortened by the presence of diphenols (Yoruk & Marshall 2003). Tyrosinase involvement was demonstrated in sclerotization in *Tribolium castaneum* (Arakane et al. 2005). Furthermore, conversion of prophenoloxidase into active phenoloxidase was associated with the activation of the immune response in *T. molitor* (Kan et al. 2008). On the other hand, laccase cannot hydroxylate monophenols, but only oxidize *para*- and *ortho*-diphenols into quinones. This enzyme (or family of enzymes) is linked to the cuticular structure and is responsible for corresponding sclerotization of insects (Andersen 2012). Tyrosine hydroxylase can only hydroxylate monophenols into diphenols, but does not oxidize diphenols further. It was shown that this enzyme is also involved in sclerotization and brown pigmentation of the insect cuticle in *T. castaneum* (Gorman & Arakane 2010). DOPA decarboxylase converts L-DOPA into L-dopamine, which is a better substrate for phenoloxidase; this enzyme process was activated as immune response in *T. molitor* upon bacterial injection (Kim et al. 2000; Sugumaran 2002). All previously mentioned enzymes use oxygen for substrate modification, whereas peroxidase oxidizes phenols only in the presence of hydrogen peroxide. Peroxidase was also demonstrated to be involved in cuticular sclerotization (Andersen 2012).

In order to assign the main enzymatic activity (or activities) responsible for enzymatic browning during grinding, an activity-guided approach was applied to the larvae of three commonly used insect species. This research focused on the relevant enzymes in the whole larvae that cause above-mentioned phenomena, impairing the use of insect species *T. molitor* (yellow mealworm), *A. diaperinus* (lesser mealworm) and *H. illucens* (black soldier fly) for food and feed applications. Furthermore, no deliberate prior activation from inactive prophenoloxidase into phenoloxidase was performed, in order to reflect the events occurring during grinding of insects for protein extraction as closely as possible.

3.2. MATERIALS AND METHODS

T. molitor and *A. diaperinus* larvae were purchased from Kreca Ento-feed BV (Ermelo, The Netherlands). *H. illucens* larvae were kindly provided by the Laboratory of Entomology, (Wageningen University, The Netherlands). Larvae were frozen using liquid nitrogen and stored at -20 °C. Water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). L-tyrosine, sodium phosphate dibasic dihydrate (Na_2HPO_4), hydrogen peroxide, tris(hydroxymethyl)aminomethane and glycerol were purchased from Merck (Darmstadt, Germany). Ultra-high-performance liquid chromatography/mass spectrometry (UHPLC/MS) grade formic acid, acetonitrile (ACN) and water were purchased from Biosolve BV (Valkenswaard, The Netherlands). Trypsin was of sequencing grade from Roche (Mannheim, Germany). All solvents for nano-chromatographic analyses were of LC-MS grade from Millipore. All other chemicals used were purchased from Sigma-Aldrich (St.-Louis, MO, USA).

3.2.1. Colour formation of insect protein extracts

Frozen larvae were ground in MilliQ water with a kitchen blender (Tomado TM-2419, Oosterhout, NL), using an insect material to solution ratio 1:4 w/v. After centrifugation at 22,000 *g* (5 min, at 4 °C), a picture was taken in a photo box.

3.2.2. Enzyme extraction at different pH values

Enzymes were extracted from insect larvae with 0.1 M citric acid / 0.2 M Na_2HPO_4 buffer with pH values in the range of pH 4-7. Frozen larvae were ground in buffer with a kitchen blender (Tomado TM-2419, Oosterhout, NL), using an insect material to solution ratio 1:4 w/v. The mixtures were centrifuged at 22,000 *g* (5 min, at 4 °C). This resulted in three layers: pellet, supernatant and fat layer. Supernatants were centrifuged again under similar conditions, and then used to assay enzymatic activities by oxygen consumption measurements.

3.2.3. Enzyme activity using oxygen consumption measurements

Oxygen consumption was measured with an Oxytherm System (Hansatech, Kings Lynn, UK). The enzyme extract (50 µL) was added to 1 mL of substrate in 0.1 M citric acid / 0.2 M Na_2HPO_4 buffer of pH 4-7, at 25 °C. The substrates used were 3 mM L-DOPA with or without 0.01% H_2O_2, 3 mM L-dopamine, 1 mM L-tyrosine and 3 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). The activity of specific enzymes on different substrates is shown in **Table 3.1**. Data acquisition and analysis were done using O_2 view 2.05 software (Hansatech, King Lynn, UK). The oxygen consumption rate was calculated using the slope of the linear part of the oxygen consumption versus time plot.

Table 3.1. Overview of reactions between different enzymes and substrates which occur (+).

	L-tyrosine	L-DOPA	ABTS	L-DOPA+H_2O_2	L-dopamine
Tyrosinase	+	+	-	+	+
Laccase	-	+	+	+	+
Tyrosine hydroxylase	+	-	-	-	-
DOPA decarboxylase	-	+	-	+	-
Peroxidase	-	-	-	+	-

3.2.4. Enzyme extraction from insects for further fractionation

For enzyme extraction, frozen insect larvae were blended with a kitchen blender (Tomado TM-2419, Oosterhout, NL) in 15 mM phosphate buffer, pH 6, using an insect material to solution ratio 1:4 w/v. These mixtures were centrifuged at 22,000 *g* (5 min, at 4 °C). Then, the supernatants were filtered through Whatman filter paper 595 ½ (Schleicher&Schnell, Dassel, Germany) and the filtrates were centrifuged at 22,000 *g* (10 min, at 4 °C). These materials constitute the protein extracts used for further enzyme fractionations. It is noted that the enzyme extract of *T. molitor* retained almost 80% of its activity for 2 weeks when stored at 4 °C. It was therefore not necessary to perform protein activation treatments.

3.2.5. Enzyme fractionation by anion exchange chromatography

Anion-exchange chromatography (AEC) was used to separate insect proteins. Insect extracts (0.5 mL) were manually injected onto a 1 mL Resource Q column (GE Healthcare, Sweden) on the ÄKTA micro (Amersham Biosciences, UK). The column was equilibrated with 15 mM phosphate buffer, pH 6 or 7. A linear gradient up to 1 M NaCl in the same buffer was applied over 20 column volumes, using a flow rate of 0.5 mL/min. Fractions of 1 mL were collected using a fraction collector (GE FRAC-950, Amersham Biosciences, UK). UV absorbance was measured at 214 nm.

3.2.6. Enzyme activity measurements using spectrophotometry

Enzyme fractions (50 µL) were incubated with 200 µL of different substrates, namely 3 mM L-DOPA, 1 mM L-tyrosine, 3 mM ABTS and 3 mM L-DOPA with 0.01% H_2O_2. A time-course absorbance measurement at 520 nm (or at 420 nm when ABTS was used as substrate) was obtained with a spectrophotometer (Tecan Infinite F500, Tecan, Switzerland).

3.2.7. Pooling anion-exchange chromatography fractions

Multiple fractions associated with the same enzyme activity were pooled. The fractions T1-3 were pooled in T_I, T4-8 in T_{II}, T9-12 in T_{III}, T13-17 in T_{IV} and T18-23 in T_V. These AEC pools were concentrated using centrifugal filter units (Amicon Ultra, 0.5 mL, 10 kDa molecular mass cut off, Millipore, Ireland) and used for further assays based on UHPLC-MS analysis and native PAGE.

3.2.8. Reversed phase-ultra high performance liquid chromatography-electrospray ionisation - mass spectrometry (RP-UHPLC-ESI-MS) analysis of enzymatic reactions

Concentrated pools (25 µL) were incubated with 200 µL substrates, *i.e.* 0.05 mg/mL L-DOPA or L-tyrosine. Standards of L-DOPA, L-dopamine and L-tyrosine were used for calibration purpose. Furthermore, a standard incubation using L-DOPA and purified mushroom phenoloxidase was used to determine the product dopachrome.

After incubation, samples were analysed with an Accela UHPLC system (Thermo Scientific, USA), which was equipped with a pump, an auto sampler and a photo-diode array detector (PDA). Each sample (5 µL) was injected onto a Hypersil Gold aQ column (2.1 x 150 mm, particle size 1.9 µm; Thermo Scientific, USA), and eluted with UHPLC-grade 0.1% v/v formic acid (eluent A) and UHPLC-grade acetonitrile, containing 0.1% v/v formic acid (eluent B). The flow rate was 400 µL/min; the temperature of the column oven and of the tray was set at 10 °C. The PDA detector was set to measure over the range of 200-600 nm. The following gradient was used: 0-5 min, isocratic on 100% v/v A; 5-19.57 min, linear gradient from 0% to 50% v/v B; 19.57-20.15 min, linear gradient from 50% to 100% v/v B; 20.15-23.06 min, isocratic on 100% v/v B; 23.06-23.64 min, linear gradient from 100% to 0% v/v B; 23.64-28.09, isocratic on 100% v/v A. Mass spectrometric data were obtained by analysing samples on a LTQ-XL (Thermo Scientific, USA) equipped with an electrospray probe coupled to the UHPLC system. The source voltage was 3.5 kV in the negative ion mode and 4 kV in the positive ion mode. The temperature of the ion transfer tube was 250 °C. The instrument was tuned using L-DOPA and L-tyrosine. Data were collected over the *m/z* range 150-300.

Tandem mass spectra were collected with a collision energy of 30%. Control of the instrument and analysis of the data were done using Xcalibur 2.2 (Thermo Scientific, USA). Quantitative data were obtained from comparison of enzymatic reactions with calibration curves of specific metabolite standards.

3.2.9. Native PAGE analysis

Native PAGE was performed under non-reducing conditions with Bio-Rad any kDa, Mini-protean TGX precast protein gel in a Mini-Protean II system (Bio-Rad laboratories), according to the manufacturer's protocol. Samples were 10 times concentrated by ultrafiltration using centrifugal filters (Amicon Ultra, 0.5 mL, 10 kDa molecular mass cut off, Millipore, Ireland) and mixed 1:1 ratio with sample buffer. For the fractions, 20 µL-samples were loaded onto the gel. For the total extract, a 10 µL-sample was loaded onto the gel. Resulting gels were stained with 3 mM L-DOPA in MilliQ for at least 2.5 h or with Instant Blue (Expedeon, UK).

3.2.10. Proteomic analysis of native PAGE bands

Visible *T. molitor* enzyme bands from native PAGE with L-DOPA staining were carefully cut, *in gel*-reduced with dithiothreitol, *S*-alkylated with iodoacetamide, and subsequently *in-gel* digested with trypsin (Shevchenko et al. 1996). Resulting peptide mixtures were desalted with µZip-TipC18 micro-columns (Millipore), and then subjected to nanoLC-ESI-LIT-MS/MS analysis. The latter was performed with a LTQ XL mass spectrometer (Thermo Scientific, USA) equipped with a Proxeon nano-spray source (Thermo Scientific, USA) connected to an UltiMate 3000 RSLC nano-liquid chromatographer (Dionex, Thermo Scientific, USA). Protein digests were resolved on a 15 cm length × 75 µm inner diameter column packed with Acclaim PepMap RSLC C18 resin (Thermo Scientific, USA). Mobile phases were 0.1% v/v formic acid in water (eluent A) and 0.1% v/v formic acid in acetonitrile/water 4/1 v/v (eluent B), running at a total flow rate of 300 nL/min. A linear gradient started 20 min after sample loading; eluent B ramped from 3% to 40% v/v over 40 min, and from 40% to 80% v/v over 5 min. Mass spectra were acquired in the range *m/z* 400-2,000. Peptides were fragmented by collision-induced dissociation and subjected to data-dependent product ion scanning, allowing dynamic exclusion (repeat count 1 and exclusion duration 60 s) over the three most abundant ions. Mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively.

Raw mass data were searched by means of MASCOT search engine (version 2.2, Matrix Science, UK) within the Proteome Discoverer software package (Thermo Scientific, USA) against the NCBI protein sequence database of all organisms belonging to the *Coleoptera* order, plus the UniProtKB entries from *T. molitor* and most common protein contaminants.

Database searches were performed by using carbamidomethylation of cysteine as fixed protein modification and oxidation of methionine as variable modification, a mass tolerance value of 1.8 Da for precursor ions and of 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, and a missed cleavage maximum value of 2. All other parameters were left as default. At least two sequenced peptides with an individual peptide expectation value less than 0.05, which corresponds to a confidence level for peptide attribution greater than 95%, determined the identification of protein candidates. In all cases, spectra visualization with manual verification of fragmentation attribution was performed to assign the protein candidates (Palazzotto et al. 2016).

3.3. RESULTS AND DISCUSSION

3.3.1. Colour formation of insect extracts

Colour formation upon grinding insects has been shown before for different insects species (Yi et al. 2013). In **Figure 3.2**, the colour of the three insects species used in this research is shown directly after grinding in MilliQ and centrifugation. *A. diaperinus* had the lightest colour, whereas *T. molitor* showed a darker brown colour and *H. illucens* was the darkest, with an almost black appearance.

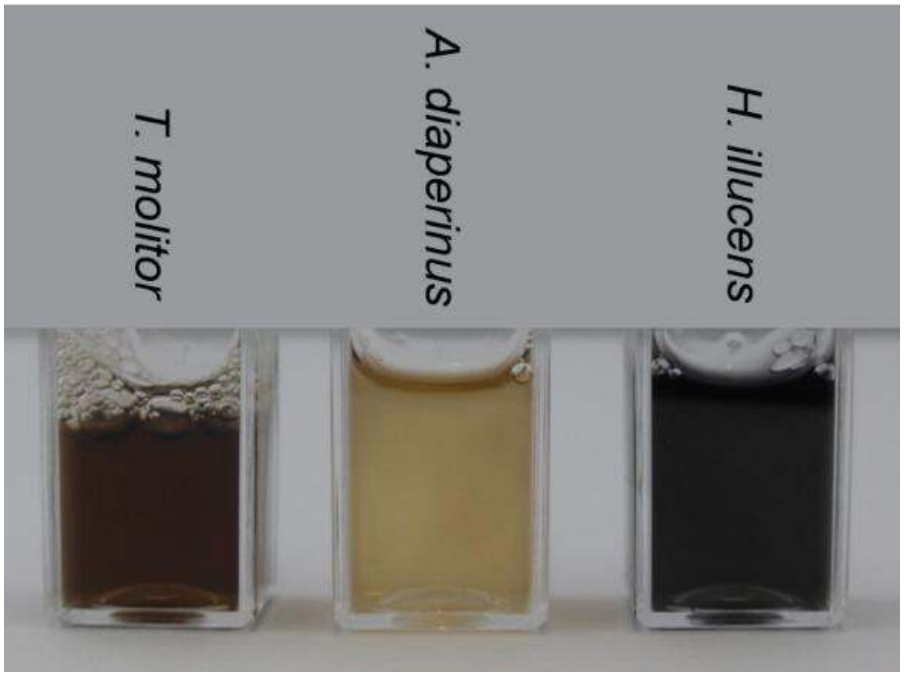


Figure 3.2. Colour formation directly after grinding in MilliQ water and centrifugation of *T. molitor*, *A. diaperinus* and *H. illucens*.

3.3.2. Optimal pH value for enzymatic reactions

Different phenolic substrates were used to get an indication of the type of enzymes contributing to browning in *T. molitor*, *A. diaperinus* or *H. illucens*, as shown in **Table 3.1**. In insects, most phenolic compounds are derived from L-tyrosine and are often modified by enzymes and/or coupled with polar substituents to increase solubility, such as phosphate, glucose or β-alanine (Kramer & Hopkins 1987). Here, the corresponding unconjugated phenolic structures were used for specific enzyme activity determinations. In particular, each enzyme activity was determined in the pH range of 4-7 for each of the substrates (**Figure 3.3**). Activities above pH 7 were not considered, as auto-oxidation of phenolic compounds might occur. Enzyme activities were assayed based on oxygen consumption during the reaction. In general, the highest enzyme activities were observed in the pH range 6-7.

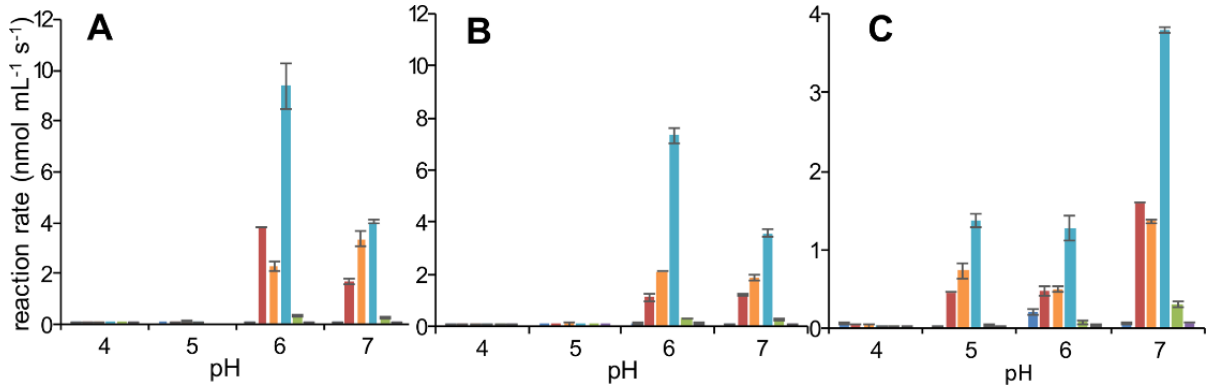


Figure 3.3. Effect of pH on specific oxidative enzyme activity (nmol $mL^{-1}s^{-1}$) of enzyme extracts from the larvae of *T. molitor* (A), *A. diaperinus* (B) and *H. illucens* (C). L-DOPA (red), L-DOPA+H_2O_2 (orange), L-dopamine (light blue), L-tyrosine (green) or ABTS (purple) were used as substrates and buffer (dark blue) as negative control (n=2, error bars represent absolute deviation).

T. molitor showed a higher oxidative enzyme activity towards L-dopamine of 9.4 nmol $mL^{-1}s^{-1}$ compared to 7.3 nmol $mL^{-1}s^{-1}$ for *A. diaperinus,* whereas *H. illucens* had lowest oxidative enzyme activity towards L-dopamine of 3.8 nmol $mL^{-1}s^{-1}$. Despite having the lowest enzyme activity, *H. illucens* showed the darkest colour as shown in **Figure 3.2**. This clearly suggests that the colour in this species is not only due to enzymatic browning, and that other factors should also be considered.

No enzymatic browning activities were observed at pH 4 for all three species. Only *H. illucens* showed activity at pH 5 and had the highest activity at pH 7, whereas *T. molitor* showed the highest activity at pH 6. For *A. diaperinus*, the highest activity was found at pH 6 for L-dopamine, whereas no significant difference was found for L-DOPA between pH 6 and 7. The highest enzyme activities were found around the physiological pH of the larvae, which were 6.5, 6.4 and 7.0 for *T. molitor*, *A. diaperinus* and *H. illucens*, respectively. As the

activation of browning enzymes might be related to an immune response in insects, a peak of activities around this pH seems to serve the animal physiology.

Regarding substrate specificity, the highest activity was observed on the diphenol L-dopamine, which was followed by L-DOPA, as shown before (Sugumaran 2002). Addition of H_2O_2 to L-DOPA substrate showed comparable activity as without at the various pH values; this indicated that H_2O_2 is not necessary for the enzyme reaction, which excluded peroxidase activity as a main contributor to enzymatic browning. Low activity was found on the monophenol L-tyrosine. The hydroxylation reaction is usually lower compared to oxidation of the *ortho*-diphenol, due to the occurrence of a lag phase (Yoruk & Marshall 2003). This is generally caused by the redox state of the copper in the enzyme's active site. Only the *oxy*-state is able to hydroxylate a monophenol into a diphenol, whereas a diphenol can be oxidized in both *meth*- and *oxy*-state (Olivares & Solano 2009). Activities on both mono- and diphenols were indicative for the presence of phenoloxidase. No activity was observed on ABTS, which indicated the lack of laccases in the insect extracts. The lack of activity assayed in the buffer without substrate showed that the oxygen consumption is correlated with the substrate added and was not due to the presence of endogenous phenolic compounds.

3.3.3. Fractionation of enzymatic activities

Based on oxygen consumption measurements, the activity on mono- and diphenols coincided, independent of pH (**Figure 3.3**). This observation indicated the presence of a phenoloxidase alone, or of multiple enzymes working in synergy to convert the above mentioned substrates. Therefore, enzyme extracts were prepared and fractionated at pH values corresponding to the highest activity with the aim to unveil possible enzymes not separated in measurements performed with crude protein extracts. Thus, enzyme extracts of *T. molitor* and *A. diaperinus* were prepared at pH 6, whereas *H. illucens* was prepared at pH 7. The fractionation profiles at 214 nm for *T. molitor* and *A. diaperinus* are shown in **Figure 3.4**. The enzymatic activities in *H. illucens* were very low and no pattern was observed (data not shown). Chromatographic fractions were collected and incubated with L-tyrosine and L-DOPA to check for coinciding activities. Each fraction with high activity towards L-DOPA also showed a (relatively) low activity towards monophenolic L-tyrosine. This indicated that the fractions contained one enzyme, phenoloxidase, with both diphenolase and monophenolase activity. Therefore, it seems unlikely that a separate L-tyrosine hydroxylase and diphenolase (or catecholase) is responsible for the enzymatic browning of *T. molitor* and *A. diaperinus*.

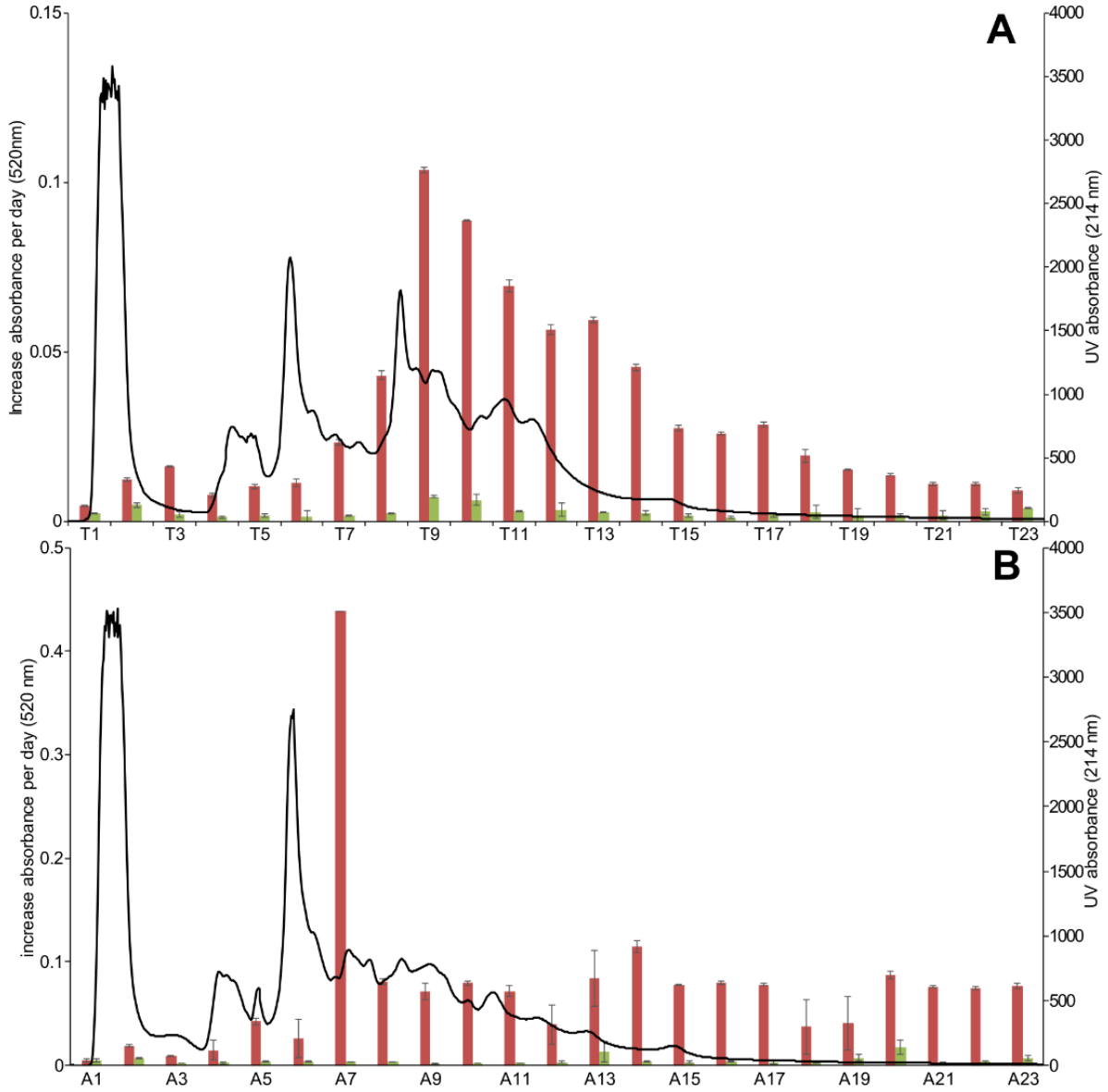


Figure 3.4. AEC fractionation pattern (black line) at 214 nm for *T. molitor* (A) and *A. diaperinus* (B). The panels also report the increase of absorbance at 520 nm per day for each fraction, when assayed with L-DOPA (red) and L-tyrosine (green) (n=2), error bars represent absolute deviation).

3.3.4. Determination of L-DOPA, L-dopamine and dopachrome formation

As reaction products L-DOPA and L-dopamine are colourless and cannot be revealed by spectrophotometry, the potential occurrence of tyrosine hydroxylase and DOPA-decarboxylase activities in *T. molitor* extracts was assayed qualitatively by UHPLC-MS analysis. The pooled fractions were incubated with L-tyrosine and L-DOPA. None of the incubations with L-DOPA resulted in the formation of L-dopamine, thus excluding the occurrence of DOPA decarboxylase activity therein. Tyrosine hydroxylase was also not found, as both L-DOPA and dopachrome were formed upon incubation of L-tyrosine pool T_{III}. Tyrosine hydroxylase was previously described in *T. castaneum* as pterin-dependent

species (Gorman & Arakane 2010). The absence of this enzyme activity in *T. molitor* might be due to the absence of tetrahydrobiopterin cofactor during enzyme extract preparation phases (Vié et al. 1999).

Furthermore, the presence of phenoloxidase activity was confirmed in pool T_{III} for *T. molitor*, as both L-DOPA and dopachrome was formed as a result of hydroxylation of L-tyrosine and further oxidation of L-DOPA, respectively. Phenoloxidase was not confirmed in the other fractions as only activity on L-DOPA and not on L-tyrosine was found for pool T_{IV} and only minor activity on L-DOPA was observed with pool T_V.

3.3.5. Presence of L-DOPA-active enzyme bands using native PAGE

To confirm the presence of phenoloxidase activity, extracts from *T. molitor*, *A. diaperinus* and *H. illucens*, and corresponding pooled fractions active on L-tyrosine and L-DOPA (**Table 3.2**), were also subjected to native PAGE, and the bands were tested for positive staining with L-DOPA. *T. molitor* samples showed the occurrence of 4 evident active bands (**Figure 3.5**), whereas *A. diaperinus* counterparts showed 2 active bands. In *H. illucens* samples, no significant enzymatic activity was observed. Parallel native PAGE experiments followed by staining with L-tyrosine and ABTS did not show positive bands (data not shown). Pooled AEC fractions showed separation of the active bands. Thus, AEC fractionation separated a number of browning-related enzyme activities. Besides, it removed some protein contaminants, which is clearly shown upon staining of the native page gel with coommassie (**Figure 3.5**).

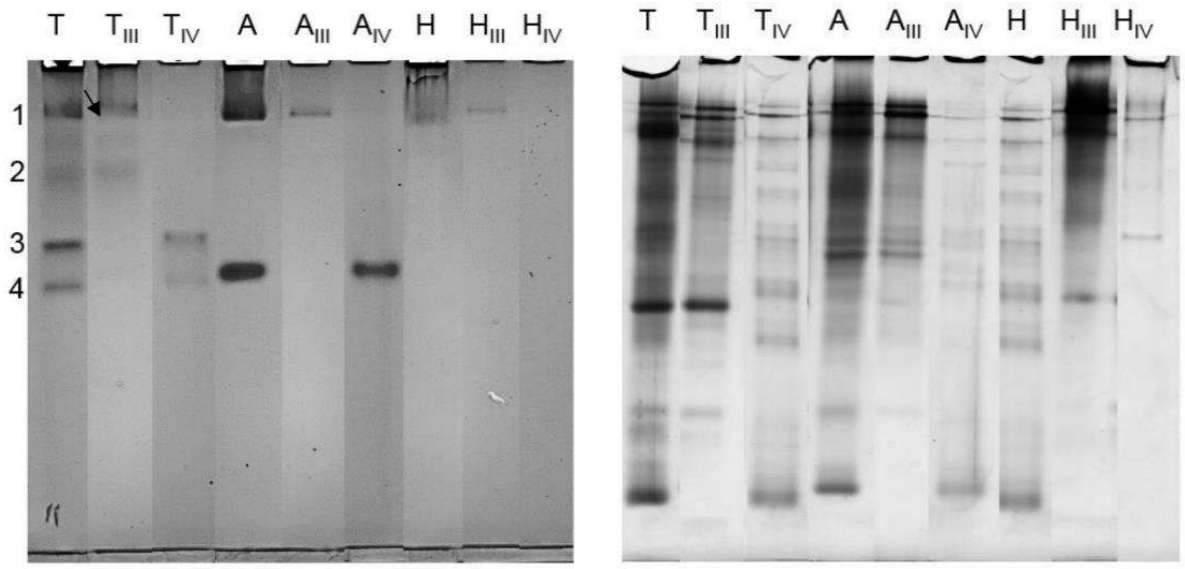


Figure 3.5. Native PAGE stained with 3 mM L-DOPA (left) showed active bands for extract of *Tenebrio molitor* (T), *Alphitobius diaperinus* (A) and *Hermetia illucens* (H). Two pooled active fractions from the same insects were subjected to PAGE analysis (T_{III}, T_{IV}, A_{III}, A_{IV}, H_{III} and H_{IV}). Numbering on the left highlights the bands excised and further subjected to proteomic analysis. A similar gel was stained with Coomassie (right).

Extra identification was done by inhibition of phenoloxidases (Lu et al. 2014). Besides extracts with corresponding active phenoloxidase, an extract was prepared in presence of a well-known phenoloxidase, namely sodium bisulphite. Sulphite is known to irreversibly inhibit the enzyme by binding in the active site of phenoloxidase (Kuijpers et al. 2013). Native PAGE of these extracts did not show any active bands as shown in supporting information **S3.1 Figure**, contrary to the extract without sodium bisulphite, confirming inhibition of insect phenoloxidase by sodium bisulphite.

Table 3.2. Reaction products formed after incubation of pooled fractions TI-V from *T. molitor* with substrates L-tyrosine and L-DOPA; + formed, × not found, = substrate constant, − substrates decreases, n/a not applicable.

Sample *T. molitor*	L-tyrosine	L-DOPA	Dopachrome	L-dopamine
T_I + L-DOPA	n/a	=	×	×
T_I + L-tyrosine	=	×	×	×
T_{II} + L-DOPA	n/a	=	×	×
T_{II} + L-tyrosine	=	×	×	×
T_{III} + L-DOPA	n/a	−	+	×
T_{III} + L-tyrosine	−	+	+	×
T_{IV} + L-DOPA	n/a	−	+	×
T_{IV} + L-tyrosine	=	×	+	×
T_V + L-DOPA	n/a	=	×	×
T_V + L-tyrosine	=	−	+	×

Due to the occurrence of reduced levels of protein contaminants as shown with Coomassie staining (**Figure 3.5**) in *T. molitor*, active PAGE bands obtained from the lanes with the pooled fractions were excised from the gel and further subjected to proteomic analysis for enzyme identification. It should be mentioned that poor and incomplete genetic data are available for the species under investigation, including enzymes involved in tyrosine metabolism. Nevertheless, proteomic analysis confirmed the occurrence in band 1 indicated with an arrow (**Figure 3.5**) of *T. molitor* phenoloxidase (also called tyrosinase) formed from pro-phenoloxidase (**Table 3.3** and **S3.1 Table**), as the inactive form was only present in the database. The phenoloxidase form was likely activated by cleaving off a 3 kDa peptide from the enzyme by a serine protease, as the native page showed a coloured band (Lee et al. 2002). This enzyme contains six histidine residues responsible for the copper binding in its active site. This active site is similar to another well studied phenoloxidase from *Bombyx mori* (silk moth), even though this species is from a different order. These phenoloxidase share a sequence identity of 58%. Band 1 also contained two other proteins related to browning processes, namely *T. molitor* early-staged encapsulation inducing protein and melanisation-related protein (**Table 3** and **S1 Table**). The first component was already described as a phenoloxidase activator that is involved in defence responses (Cho et al. 1999). The second one is a vitellogenin-like protein that was demonstrated to enhance melanin biosynthesis (Lee et al. 2000); it seems to react with oxidized phenolics generated as result of phenoloxidase action, but the mechanistic details underlying this process are still unclear. It is worth mentioning that three browning-related enzymes mentioned above occurred within the same native PAGE band; further studies are necessary

to determine if this finding was a coincidence or was due to the occurrence of a stable protein complex of these proteins in band 1.

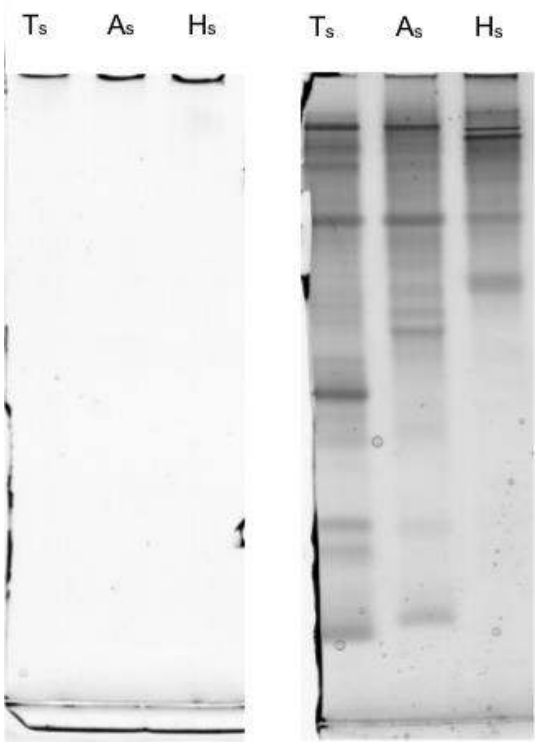
Proteomic analysis also ascertained the occurrence of *T. molitor* DOPA decarboxylase in band 2 (**Figure 3.5**) (**Table 3.3** and **S3.1 Table**). This enzyme was previously reported in *T. molitor* (Kim et al. 2000); however, its enzyme activity was detected only after bacterial infection in the larvae and when the cofactor pyridoxal phosphate was added to the enzyme incubation (Kim et al. 2000), contrarily to what was done in this study. As this cofactor is necessary for activation, DOPA decarboxylase is not responsible for the browning during protein extraction and it cannot be responsible for band staining. Moreover, it was shown by UHPLC-MS that dopachrome and no L-dopamine was formed during incubation with L-DOPA. In addition to this, the bands on native page were also formed with L-dopamine as substrates, confirming the oxidative activity towards diphenolic substrates. No proteins related to enzymatic browning were identified in bands 3 and 4 (**S3.1 Table**). This might be due to the above-mentioned lack of complete information on *T. molitor* genome.

Table 3.3. Browning related proteins identified in *T. molitor* bands from native PAGE (Figure 3.4).

	Band 1			**Band 2**
NCBI or UniProtKB accession code	Q9Y1W5	Q9NDN7	L7US91	Q9NL84
***T. molitor* protein**	86 kDa early-staged encapsulation inducing protein	Melanisation-related protein	Pro-phenoloxidase	Dopa decarboxylase
Amino acids	754	1439	684	475
Theoretical mass [kDa]	90.6	167.7	79.1	53.5
Theoretical pI	7.09	6.86	8.25	6.15
Sequence coverage (%)	14.99	3.68	5.99	12.63
Unique Peptides	9	4	3	4
Mascot score	223.41	114.59	124.81	98.65

This research aimed at investigating the relevant enzymes that cause browning during the grinding of insects using different specific substrates. Identification of the responsible enzyme is a prerequisite to develop targeted strategies to inhibit undesired browning. All together our results indicated that phenoloxidase is an important enzyme in causing browning during grinding of *Tenebrio molitor* and most likely *Alphitobius diaperinus*.

3.4. SUPPORTING INFORMATION



S3.1. Figure Native PAGE stained with 3 mM L-DOPA (left) showed no active bands for extracts treated with sodium bisulphite from *Tenebrio molitor* (T_s), *Alphitobius diaperinus* (A_s) and *Hermetia illucens* (H_s). A similar gel was stained with Coomassie (right).

S3.1. Table. Results of proteomic analyses performed on native PAGE bands from *T. molitor* showing positive staining with L-DOPA. The corresponding NCBI or UniProtKB accession code, protein name, number of amino acids, theoretical molecular mass and pI value, sequence coverage (%), detected unique peptides and Mascot score are reported.

Accession	Description	# AAs	Theor. Mass (kDa)	Theor. pI	Coverage (%)	# Unique Peptides	# PSMs	Score
Band 1								
642931317	Myosin heavy chain, muscle isoform X28 [*Tribolium castaneum*]	1948	222.3	6.09	10.93	16	33	377.36
Q9Y1W5	86 kDa early-staged encapsulation inducing protein OS=*Tenebrio molitor* GN=enp-6	754	90.6	7.09	14.99	9	22	223.41
91082465	60 kDa heat shock protein, mitochondrial [Tribolium castaneum]	574	61.1	5.55	19.86	7	16	308.35
Q95PI7	Hexamerin 2 OS=*Tenebrio molitor*	702	84.5	6.68	7.41	4	9	119.22
642919317	Alpha-actinin, sarcomeric isoform X1 [Tribolium castaneum]	897	103.7	5.87	7.47	5	9	146.56
642926018	ATP-citrate synthase isoform X2 [*Tribolium castaneum*]	1089	119.5	7.28	5.97	4	8	92.19
550249776	Heat shock protein, mitochondrial [*Anoplophora glabripennis*]	366	39.4	7.20	15.30	3	8	165.88
Q9NDN7	Melanization-related protein OS=*Tenebrio molitor* GN=160 kDa MRP [Q9NDN7_TENMO]	1439	167.7	6.86	3.68	4	6	114.59
237681135	Catalase-like [*Tribolium castaneum*]	501	56.1	8.19	8.98	3	4	84.59
A0A0B5IPQ8	C1 family cathepsin B33 OS=*Tenebrio molitor*	335	36.4	5.01	8.66	2	4	100.56
642926112	Apolipophorins [*Tribolium castaneum*]	3334	370.2	8.31	0.69	2	3	73.83
5902775	Alpha-amylase [*Tenebrio molitor*]	471	51.2	4.74	4.25	2	3	64.23
91088023	Glyceraldehyde-3-phosphate dehydrogenase 2 [*Tribolium castaneum*]	334	35.4	8.25	9.58	2	3	76.89
189237685	Proteasome subunit alpha type-7-1 [*Tribolium castaneum*]	249	28.1	8.18	11.65	2	3	96.15
550249068	Glutamate dehydrogenase, mitochondrial [*Anoplophora glabripennis*]	548	60.9	8.53	5.47	2	3	86.35
L7US91	Prophenoloxidase OS=*Tenebrio molitor* GN=PPO	684	79.1	8.25	5.99	3	3	124.81
546686306	Hypothetical protein D910_00056 [*Dendroctonus ponderosae*]	960	103.7	5.30	3.13	3	3	122.47
546680166	Hypothetical protein D910_07850 [*Dendroctonus ponderosae*]	365	40.6	7.99	8.22	2	2	83.61
546679426	Hypothetical protein D910_07249 [*Dendroctonus ponderosae*]	259	28.8	5.82	8.88	2	2	56.84
Q8I6J9	Masquerade-like serine proteinase homologue OS=*Tenebrio molitor*	444	48.8	6.30	5.86	2	2	50.78
Band 2								
550249393	Heat shock 70 protein cognate 3 [*Anoplophora glabripennis*]	657	72.8	5.19	15.98	8	17	158.61
5902775	Alpha-amylase [*Tenebrio molitor*]	471	51.2	4.74	15.07	5	10	169.18

Q9NL84	Dopa decarboxylase OS=*Tenebrio molitor* GN=dopa decarboxylase	475	53.5	6.15	12.63	4	7	98.65
642937729	Tropomyosin-2 isoform X15 [*Tribolium castaneum*]	283	32.7	4.73	12.72	3	6	93.09
Q27013	28 kDa desiccation stress protein OS=*Tenebrio molitor* [Q27013_TENMO]	225	24.8	5.53	20.89	5	6	104.98
91080775	Rab GDP dissociation inhibitor alpha [*Tribolium castaneum*]	443	49.9	5.60	5.19	2	5	77.65
724090709	Tropomyosin-1, partial [*Monochamus alternatus*]	256	29.1	4.93	11.33	2	5	85.48
Q8MPF2	Triosephosphate isomerase OS=*Tenebrio molitor* GN=tpi	247	26.7	6.35	10.12	2	5	57.01
625295138	Heat shock cognate protein 70, partial [*Propylea japonica*]	209	22.7	5.33	19.14	3	4	69.34
478257952	Hypothetical protein YQE_05244, partial [*Dendroctonus ponderosae*]	432	48.6	4.78	7.41	2	4	145.15
642923291	Uncharacterized protein C05D11.1-like [*Tribolium castaneum*]	1022	115.9	5.50	2.15	2	4	68.37
219873007	Heat shock protein 90 [*Harmonia axyridis*]	717	82.2	**5.02**	5.72	3	4	78.38
270002786	Alpha spectrin [*Tribolium castaneum*]	2415	278.5	5.20	1.49	3	4	79.00
730042794	Histone 4, partial [*Sternopriscus wallumphilia*]	48	5.6	9.60	62.50	3	4	79.51
381414109	Actin, partial [*Merizodus soledadinus*]	339	37.6	5.36	12.09	3	3	89.58
157102538	Histone H3, partial [*Deronectes aubei aubei*]	102	11.4	10.43	13.73	3	3	59.72
91088023	Glyceraldehyde-3-phosphate dehydrogenase 2 [*Tribolium castaneum*]	334	35.4	8.25	9.58	2	3	93.58
C5H0E3	Prolyl carboxypeptidase OS=*Tenebrio molitor*	488	55.1	5.05	5.53	2	2	61.83
Band 3								
5902775	Alpha-amylase [*Tenebrio molitor*]	471	51.2	4.74	4.67	2	4	58.62
91089297	Membrane-bound alkaline phosphatase [*Tribolium castaneum*]	503	55.1	5.58	5.37	2	4	82.96
Q27013	28 kDa desiccation stress protein OS=*Tenebrio molitor*	225	24.8	5.53	15.11	3	3	74.60
Band 4								
5902775	Alpha-amylase [*Tenebrio molitor*]	471	51.2	4.74	8.92	4	7	73.99
Q7YZB9	Chitinase OS=*Tenebrio molitor*	367	39.5	4.55	7.90	2	3	83.50
Q9GSE6	Beta-glucosidase (Fragment) OS=*Tenebrio molitor*	502	57.7	4.59	4.58	2	3	71.25

4

Iron-polyphenol complexes cause blackening upon grinding *Hermetia illucens* (black soldier fly) larvae

This chapter has been submitted as Janssen RH, Canelli G, Sanders MG, Bakx E, Lakemond CMM, Fogliano V, Vincken J-P. Iron-polyphenol complexes cause blackening upon grinding *Hermetia illucens* (black soldier fly) larvae.

ABSTRACT

Insects are a promising alternative protein source. One of the bottlenecks in applying insects in food is the fast darkening initiated during grinding. Besides enzymatic browning, non-enzymatic factors can cause off-colour formation, which differs between species. This study investigates the impact of iron, phenoloxidase, and polyphenols on off-colour formation in insect larvae. *Hermetia illucens* showed a blackish colour, whereas *Tenebrio molitor* turned brown and *Alphitobius diaperinus* remained the lightest. This off-colour formation appeared correlated with the iron content in the larvae, which was 61±9.71, 54±1.72 and 221±6.07 mg/kg dm for *T. molitor*, *A. diaperinus* and *H. illucens*, respectively. In model systems, the formation of iron-L-3,4-dihydroxyphenylalanine (L-DOPA) bis- and tris-complexes were evidenced by direct injection into ESI-TOF-MS, based on their charges combined with iron isotope patterns. The reversibility of the binding of iron to phenolics, and thereby loss of blackening, was confirmed by EDTA addition. Besides complex formation, oxidation of L-DOPA by redox reactions with iron occurred mainly at low pH, whereas auto oxidation of L-DOPA mainly occurred at pH 10. Tyrosinase (i.e. phenoloxidase) activity did not change complex formation. The similarity in off-colour formation between the model system and insects indicated an important role for iron-phenolic complexation in blackening.

4.1. INTRODUCTION

Insects are nowadays investigated as alternative protein source to meet the growing protein demand in the future (Alexandratos & Bruinsma 2012). Insects can be a protein source for feed because of their well-balanced amino acid profile and the sustainability of their rearing (Rumpold & Schlüter 2013a; van Huis et al. 2013). Larvae from *T. molitor* (yellow mealworm) and *A. diaperinus* (lesser mealworm) are under investigation, as they are reared for food and feed in The Netherlands; while *H. illucens* larvae (black soldier fly) are mainly used for feed. To use insects as ingredient in food and feed, grinding is often necessary. Fast off-colour formation occurs during this treatment, which is partly caused by enzymatic browning catalysed by phenoloxidase, also called tyrosinase (Janssen et al. 2017b). Nevertheless, the oxidative enzyme activity of the different species cannot explain the differences in off-colour formation of *Hermetia illucens* compared to that of *Tenebrio molitor* and *Alphitobius diaperinus*. *H. illucens* showed a marked black colour after grinding, whereas *T. molitor* was deep brown, and *A. diaperinus* yellowish. The highest enzyme activity was found for *T. molitor*, then *A. diaperinus* and the lowest activity was found for *H. illucens*, based on oxygen consumption measurements with L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate (Janssen et al. 2017b).

Multiple insect species have been shown to contain a high iron content (Bukkens 1997; Rumpold & Schlüter 2013a). Minerals like iron are important as micronutrient source in food and feed. Iron deficiency is the most common nutritional disorder in the world, according to The World Health Organization (FAO & WHO 2001). Nevertheless, iron ions are reactive and are known to induce off-colour formation in fortified foods, often caused by their interaction with polyphenols (Mellican et al. 2003; Habeych et al. 2016).

In insects, iron is both an essential nutrient and a strong toxin. Insects contain transferrin proteins to transport iron in serum and ferritin proteins to store it. Other proteins that contain iron are haemoglobin and myoglobin, which both transport and store oxygen (Nichol et al. 2002). Transferrin binds only one ferric iron (Fe^{3+}). Ferritin can bind multiple ferrous iron (Fe^{2+}) which is then converted into the ferric form and stored as such in the oxoferrihydrite core (Nichol et al. 2002).

Polyphenols in insects are substrates for oxidative enzymes contributing to the immune response, wound healing and sclerotization of the cuticle (Andersen 2012; Sugumaran 2002). Polyphenols in insects usually reside in the haemolymph in the form of L-tyrosine (Clark & Strand 2013), besides small amounts of L-tyrosine derivatives like L-dopamine and L-DOPA, often in phosphorylated or glycosylated form (Andersen 2012). These polyphenols are prone to oxidation by endogenous enzymes or iron, although the

latter has never been studied in insects. Iron oxidation is mediated by redox reactions, in which dihydroxyphenolic compounds can serve as reducing agents (Mellican et al. 2003), as shown in **Equation 4.1** (Perron & Brumaghim 2009). The product DOPAquinone can react further non-enzymatically to form melanins or crosslinks with proteins (Bittner 2006).

$$\boldsymbol{2Fe^{3+} + DOPA \rightarrow 2Fe^{2+} + DOPAquinone + 2H^{+}} \quad (4.1)$$

Polyphenols with low redox potentials are most easily oxidized by ferric iron, causing off-colour (Mellican et al. 2003). Ferrous iron needs to be converted into ferric iron by e.g. oxygen to form a complex with, and/or oxidize, these phenolics (Mellican et al. 2003; Mira et al. 2002).

Besides oxidation, *ortho*-hydroxy polyphenols can also form complexes with minerals, e.g. iron (Mellican et al. 2003). For example, L-DOPA has been described to form complexes with ferric iron (Fe^{3+}) in marine mussel threads. Mussel foot proteins contain large quantity of L-DOPA and in presence of iron, these L-DOPA residues react by intermolecular crosslinking (Hight & Wilker 2007; Yang et al. 2014). Black colour formation is observed when the L-DOPA residues in the proteins chelate Fe^{3+} into this intermolecular complex (Hight & Wilker 2007). Ferric iron shows approximately ten times more intense colour formation with catechol compared to ferrous iron (Fe^{2+}), underlining the importance of iron to be in the oxidized form (Mellican et al. 2003).

The chelating ability increases with increasing pH, as a larger proportion of the hydroxyl groups resides in the dissociated form (Mira et al. 2002). Below pH 5, iron is complexed with only one L-DOPA, between 5.6-9.1 the bis form is dominant, and above 9.1, the tris form (Holten-Andersen et al. 2011).

The aim of this research was to understand the differences in colour formation upon grinding between *Hermetia illucens* in comparison to *T. molitor* and *A. diaperinus* with emphasis on the impact of tyrosinase, phenolics and minerals. To investigate this, a model system was developed, containing L-DOPA, iron and tyrosinase, all parameters that potentially play a role in off-colour formation upon insect processing. It was hypothesized that, besides enzymatic browning, iron-DOPA complexes play a role in off-colour formation in insects, and explain the blackish colour in *H. illucens*.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Hermetia illucens larvae were kindly provided by the Laboratory of Entomology (Wageningen University, The Netherlands). *H. illucens* larvae were washed and dried before freezing. *Tenebrio molitor* larvae were purchased from Insectenkwekerij Van de Ven (Deurne, The Netherlands). *Alphitobius diaperinus* larvae were purchased from Kreca Ento-Feed BV (Ermelo, The Netherlands). Larvae were frozen with liquid nitrogen and stored at -22 °C.

Ultra-high-performance liquid chromatography/mass spectrometry (UHPLC-MS) grade formic acid, methanol, hexane, acetonitrile (ACN) and water were purchased from Biosolve BV (Valkenswaard, The Netherlands). Ethylenediaminetetraacetic acid (EDTA) was purchased from Arcros organics (New Jersey, USA).

All other chemicals used were purchased from Sigma Aldrich (St. Louis, USA) or Merck Millipore (Billerica, USA). Water was acquired using a Milli-Q water purification system (Millipore, Billerica, USA). Ferric iron was used as $FeCl_3$ (purity ≥ 99.0%), unless stated otherwise. Tyrosinase originated from *Agaricus bisporus*, which was purified to 3000 U/mL as described before (Kuijpers et al. 2012).

4.2.2. Colour assessment of insect extracts under different conditions

The larvae of *T. molitor*, *A. diaperinus* and *H. illucens* were blended in MilliQ with 0.1% (w/w) formic acid for pH 3 or 100 mM bicarbonate buffer for pH 7 and 10. Different conditions were tested at pH 7 by adding 1 mM $FeCl_3$, 24 mM EDTA or sodium bisulphite (2 g/L) (Janssen et al. 2017b) directly during blending. After centrifugation for 5 min at 12,500 *g*, the colour was assessed by taking pictures in a photo box and / or using spectrophotometric analysis (Shimadzu UV-1800, Kyoto, Japan). The spectrum was measured between 300-800 nm in quartz cuvettes and samples were diluted when necessary.

4.2.3. Colour assessment of L-DOPA and iron under different conditions

The effect of pH on colour formation was investigated with equimolar concentrations of 3 mM L-DOPA and 3 mM Fe^{3+} in MilliQ with 0.1% (w/w) formic acid for pH 3 or 100 mM bicarbonate buffer for pH 7 and 10. Only at pH 7, different ratios ranging from 1-3 mM L-DOPA and 0.3-30 mM $FeCl_3$ were tested. Also for pH 7, an excess of EDTA was added to test the reversibility of colour formation.

The effect of tyrosinase on colour formation was tested at pH 7 using 0.1 M citric acid − 0.2 M phosphate buffer. Tyrosinase from *Agaricus bisporus* was added at 150 units/mL to 3 mM L-DOPA with and without 3 mM $FeCl_3$. Colour formation in this solution was monitored for 1 h, and compared to a similar solution without enzyme addition. The colour was assessed by taking pictures in a photo box and / or using spectrophotometric analysis (Shimadzu UV-1800, Kyoto, Japan). The spectrum was measured between 300-800 nm in quartz cuvettes and samples were diluted when necessary.

4.2.4. Mineral composition of larvae and insect protein extracts with ICP-AES

Freeze-dried larvae were dried overnight at 70 °C and hydrolysed using concentrated 65% nitric acid and 37% hydrochloric acid by microwave digestion (MARS-X, CEM, USA). Hydrogen peroxide was added to remove the nitrous vapours. The minerals calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, sulphur and zinc were analysed using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) (Varian Vista Pro Radial, Varian Inc., USA), according to the guidelines NPR-6425 and NEN-6966.

4.2.5. Extraction of polyphenols from insect larvae

A brown and light insect extract for each of the three insects species studied was prepared, in order to investigate the polyphenols changing upon browning. For the light extract, insect larvae were freeze-dried and milled using a 0.5 mm sieve (Ultra centrifugal Mill ZM 200, Retsch, Haan, Germany). Hexane was subsequently used to remove fat. The 10 mg/mL defatted extract was solubilized in aqueous MeOH (50% w/w) with 0.1% (v/v) formic acid. After centrifugation at 12,500 *g* (5 min, at 20 °C), this extract was considered light extract and diluted for analysis.

The brown extract was prepared by grinding (0.5 mm sieve) frozen insects larvae before freeze-drying. The freeze-dried insects were defatted using hexane. The defatted extract was solubilized in water and mixed for 1 h. MeOH with 0.2% (v/v) formic acid was added in ratio 1:1 to stop the browning and obtain the final concentration of 10 mg/mL. After centrifugation at 12,500 *g* (5 min, at 20 °C), this extract was considered brown extract and diluted for analysis.

4.2.6. Phenolic analysis using RP-UHPLC-UV-MS

Samples were analysed using a Vanquish UHPLC which was equipped with a pump, an auto sampler and a photo-diode array detector (PDA) (Thermo Fischer Scientific, San Jose, CA,

USA). Each sample (1 µL) was injected onto an Acquity UHPLC BEH RP18 column (2.1 x 150 mm, particle size 1.7 µm; Waters, Milford, MA, USA), and eluted with UHPLC-MS grade 0.1% v/v formic acid (eluent A) and UHPLC-MS grade acetonitrile, containing 0.1% v/v formic acid (eluent B). The flow rate was 400 µL/min; the temperature of the column oven was 45 °C, with a post column cooler at 40 °C. The sample tray was set at 5 °C. The PDA detector was set to measure over the range of 190-690 nm. The following gradient was used: 0-1.1 min, isocratic on 99% v/v A; 1.1-37.3 min, linear gradient from 1% to 99% v/v B; 37.3-42.8 min, isocratic on 99% v/v B; 42.8-43.9 min, linear gradient from 99% to 1% v/v B; 43.9-50 min, isocratic on 99% v/v A.

Mass spectrometric data were obtained by analysing samples on Q-executive Focus Hybrid Quadrupole-Orbitrap Mass spectrometer (Thermo Fischer Scientific, San Jose, CA, USA), coupled to the UHPLC system. The source voltage was 2.5 kV in the negative ion mode and 4 kV in the positive ion mode. The temperature of the ion transfer tube was 250 °C. Data were collected over the *m/z* range 150-1,500. MS/MS analysis was done on a light or brown mixture of the three species.

Control of the instrument and analysis of the data were done using Xcalibur 2.2 (Thermo Scientific, USA). Analysis was done based on the comparison of light and brown extracts from the three species using compound discoverer v2.1 (Thermo Fisher Scientific, San Jose, CA, USA). To investigate the key components in browning, light and brown extracts were compared within the same species. The phenolics were taken into account when their intensity in the brown extract compared to the light extract was decreased two-fold, and when their intensity in the light extract was above 10^6. Quantification was done based on standards.

4.2.7. Sample preparation of complex formation analysis for ESI-Q-TOF-MS

Various parameters (pH, ratio [DOPA]:[Fe] and iron oxidative state), were tested to establish the effect on complex formation. Formic acid in a concentration of 0.1% (w/w) was used for pH 3 and 100 mM ammonium bicarbonate buffer for pH 7 and 10. All experiments were performed with equimolar quantities of L-DOPA and $FeCl_3$ (3 mM) unless stated otherwise. A control with EDTA addition in excess to the samples was prepared in order to chelate metal ions present. Each sample was diluted ten times in the buffer at respective pH and centrifuged for 5 min at 12,500 *g* and 15 °C.

4.2.8. Complex formation and dopachrome formation by Electron Spray Ionization Time of Flight Mass Spectrometry (ESI-Q-TOF-MS)

The samples prepared as previously explained were tested by ESI-Q-TOF-MS to examine the structure of the molecules and complexes responsible for the colour formation. The samples were introduced by direct infusion (µL/min) on Synapt G2-Si high definition mass spectrometer, equipped with a z-spray electrospray ionization (ESI) source, a hybrid quadrupole and an orthogonal time-of-flight (Q-TOF) (Waters, Milford, USA). The intensity of the peaks corresponding to possible iron complexes was annotated in negative mode (NI). Peaks corresponding to dopachrome were annotated in positive mode (PI). L-DOPA was annotated both in PI and NI. The capillary voltage was set to 3.0 kV and 1.8 kV with the source respectively in PI and NI. The source temperature was 150 °C and the sample cone was operated at 30 V in PI and 40 V in NI. MS and MS/MS were performed between *m/z* 25-800 with a 0.3 s scan time and the data was collected for 2 min. The trap collision energy was set at 6 V in single MS mode and optimized in the range between 20 to 30 V in MS/MS mode. Data were acquired and analysed by MassLynx v4.1 (Waters, Milford, USA).

4.2.9. Significance

Significance between treatments and species were statistically evaluated using t-test with the SPSS 23 program.

4.3. RESULTS AND DISCUSSION

4.3.1. Off-colour formation upon grinding of insect larvae under different conditions

Off-colour formation was observed in extracts from larvae of three insect species at different conditions (**Table 4.1**). Dark brown or black colour was observed at pH 7, whereas at pH 3 the colour formation was limited for all species, probably because phenoloxidase was inactive at low pH (Janssen et al. 2017b). At pH 10, the dark brownish colour was attributed to auto-oxidation of polyphenols, which is known to be enhanced at high pH (Yang et al. 2014).

The extracts from the various species responded differently to the various conditions, especially at neutral pH. The least off-colour formation was found for *A. diaperinus*, whereas *T. molitor* showed darker brown colour and *H. illucens* black colour.

To investigate the effect of phenoloxidase on colour formation of the different species, sulphite was added. Sulphite is known to inhibit phenoloxidase irreversibly in a time-dependent manner (Kuijpers et al. 2013). Besides inhibition of phenoloxidase, sulphite can act as reducing agent by reducing ferric ion back to ferrous ion (Mellican et al. 2003). All extracts became lighter upon sulphite addition, indicating that phenoloxidase caused part of the off-colour formation. Nevertheless, only phenoloxidase could not explain differences between species in discolouration (Janssen et al. 2017b).

To investigate the difference in off-colour formation between species at pH 7, extra iron ($FeCl_3$) was added during grinding. The colour of *T. molitor* and *A. diaperinus* extracts darkened upon iron addition and became more similar (blackish) to the colour of *H. illucens* without iron addition, indicating a role for iron in colour change. The addition of the chelating agent EDTA resulted in lighter colour of all insect extracts at pH 7, and provided further support for this theory (see **Table 4.1**) (El Hajji et al. 2006; Andjelkovic et al. 2006). As part of the off-colour could be removed upon EDTA addition, it was hypothesized that iron was chelated by L-DOPA and not by quinones that had reacted further into coloured products.

Table 4.1. Off-colour formation at pH 3, 7 and 10 of extracts from *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* 1 h after grinding. At pH 7 sodium bisulphite (1 mM), FeCl3 (3 mM) and EDTA (1 mM) were added. Extra EDTA was added to *H. illucens* to obtain a lighter colour (~4 mM).

	pH 3	pH 7	pH 7 + Fe	pH 7 + EDTA	pH 7 + HSO_3^-	pH 10
T. molitor						
A. diaperinus						
H. illucens						

4.3.2. Mineral analysis of insect larvae

The mineral composition of *T. molitor*, *A. diaperinus* and *H. illucens* was investigated in order to correlate this to the off-colour formation observed in insect extracts (**Table 4.2**). Both *T. molitor* and *A. diaperinus* showed comparable mineral composition, whereas *H. illucens* gave significant differences in the content of the minerals calcium, iron, manganese, sodium and zinc. The iron content of *H. illucens* was four times higher than that of the other species. Interestingly, the darkest colour was formed with *H. illucens*, despite the fact that it had the lowest tyrosinase activity (Janssen et al. 2017b). Differences in iron content, in particular, might explain differences in colour formation between species, as iron is known for off-colour formation in e.g. fortified products (Habeych et al. 2016) and iron addition was observed to darken the extracts in **Table 4.1**.

Table 4.2. Mineral composition of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* larvae based on dry matter (n=3, mean ±S.D.).

Larvae	Ca [g/kg]	Cu [mg/kg]	Fe [mg/kg]	K [g/kg]	Mg [g/kg]	Mn [mg/kg]	Na [mg/kg]	P [g/kg]	S [g/kg]	Zn [mg/kg]
T. molitor	**1** ± 0.3	**13.4** ± 0.5	**61.0** ± 9.7	**9.7** ± 0.4	**2.2** ± 0.3	**10.8** ± 2.1	**1737.3** ± 106.0	**7.6** ± 0.7	**3.5** ± 0.2	**111.0** ± 13.1
A. diaperinus	**0.5** ± 0.0	**21.9** ± 0.4	**53.5** ± 1.7	**10.0** ± 0.2	**1.3** ± 0.0	**5.4** ± 0.3	**2270** ± 116.8	**8.4** ± 0.4	**4.3** ± 0.2	**140.6** ± 4.3
H. illucens	**26.6** ± 1.8	**13.2** ± 1.0	**220.7** ± 6.1	**8.9** ± 0.3	**2.0** ± 0.2	**338.0** ± 49.1	**673.7** ± 25.9	**6.9** ± 0.3	**3.1** ± 0.0	**93.0** ± 3.1

4.3.3. Phenolics involved in off-colour formation of insects larvae from *T. molitor, A. diaperinus* and *H. illucens*

To investigate which phenolics play a role in off-colour formation of insects, darkened and light extracts were investigated (**Table 4.3**). Decreases in phenolics of at least two-fold in the brown extract compared to the light extract were considered diagnostic for participation of these phenolics in off-colour formation. Furthermore, only abundant phenolics with an area of at least 10^6 in the light extracts were taken into account.

Table 4.3. Phenolics involved in browning of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* during grinding and their decrease upon darkening, per kg dry matter (n=3, ±SD). The molar ratio L-tyrosine and iron was included as well.

	Extract	*T. molitor*	*A. diaperinus*	*H. illucens*
L-tyrosine (mg/kg)	Light	2557 ± 190	1912 ± 263	2674 ± 253
	Dark	651 ± 140	205 ± 8	105 ± 13
L-DOPA (mg/kg)	Light	40 ± 7	27 ± 4	21 ± 2
	Dark	36 ± 1	10 ± 4	3 ± 1
	Molar ratio L-tyrosine : Fe	13	11	4

Table 4.3 shows that L-tyrosine had a high initial concentration and the largest decrease upon darkening for all three species. L-tyrosine is known to be present in insect haemolymph (Clark & Strand 2013). Upon browning, the largest decrease of tyrosine was 25 times for *H. illucens*, whereas a nine and four times decrease was found for *A. diaperinus* and *T. molitor*, respectively.

L-tyrosine itself cannot form a complex with iron, as two *ortho*-hydroxyl groups are necessary. L-tyrosine should therefore be first hydroxylated into L-DOPA (Mellican et al. 2003). The concentration of L-DOPA (mg/kg) was found to be low in both dark and light insect extracts compared to L-tyrosine. Likely, L-DOPA, formed from L-tyrosine, was either oxidized into quinones upon reaction with endogenous phenoloxidase, or complexed with minerals. The most important precursor in darkening substrates was L-tyrosine. The ratio of reactive phenolics, in this case L-tyrosine, and iron was four times lower for *H. illucens* compared to *A. diaperinus* and *T. molitor*. Given the observation that the L-tyrosine concentration in light extracts is similar for the three

different insect extracts, the differences in iron content might explain the difference in off-colour formation between the species.

4.3.4. Factors affecting colour formation with diphenolics structures

4.3.4.1 Ferric iron interacts with diphenolics structures causing colour formation

To investigate the impact of phenolics and minerals on colour formation, a model system was set up. As *ortho*-hydroxyl groups are necessary for complex formation, (Mellican et al. 2003) L-DOPA was used in the model system instead of its precursor L-tyrosine.

First, L-DOPA was combined with one mineral at a time. Colour formation was only observed with iron and not with any of the other minerals tested (**Supplementary Figure S4.1**), of which the content that different among the three insect species as shown before in **Table 4.2**.

To investigate the effect of oxygen on colour formation, ferric and ferrous iron were combined with L-DOPA with and without reduced oxygen levels. Ferrous iron only formed colour in presence of oxygen, whereas ferric iron also gave colour without oxygen (**Supplementary Figure S4.2**). Thus, oxygen was necessary to oxidize iron into the ferric form to be able to react with L-DOPA. This was confirmed by mass spectrometric analysis of L-DOPA-iron complexes (**Supplementary Figure S4.3**, see further). Chloride was used as counter ion, as this counter ion did not influence oxidation as shown before (Welch et al. 2002). Therefore, ferric iron chloride ($FeCl_3$) was used in subsequent experiments.

4.3.4.2 Effect of pH on colour formation

Insect extracts showed different colour at different pH values. The colour of the model system with L-DOPA after addition of iron at different pH values is shown in **Figure 4.1A**. Although similar black colour was formed after addition of iron at pH 7 and 10, differences are observed after dilution. The diluted model system at pH 7 had a more purple colour, whereas that at pH 10 was more reddish corresponding to a hypsochromic shift in the visible light spectrum from 570 nm at pH 7 to 520 nm at pH 10 (**Figure 4.1A**). The absorbance peak at 570 nm for pH 7 has been reported before to correspond to an iron-L-DOPA complex (Hight & Wilker 2007; Perron & Brumaghim 2009). The chelating agent EDTA lightened the colour, indicating that iron and L-DOPA interacted non-covalently with each other. Increased absorption at 520 nm was suggested to represent dopachrome formation upon auto-oxidation of L-DOPA at basic pH (Kuijpers et al. 2013). The least colour formation was

observed at pH 3, suggesting no complex formation between L-DOPA and iron. This might be due to the competition between protons and metal ion for the L-DOPA binding site, which is more favourable for protons at low pH.(El Hajji et al. 2006) However, a peculiar green colour formation was observed at pH 3 a few minutes after combining L-DOPA and Fe^{3+}. This colour was attributed to Fe^{2+}-semiquinone complexes, where the semiquinone radical is stabilized by the aromatic ring. Fe^{2+} was formed from Fe^{3+} by redox reaction as shown in **Equation 4.1**. Subsequently, the semiquinone reduced another equivalent of Fe^{3+} to Fe^{2+} leading to a simultaneous oxidation of the semiquinone to quinone (Perron & Brumaghim 2009). This changed the green colour to a light brown colour, which was probably due to products obtained by e.g. oxidative coupling of quinones and phenolics (Vissers et al. 2017).

4.3.4.3 Effect of ratio L-DOPA : Fe^{3+} on colour

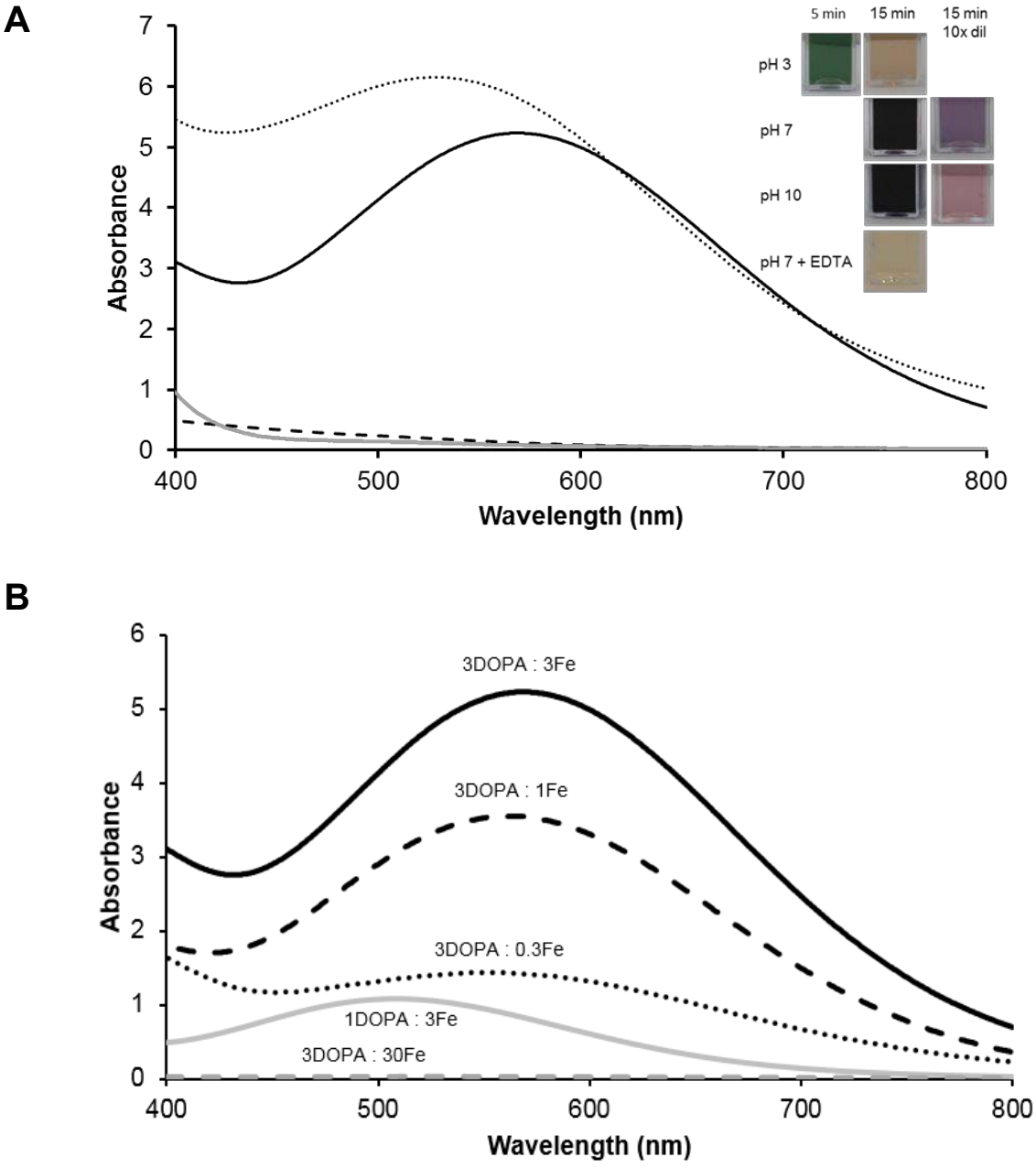
The effect of different ratios of L-DOPA and iron concentrations on colour formation were tested at pH 7. **Figure 4.1B** shows the absorbance of L-DOPA (3 mM) and different concentration of Fe^{3+} (0.3, 1, 3, 30 mM), as well as L-DOPA (1 mM) and Fe^{3+} (3 mM). Samples with excess of L-DOPA showed the highest absorbance at 570 nm, whereas excess of iron showed a shift towards 520 nm. Thus, iron excess promoted L-DOPA oxidation by redox reaction, rather than iron-phenolics complexation. This was further indicated by the substantial precipitation of material after centrifuging samples from incubations with L-DOPA (3 mM) and iron (30 mM). The precipitate probably contained insoluble products formed upon L-DOPA oxidation in presence of high amount of iron by redox reaction.

4.3.4.4 Effect of tyrosinase on colour formation

In insects, not only iron, but also phenoloxidase can react with phenolics. Phenoloxidase can oxidize diphenols into quinones. The effect of tyrosinase (phenoloxidase from fungal source) on colour formation with L-DOPA in presence of iron was investigated over time in a model system. The complexation of L-DOPA and iron was fast and did not significantly (P<0.05) change over time at pH 7 (**Figure 4.1C**). Oxidation of L-DOPA by tyrosinase on the other hand increased slowly over time observed at 520 nm (data not shown). A similar trend was observed at 570 nm, the absorbance where DOPA-iron complexes were observed (**Figure 4.1A-B**), as also described in literature (Perron & Brumaghim 2009; Hight & Wilker 2007). Thus, absorbance at 570 nm did not solely reflect complexation of iron and L-DOPA, but also oxidation of phenolics.

The dark colour also increased over time in presence of both tyrosinase and iron. This increase in colour formation was mainly due to oxidation, as the absorbance of L-DOPA plus tyrosinase showed a similar absorbance to the reaction with all combined. No synergy

between iron and tyrosinase was found indicating that it is unlikely that iron-quinone complexes caused the colour formation. This was in accordance with the lightening of the mixture after EDTA addition, showing the reversibility of off-colour formation in insects (**Table 4.1**) and in the model system with EDTA and equimolar iron and L-DOPA (**Figure 4.1A**). The colour formation at pH 7 was an interplay between L-DOPA complexation with iron (instant) and oxidation by tyrosinase (over time).



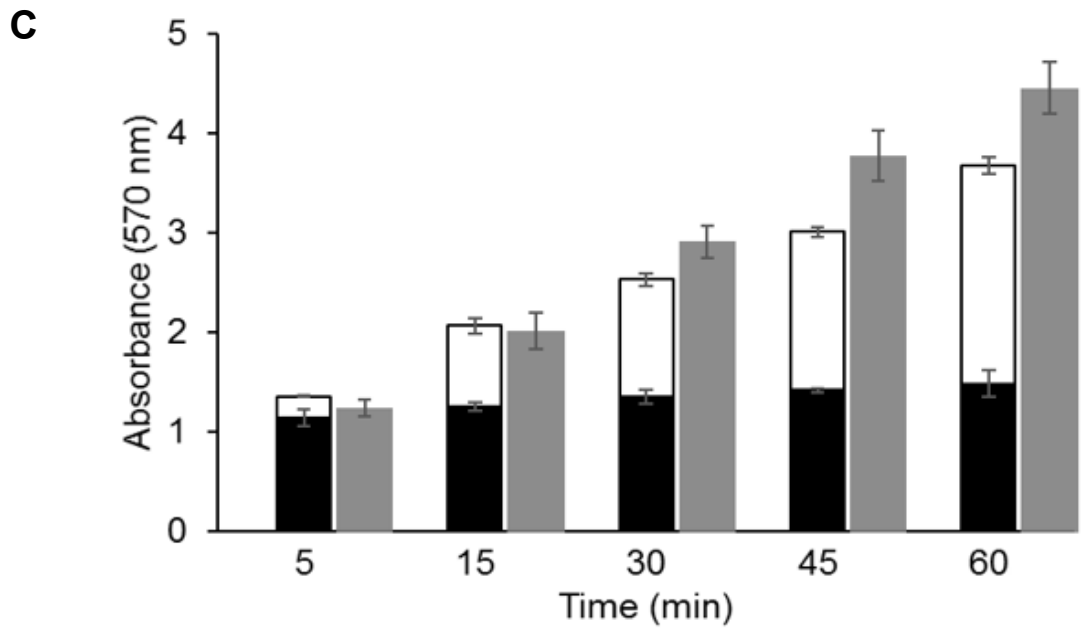


Figure 4.1. Panel A: Absorbance spectra at pH 3 (dashed line), 7 (solid black line), 7 + EDTA (solid grey line) and 10 (dotted line) on absorbance of equimolar (3 mM) L-DOPA and $FeCl_3$ with hypsochromic shift from pH 7 to 10. The colours are shown at pH 3, 7 and 10, as such and 10 times diluted if necessary. The green colour at pH 3 was directly formed after addition of iron and L-DOPA and the absorbance was measured after 15 min without dilution. The sample containing EDTA was also measured without dilution. Panel B: Absorbance of sample containing different combinations of L-DOPA + ferric iron: respectively 1 and 3 mM (grey line), 3 and 30 mM (grey dashed line), 3 and 0.3 mM (black dotted line), 3 and 1 mM (black dashed line), 3 and 3 mM (black line). Panel C: Colour formation of L-DOPA and iron for molar ratio 1:1 (black), L-DOPA with tyrosinase (white) and L-DOPA with iron and tyrosinase (grey) at 570 nm over time (n=3).

4.3.4.5 Iron-DOPA complex formation and oxidation analysed by ESI-TOF-MS

To elucidate the structures and intensity of the complexes at different pH values, samples were measured in negative mode mass spectrometry. This was done using direct injection into the mass spectrometer to avoid losing the non-covalently bound iron-L-DOPA complexes upon chromatographic separation. Also, the total intensity of L-DOPA in positive mode was determined in this way.

The sample containing equimolar concentration of L-DOPA and iron (3 mM) at pH 7 showed the most intense colour formation in the preliminary experiments. Potential complexes with iron were found at *m/z* 386.05, 446.06, 482.03 and 643.13. These signals were not observed in the control with only L-DOPA and upon iron chelation by EDTA, indicating once more the involvement of iron in complex formation, and confirming that complex formation is reversible (**Supplementary Figure S4.4)**. Moreover, at *m/z* 344.01 a large peak was observed after EDTA addition, indicating the presence of EDTA-iron complex $[(EDTA\text{-}4H^{+})+Fe^{3+}]^{-}$ upon chelation (Wu & Chen 2006).

The presence of iron in the identified complexes was confirmed by the isotope pattern, as this is specific for iron with 5.85% ^{54}Fe and 91.75% ^{56}Fe. Besides that, also 2.12% ^{57}Fe and 0.28% ^{58}Fe was present. **Table 4.4** shows the proposed structures with the theoretical and experimental isotope abundance. The structures were proposed according to the *m/z* value, fragmentation pattern and isotope abundance in comparison to the theoretical abundance.

Peaks at *m/z* 446.06, 482.03 and 643.13 were tentatively annotated as respectively $[2(DOPA\text{-}2H^+)+Fe^{3+}]^-$, $[2(DOPA\text{-}2H^+)+Fe^{3+}+2H_2O]^-$ and $[3DOPA+Fe^{3+}\text{-}4H^+]^-$, as shown in **Table 4.4** based on MS^2 fragmentation. **Supplementary Figure S4.5** shows the MS^2 spectrum of $[2(DOPA\text{-}2H^+)+Fe^{3+}+2H_2O]^-$ with *m/z* 482.03, with the positions of fragmentation (leading to the daughter ions) projected on the proposed chemical structure. Upon fragmentation, two water molecules were removed (*m/z* 35.97) and a loss of $[DOPA\text{-}H^+]^-$ (*m/z* 196.07) occurred, confirming the presence of L-DOPA in the complex and not the quinones.

The peaks *m/z* 386.06 and 536.05 were also removed upon EDTA addition, and showed the specific iron isotope pattern. As *m/z* 386.06 was found in the fragmentation pattern of *m/z* 446.04 and *m/z* 482.03, this peak likely originated from in-source fragmentation. The structure of *m/z* 536.05 remains unclear, but it is also fragmented, among others, to *m/z* 446.03. Both peaks were therefore regarded as iron-phenolic complexes and they were accounted for when estimating the abundance of iron-phenolic complexes.

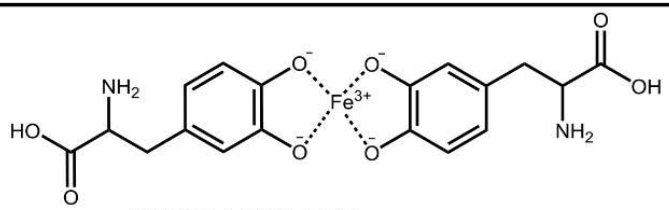
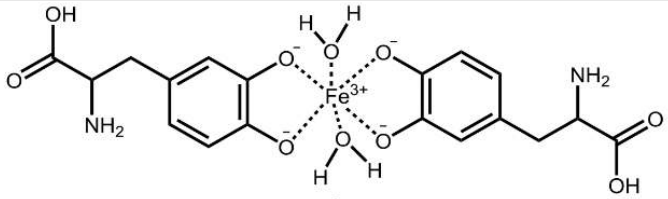
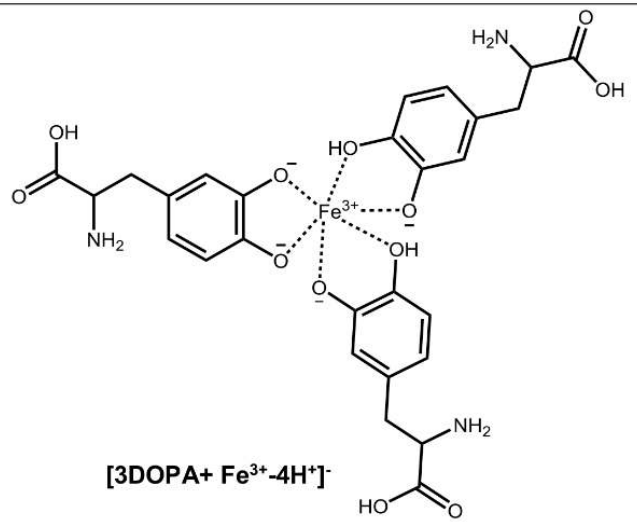
4.3.4.6 Ferric iron in complex

The complex at *m/z* 446.06 was proposed as two L-DOPA molecules chelating Fe^{3+}, $[2(DOPA\text{-}2H^+)+Fe^{3+}]^-$. Although the pKa of the hydroxyl groups in L-DOPA is known to be around 9-10, it is possible that suitable cations, like Fe^{3+}, can displace protons at lower pH values, e.g. 5.0-8.0 (Hider et al. 2001). Therefore, it is likely that the iron interacted with the negatively charged hydroxyl groups of L-DOPA. Moreover, as the complex was detected with an overall charge of -1 in negative mode, it was suggested that two carboxyl groups were protonated.

In all identified complexes, the iron ion in the centre was likely to be in the ferric form, based on the overall charge. This is consistent with a previous report, which also described Fe^{3+}-catecholate and Fe^{3+}-gallate complexes (Perron & Brumaghim 2009). As complexes with Fe^{3+} are more stable than complexes with Fe^{2+}, complexes with Fe^{2+} rapidly oxidize to complexes with Fe^{3+} via auto-oxidation (Perron & Brumaghim 2009). This was confirmed by the results in **Supplement Figure S4.2**, showing the necessity of oxygen to convert ferrous iron into ferric iron to form colour. Also, less iron-DOPA complexes were formed with ferrous iron compared to ferric iron (**Supplement Figure S4.3**).

Taken together, our data suggested that iron acted as a metal-to-ligand-charge-transfer (MLCT), allowing the electrons of the negatively charged hydroxyl groups to be delocalized over both ligands (Barreto et al. 2008; Alberding et al. 2011). Thus, the enlarged electronic system caused black colour formation and UV absorbance at 570 nm.

Table 4.4. Tentative structures of [2(DOPA-2H⁺)+Fe³⁺] ⁻, [2(DOPA-2H⁺)+Fe³⁺+2H₂O]⁻, [3DOPA+Fe³⁺-4H⁺]⁻ with corresponding experimental and theoretical relative isotopic intensity measured in negative mode.

Tentative structure	Isotopes	*m/z*		Isotope abundance (%)	
		Exp.	Theoretical	Exp.	Theoretical
 [2(DOPA-2H⁺)+Fe³⁺]⁻	$^{12}C_{18}H_{18}N_2O_8{}^{54}Fe$	444.06	444.05	3.2	6.4
	$^{12}C_{17}{}^{13}C_1H_{18}N_2O_8{}^{54}Fe$	445.05	445.05	0.6	1.2
	$^{12}C_{18}H_{18}N_2O_8{}^{56}Fe$	446.06	446.06	100	100
	$^{12}C_{17}{}^{13}CH_{18}N_2O_8{}^{56}Fe$ or $^{12}C_{18}H_{18}N_2O_8{}^{57}Fe$	447.06	447.04	16.0	21.8
	$^{12}C_{16}{}^{13}C_2H_{18}N_2O_8{}^{56}Fe$ or $^{12}C_{18}H_{18}N_2O_8{}^{58}Fe$	448.06	448.05	1.2	3.4
 [2(DOPA-2H⁺)+Fe³⁺+	$^{12}C_{18}H_{22}N_2O_{10}{}^{54}Fe$	480.03	480.07	5.5	6.4
	$^{12}C_{17}{}^{13}C_1H_{22}N_2O_{10}{}^{55}Fe$	481.02	481.07	1.6	1.2
	$^{12}C_{18}H_{22}N_2O_{10}{}^{56}Fe$	482.03	482.06	100	100
	$^{12}C_{17}{}^{13}CH_{22}N_2O_{10}{}^{56}Fe$ or $^{12}C_{18}H_{22}N_2O_{10}{}^{57}Fe$	483.04	483.07	23.0	21.8
	$^{12}C_{16}{}^{13}C_2H_{22}N_2O_{10}{}^{56}Fe$ or $^{12}C_{18}H_{22}N_2O_{10}{}^{58}Fe$	484.03	484.07	33.7	3.9
 [3DOPA+ Fe³⁺-4H⁺]⁻	$^{12}C_{27}H_{29}N_3O_{12}{}^{54}Fe$	641.13	641.12	3.8	6.3
	$^{12}C_{26}{}^{13}C_1H_{29}N_3O_{12}{}^{54}Fe$	642.13	642.12	1.6	2.0
	$^{12}C_{27}H_{29}N_3O_{12}{}^{56}Fe$	643.13	643.11	100	100
	$^{12}C_{26}{}^{13}CH_{29}N_3O_{12}{}^{56}Fe$ or $^{12}C_{27}H_{29}N_3O_{12}{}^{57}Fe$	644.14	644.11	26.8	33.3
	$^{12}C_{25}{}^{13}C_2H_{29}N_3O_{12}{}^{56}Fe$ or $^{12}C_{27}H_{29}N_3O_{12}{}^{58}Fe$	645.15	645.12	6.1	8.1

4.3.4.7 Effect of pH and ratio L-DOPA : Fe on complex formation

After identification of the complexes, the complex formation was investigated at different pH and with various L-DOPA : Fe ratios. The total absolute intensity of the different complexes is shown in **Figure 4.2A** for pH 3, 7 and 10 at equimolar ratio and at pH 7 with three different ratios of L-DOPA : Fe.

Few complexes were formed at pH 3 as shown in **Figure 4.2A**, whereas they were formed at higher pH in line with the colour formation (**Figure 4.1**). No mono-complexes (one phenolic combined to iron) were found at low pH, as has been suggested in literature before (Holten-Andersen et al. 2011). Protons were more favourite than iron to bind to L-DOPA hydroxyl groups at low pH (El Hajji et al. 2006). Despite the large variation, complexes at pH 7 and 10 showed comparable intensity in **Figure 4.2A**. In literature, it was reported that the concentration of tris-complexes (three phenolics coordinated by one central iron) were increased at higher pH (Sever et al. 2004; Yang et al. 2014). In this research, pH 7 showed the highest intensity of tris-complexes (*m/z* 643.13), as well as in total intensity of the complexes. At pH 10, part of the variation was likely due to the high reactivity of phenolics at this pH caused by its decreased redox potential (Krishtalik 2003).

Formation of L-DOPA-iron complexes at pH 7 was increased by a higher concentration of L-DOPA added, as the summarized signal intensity with 1 mM DOPA:3 mM Fe was lower than that with 3 mM L-DOPA: 3 mM Fe (**Figure 4.2A**). This was in line with the decreased darkening as observed before. The total complex formation with 3 mM L-DOPA : 1 mM Fe (with high standard deviation) was similar to that with 3 mM L-DOPA : 3 mM Fe.

Figure 4.2B shows the effect of different L-DOPA : Fe ratios on the remaining L-DOPA concentration. L-DOPA without iron was assumed to be stable at pH 3, whereas at pH 10 the amount of L-DOPA decreased, likely due to auto-oxidation. Addition of iron to L-DOPA at pH 3 decreased L-DOPA, due to the redox reaction. Moreover, iron also likely prevented part of the L-DOPA oxidation compared to L-DOPA without iron at pH 7. This prevention of L-DOPA oxidation after iron addition was also found at pH 10, indicating that complexation could partially prevent L-DOPA auto-oxidation (**Figure 4.2B**). This was also described by Xu et al. (2012).

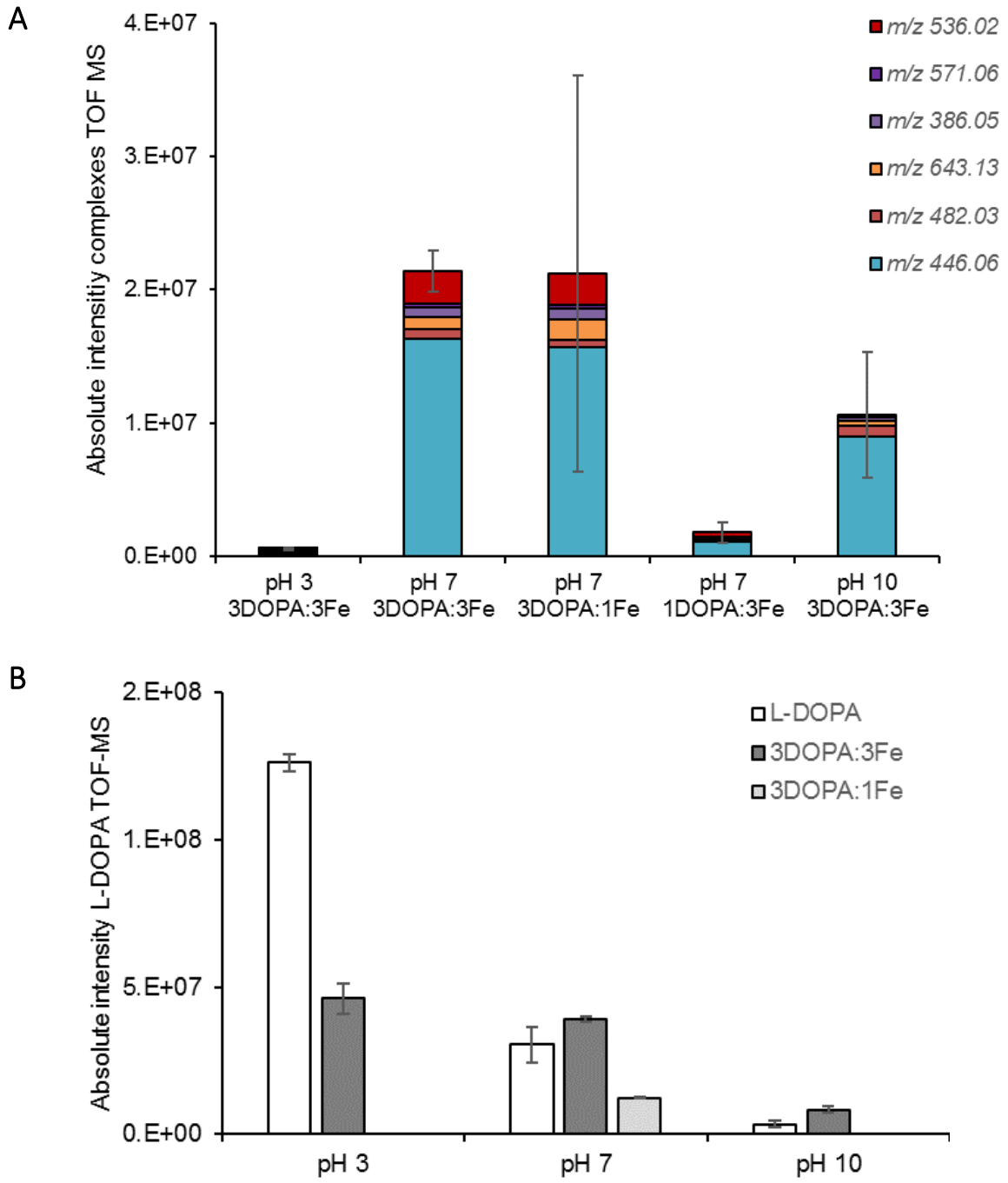


Figure 4.2. Panel A: Absolute intensity after direct injection in TOF-MS of different iron-L-DOPA complexes (different m/z values) at pH 3, 7 and 10 using equimolar and different ratios of L-DOPA and iron. Panel B: absolute intensity of L-DOPA without iron and equimolar addition of iron at pH 3, 7 and 10 and ratio 3L-DOPA:1Fe at pH 7 (n=2, error bars represent absolute deviation).

4.3.5 Pattern of oxidation and complexation of diphenolic structures and iron in relation to colour formation in insect

Our results suggested that reversible complexation of L-DOPA by iron and irreversible L-DOPA oxidation were two independent parallel mechanisms as shown for the model system. Factors such as pH and ratio L-DOPA to iron were important determinants in complexation or oxidation. Oxidized phenolics are reactive and can covalently crosslink by e.g. oxidative coupling, forming larger coloured structures that can precipitate. **Figure 4.3** proposes an overall scheme about the fate of L-DOPA under different conditions.

Figure 4.3 shows the effect of pH on reactions with L-DOPA with or without iron or in presence of phenoloxidase. The reactions playing a role are (i) PO oxidation, (ii) redox oxidation involving iron and/or oxygen and (iii) formation of complexes comprising diphenolic structures and iron. All reactions combined lead to quinones with subsequent covalent crosslinking or formation of complexes comprising diphenolic structures and iron.

At pH 3, a light colour was observed and only limited redox reaction occurred between iron and L-DOPA. Phenoloxidases are inactive at this pH and no iron-L-DOPA complexes were formed. At pH 7, a dark colour was observed caused by the formation of iron-L-DOPA complexation as well as oxidation. Limited auto-oxidation of L-DOPA occurred, but more extensive oxidation was observed when phenoloxidases were added at this pH. At pH 10, iron-L-DOPA complexes were formed and auto-oxidation was more prominent at this pH than at pH 7. Auto-oxidation of L-DOPA at pH 7 and 10 was partly prevented by addition of iron. After oxidation, quinones likely participated in oxidative coupling reactions leading to irreversible covalent cross-linking.(Yang et al. 2014) Dissociation of iron-L-DOPA complexes upon EDTA addition and fragmentation of the complexes suggested the presence L-DOPA in the complex rather than L-DOPA quinone. The higher the iron concentration, the darker the colour that was formed when L-DOPA was in excess. In insects, this trend in off-colour formation was also observed, as excess L-tyrosine (and therewith L-DOPA) was present compared to iron. Extra addition of iron blackened the colour of *T. molitor* and *A. diaperinus* extracts, similar to the colour in *H. illucens*, which had already a four times higher iron content than the other two species. Even though the model system and insect extracts showed the same trend in colour formation, the iron-phenolic complexes were not evidenced in the insect extracts. This might be due to formation of larger structures with e.g. proteins, which are difficult to detect by mass spectrometry. Based on the similarities in colour formation under different conditions between the model system and during grinding of *H. illucens* larvae, we postulate that off-colour formation in *H. illucens* followed similar mechanisms as in the model system.

This is the first time that a link between off-colour formation and the presence of iron in insects was made. Iron complexation with phenolic structures and off-colour formation was found before in, for example, iron fortified foods.(Habeych et al. 2016) By identifying colour formation mechanisms in the model system, insight was obtained in the mechanisms playing a role in off-colour formation when processing insects. This understanding in colour formation can help in designing future application of insects for food and feed.

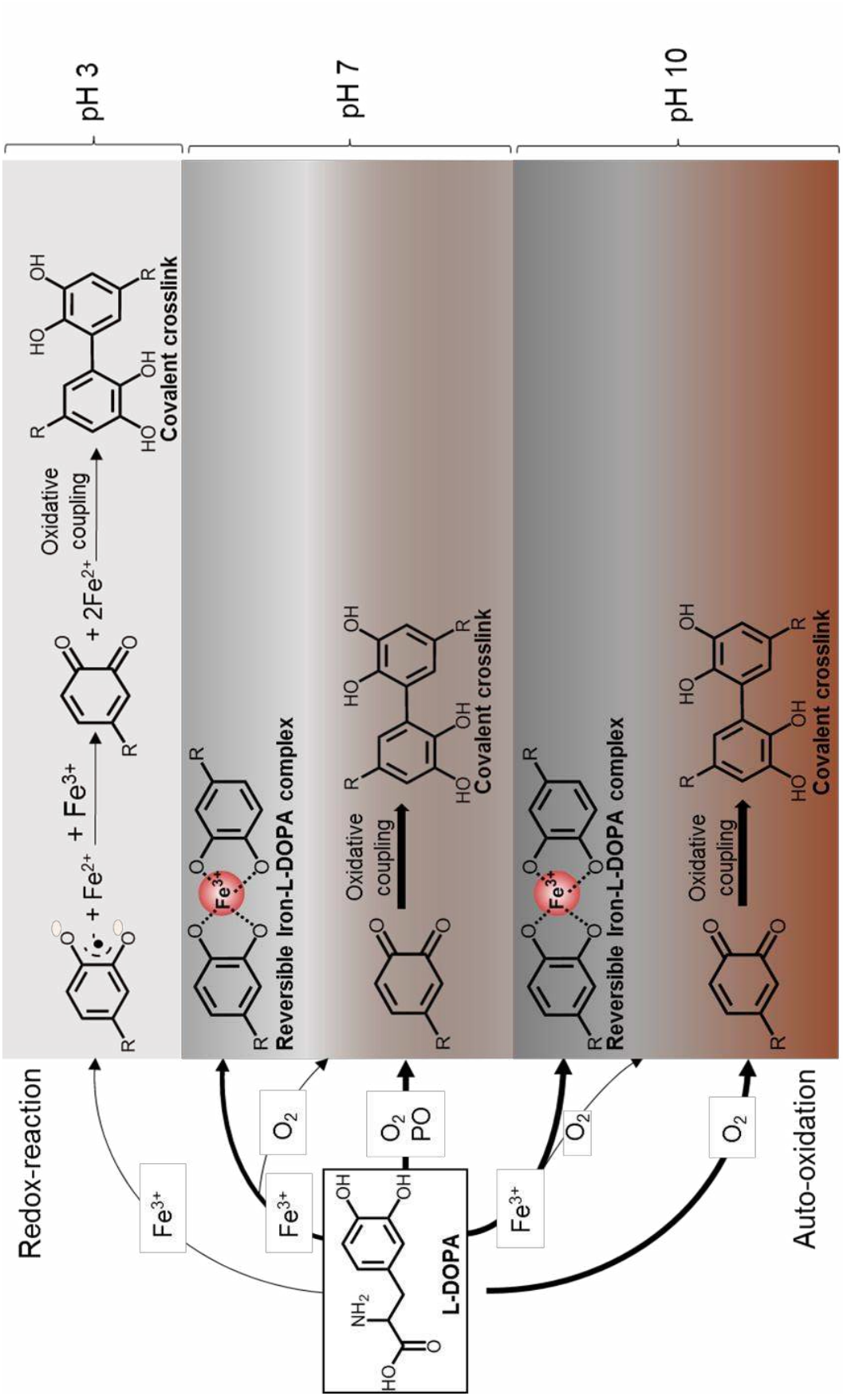


Figure 4.3. Schematic representation of interactions and reactions between 3 mM L-DOPA and 3 mM Fe^{3+} at different conditions. The thickness of the arrows indicates the extent of the reaction at its pH. R=CH_2CHNH_2COOH. Colour shading behind the structures is indicative for the colour shade and extent of colour formation after reaction.

4.4. SUPPORTING INFORMATION

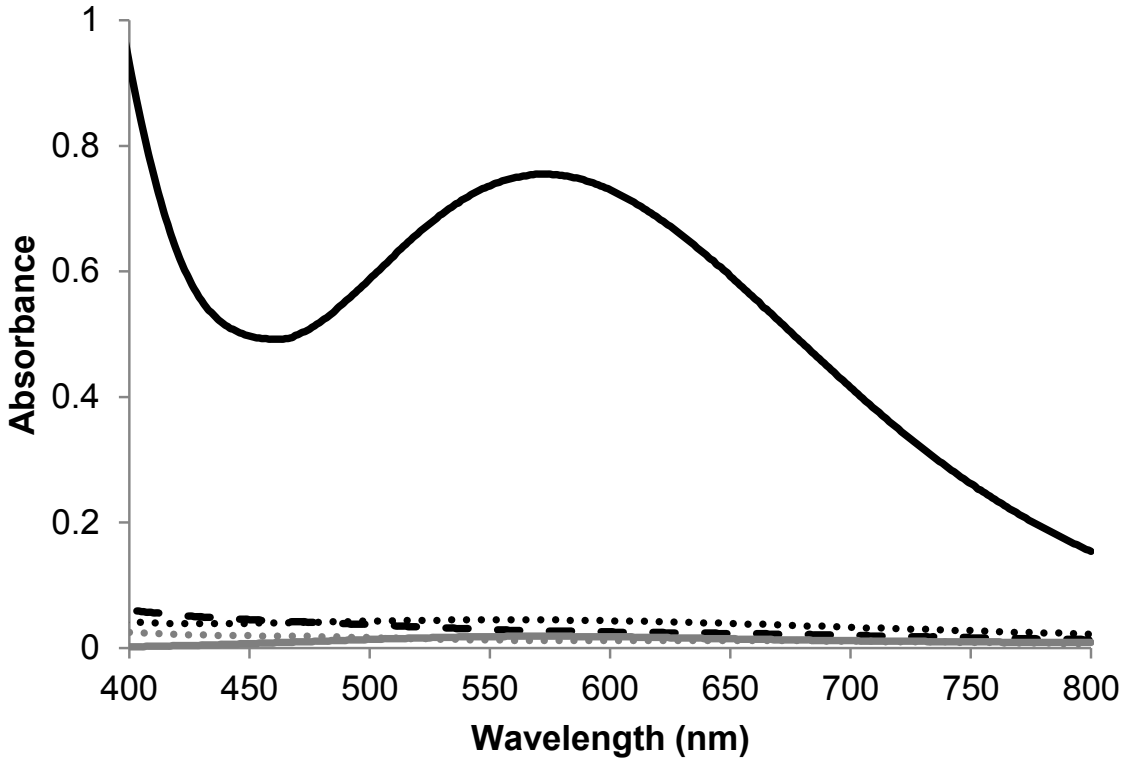


Figure S4.1. Absorbance of L-DOPA with different minerals: calcium (black striped), manganese (grey dotted), sodium (black dotted), zinc (grey line) and iron (black line). The content of these minerals was significantly different between insect species.

Method:

Effect of minerals (3 mM $CaCl_2$, $MnCl_2$, NaCl, $ZnSO_4$ or $FeCl_2$) was tested at pH 7 using 0.1 M citric acid − 0.2 M phosphate buffer in addition to 3 mM L-DOPA. The colour was assessed using spectrophotometric analysis (Shimadzu UV-1800, Kyoto, Japan). The spectrum was measured between 300-800 nm in quartz cuvettes.

A	B	C	D
Ferrous iron	Ferric iron	Ferrous iron	Ferric iron
20% Oxygen	20% oxygen	100% oxygen	100% oxygen

Figure S4.2. Colour formation in low-oxygen-level L-DOPA solution with ferrous (A) or ferric (B) iron and normally oxygenated L-DOPA solution with ferrous (C) or ferric (D) iron.

Method: Water was boiled to remove the oxygen. To confirm the removal of oxygen, the concentration was measured using oxytherm system. Eighty % of the oxygen was removed. DOPA and iron were subsequently solubilized and kept under nitrogen flow.

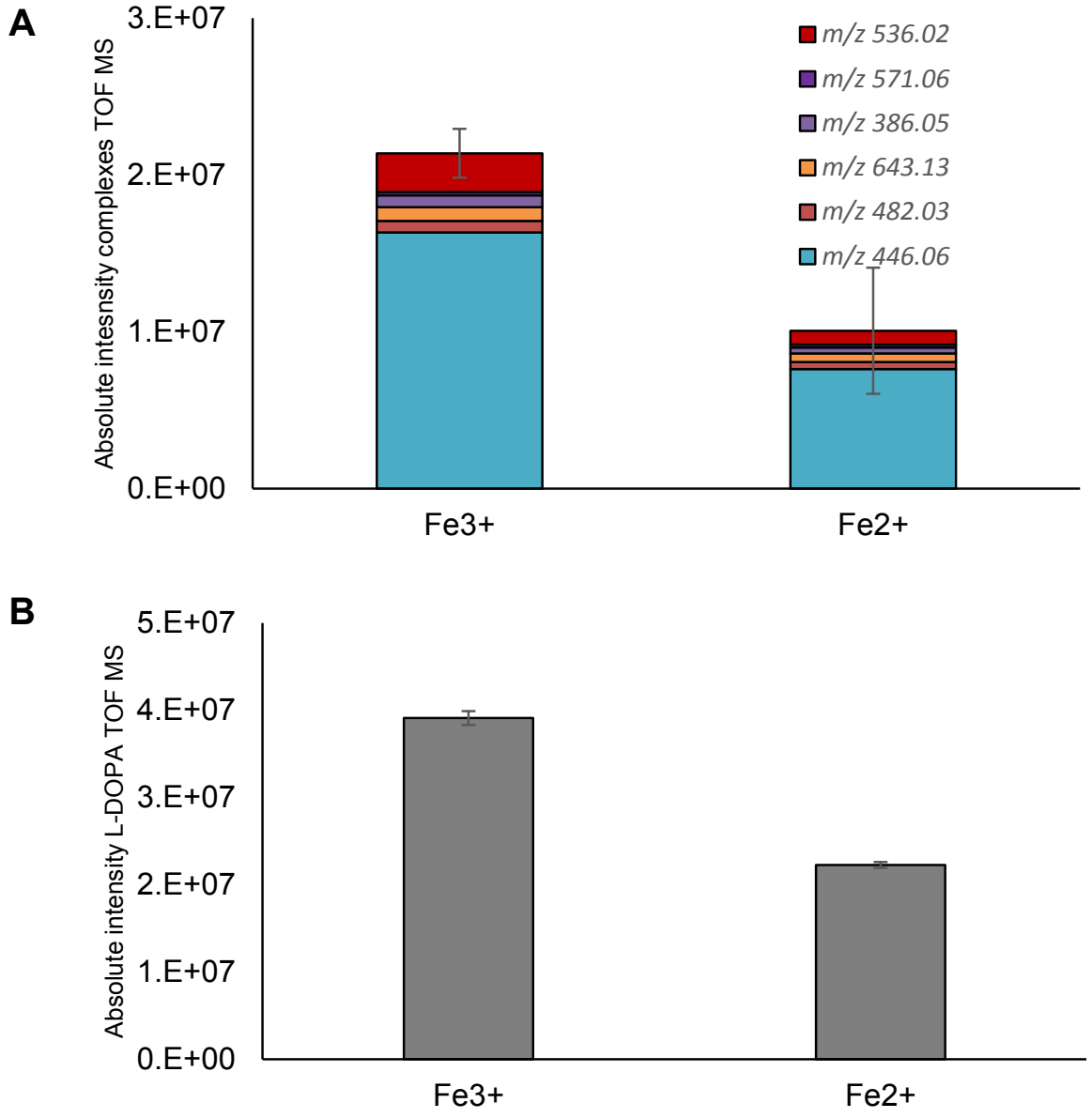


Figure S4.3. Panel A: Absolute intensity of iron-L-DOPA complexes after addition of 3 mM L-DOPA with 3 mM iron in ferrous or ferric form. Panel B: Absolute intensity of L-DOPA of 3 mM DOPA with 3mM iron ferric or ferrous form (n=2, error bars represent absolute deviation).

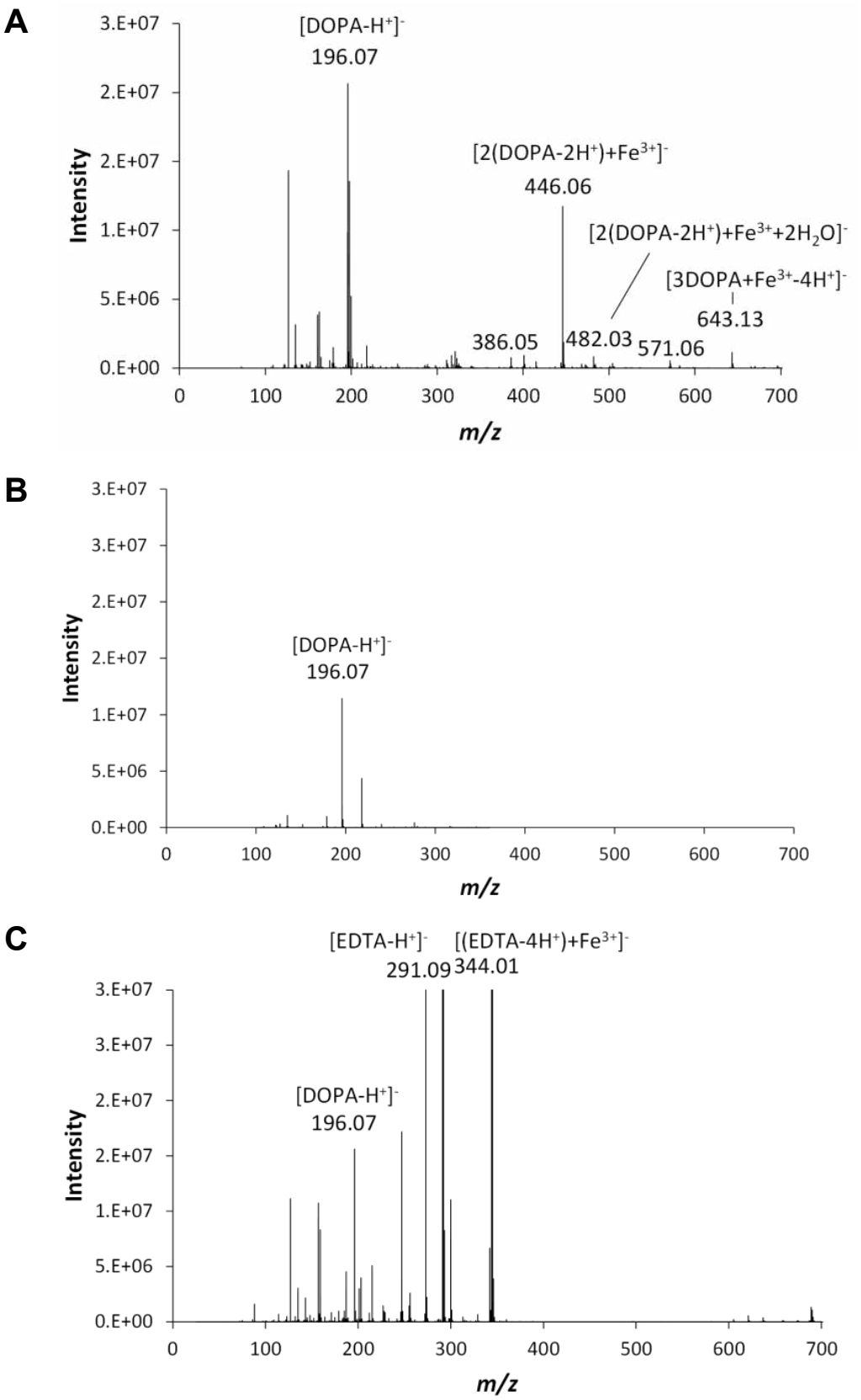

A
[DOPA-H+]-
196.07
[2(DOPA-2H+)+Fe3+]-
446.06
[2(DOPA-2H+)+Fe3++2H2O]-
[3DOPA+Fe3+-4H+]-
643.13
386.05
482.03
571.06
Intensity
m/z
B
[DOPA-H+]-
196.07
Intensity
m/z
C
[EDTA-H+]-
291.09
[(EDTA-4H+)+Fe3+]-
344.01
[DOPA-H+]-
196.07
Intensity
m/z

Figure S4.4 Panel A: Electrospray mass spectrum of a solution of L-DOPA (3 mM) + $FeCl_3$ (3 mM) at pH 7 in NI. The peaks at m/z 196.07, 446.06, 482.03, 643.13 were identified as $[DOPA\text{-}H^+]^-$, $[2(DOPA\text{-}2H^+)+Fe^{3+}]^-$, $[2(DOPA\text{-}2H^+)+Fe^{3+}+2H_2O]^-$ and $[3DOPA+Fe^{3+}\text{-}4H^+]^-$, respectively. Panel B: Electrospray mass spectrum of a solution of L-DOPA (3 mM) at pH 7 in NI. The peak at m/z 196.07 was identified as $[DOPA\text{-}H^+]^-$. Panel C: Zoom of electrospray mass spectrum of a solution of L-DOPA (3 mM) + $FeCl_3$ (3 mM) + EDTA (24 mM) at pH 7 in NI. The peak at m/z 273.09, 291.09 and 344.01 showed an intensity of respectively $4.1*10^7$, $4.09*10^8$ and $1.84*10^8$. The peaks at m/z 196.07, 291.09 and 344.01 were identified as $[DOPA\text{-}H^+]^-$, $[EDTA\text{-}H^+]^-$ and $[(EDTA\text{-}4H^+)+Fe^{3+}]^-$, respectively.

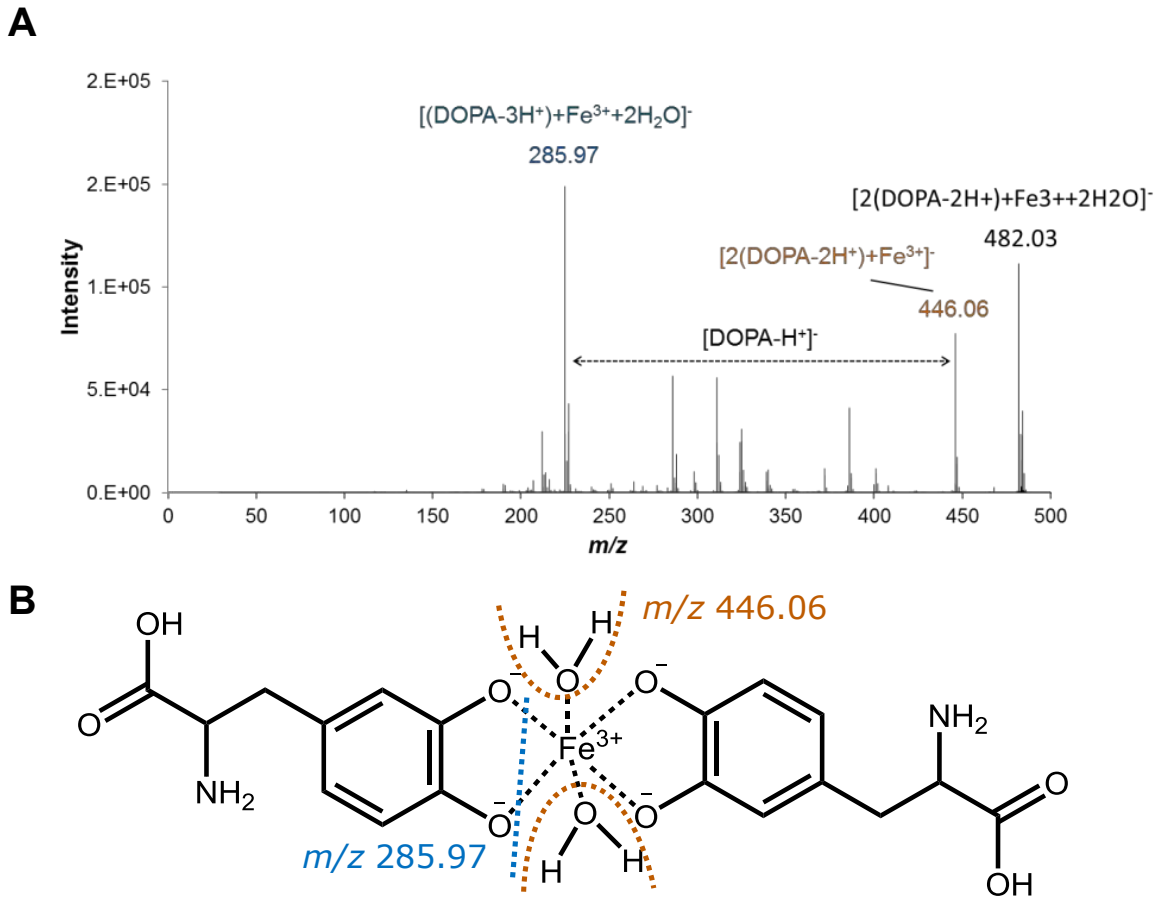


Figure S4.5. Panel A: MS^2 spectrum of the $[2(DOPA\text{-}2H^+)+Fe^{3+}+2H_2O]^-$. A loss of $[2H_2O]$ (blue) from the parental molecule resulted in daughter ion $[2(DOPA\text{-}2H^+)+Fe^{3+}]^-$ (m/z 446.06), whereas loss of $[DOPA\text{-}H^+]^-$ (orange) resulted in daughter ion $[(DOPA\text{-}3H^+)+Fe^{3+}+2H_2O]^-$ (m/z 285.97). Panel B: Schematic representation of the main MS^2 fragmentation pattern of $[2(DOPA\text{-}2H^+)+Fe^{3+}+2H_2O]^-$.

5

Effects of the endogenous phenoloxidase on protein solubility and digestibility after processing of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*

This chapter has been submitted as Janssen RH, Vincken J-P, Arts NJG, Fogliano V, Lakemond CMM, Effect of endogenous phenoloxidase on protein solubility and digestibility after processing of *Tenebrio molitor, Alphitobius diaperinus* and *Hermetia illucens*.

ABSTRACT

Upon extracting soluble proteins from insects as potential food ingredient, endogenous enzymes, such as phenoloxidases, are expected to negatively affect protein properties. The effect of phenoloxidases on solubility and digestibility was investigated for larvae of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. Phenoloxidase inhibition was done using blanching (50 s, 90°C) before extraction or extracting in presence of sulphite.

Similar soluble protein yields and compositions were found without and with sulphite addition, whereas blanching decreased soluble protein yield. Upon *in-vitro* hydrolysis by pepsin and trypsin, soluble proteins from *H. illucens* were more digestible than those of *T. molitor* and *A. diaperinus*. Phenoloxidase activity during processing negatively affected *in-vitro* pepsin hydrolysis.

Besides phenoloxidase activity, also endogenous proteases were shown to remain active at pH 8 in extracts containing sulphite and after blanching of larvae. This stresses that protease activity needs to be carefully controlled in the design of insect based ingredients.

5.1. INTRODUCTION

Insects are investigated as alternative protein source for food and feed due to their nutritional composition and ease of rearing (Ghaly & Alkoaik 2009). Although insects are already eaten, most consumers dislike to eat insects as such (Schösler et al. 2012; van Huis et al. 2013). Acceptance increases when insects are not visible anymore and used as ingredients in food products (Schösler et al. 2012; van Huis et al. 2013). To make insects invisible, they are ground as a whole, which means that endogenous enzymes from the insects itself are released from the various tissues and subcellular compartments, and contact substrates leading to quality defects in the ingredients.

Endogenous phenoloxidases (POs), can cause undesired browning during grinding of insects (Janssen et al. 2017b). Insect POs are responsible for defence reactions, sclerotization and melanisation in living insects (Terwilliger 1999). During this enzymatic browning, mono- and diphenols are oxidized by phenoloxidases to form *o*-quinones. *O*-quinones can polymerize into brown melanin pigments or react with several functional groups of proteins or amino acids to form crosslinked structures (Friedman 1997; Bittner 2006). This enzymatic browning can be detrimental for protein quality, e.g. solubility and digestibility (Matheis & Whitaker 1984; Prigent et al. 2007; Kroll et al. 2003). Enzymatic browning is expected to reduce insect protein digestibility, as was observed in previous studies for plant proteins (Matheis & Whitaker 1984). However, this effect depends on the type of proteins and phenolics (Kroll et al. 2003). For insect proteins, no such studies have been performed so far. Besides digestibility, browning might also change techno-functional properties of proteins like solubility, water holding capacity or foaming (Prigent et al. 2007; Kroll et al. 2003).

Besides PO activity, also proteolytic activity might be present in the insect protein extracts. In fact, insects are not eviscerated before consumption and their gut has serine and cysteine-like proteases (Thie & Houseman 1990). The possible occurrence of protease activity has so far been neglected when processing insects into ingredients, despite the fact that this might largely alter protein functionality.

The aim of this research was to investigate the effect of endogenous phenoloxidase on the solubility and digestibility of proteins extracted from the three insect species: *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. Solubility and digestibility were measured after protein extraction using three pre-treatments: (i) a control made without prevention of enzymatic browning, (ii) an extract in presence of sulphite and (iii) an extract from blanched insects to prevent enzymatic browning. It was expected that phenoloxidase activity would decrease protein solubility and digestibility. Besides phenoloxidase, also

endogenous protease activity was expected to remain active after extraction in presence of sulphite.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Larvae from *Tenebrio molitor* and *Alphitobius diaperinus* were purchased by Kreca Ento-Feed BV (Ermelo, The Netherlands). *Hermetia illucens* larvae were kindly provided by laboratory of Entomology (Wageningen University, The Netherlands). Larvae were frozen using liquid nitrogen and stored at -22 °C.

Sodium dodecyl sulfate was purchased from Carl Roth (Karlsruhe, Germany). Instant blue was purchased from Expedeon (San Diego, CA, USA) and glycerol from VWR International (Radnor, PA, USA). Purified water was prepared using a Milli-Q water purification system (Millipore, Billerica, MA, USA). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

5.2.2. Soluble protein extraction and purification

Insect proteins were extracted to investigate the effect phenoloxidase on the solubility and digestibility. Therefore, three different protein extracts were made in which the soluble proteins were dialyzed (12-14 kDa cut-off) after extraction: (i) Untreated extract was prepared according to Janssen et al. (2017a), (ii) sulphite-containing extract was prepared similarly as the untreated extract with addition of sulphite by dissolving 2 g/L sodium bisulphite into 0.1 M citric acid / 0.2 M phosphate buffer pH 6 before blending and (iii) blanched extract was obtained by blanching frozen larvae for 50 s at 90 °C in excess of demi water. Larvae were drained and directly blended in cold 0.1 M citric acid / 0.2 M phosphate buffer pH 6.

5.2.3. Phenoloxidase activity

Phenoloxidase activity was estimated by oxygen consumption assay as described previously (Janssen et al. 2017b) using Oxytherm System (Hansatech, Kings Lynn, UK). Briefly, extracts (50 µL) were incubated with 1 mL 3 mM L-DOPA with 0.1 M citric acid / 0.2 M phosphate buffer pH 6 at 25 °C. The activity was measured within 1 h after extraction. Data were acquired using O2-view software (Hansatech, Kings Lynn, UK). Oxygen consumption rate was calculated using the slope of the linear part of the oxygen consumption curve.

5.2.4. Amino acid determination and protein content

The amino acid composition was determined using the ISO13903:2005 method, adjusted for micro-scale. The amide nitrogen from Asn and Gln was measured together with respectively Asp and Glu (Janssen et al. 2017a).

The total nitrogen content (Nt) was determined by the Dumas method using Flash EA 1112 NC analyser (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol using methionine as standard.

5.2.5. SDS-PAGE

The protein composition was determined using SDS-PAGE under reducing conditions on a Bio-Rad mini-protean II system (Hercules, California, USA). Electrophoresis was performed using commercially prepared polyacrylamide gels (Mini-PROTEAN TGX Stain-Free Any kD precast polyacrylamide gels, Bio-Rad Laboratories, Hercules, California, USA) according to manufacturer's protocol.

Protein extract (3 mg/mL) was dissolved in sample buffer consisting of 0.1 M Tris, 2% SDS, 20% Glycerol and ~ 0.5 mg/mL bromophenolblue. From this, 10 µL was added to 20 µL sample buffer with 100 mM DTT. The marker used was Precision plus protein standard 10-250 kDa (Bio-Rad, Hercules, California, USA). Protein bands were stained using Coomassie blue stain according to the manufacturer's protocol (InstantBlue, Expedeon, San Diego, CA, USA).

5.3.6. Degree of protein hydrolysis

Enzymatic endogenous protein hydrolysis was determined by real-time monitoring using a pH-stat device (Metrohm AG, Herisau, Switzerland). Freeze-dried protein extracts were dissolved in 10 mL MilliQ water in a concentration 0.9% (w/v) protein content, based on Dumas using nitrogen-to-protein conversion factor of 5.60 (Janssen et al. 2017a).

The degree of hydrolysis (DH) by endogenous proteases was directly estimated from the amount of 0.05 M NaOH added to keep pH constant at pH 8 at 37 °C for 2 h (Minekus et al. 2014).

Exogenous enzymes were added to perform an *in vitro* digestion. This was done in a two-step hydrolysis in which 2 % (w/v) pepsin from porcine gastric mucosa (EC 3.4.23.1) (601 U/mg) was dissolved in 10 mM HCl. Hundred µL was added to the protein solution at pH 3 and incubated for 2 h at 37 °C. After pepsin hydrolysis, the pH was adjusted to pH 8 using 1 M NaOH. The pepsin hydrolysis was followed by trypsin hydrolysis. Hydrolysis was

performed using 2 % (w/v) trypsin from bovine pancreas (EC 3.4.21.4) (≥ 10,000 BAEE units/mg) dissolved in MQ, and 100 µL was added to the protein solution. The reaction was stopped after 2 h of hydrolysis at 37 °C.

The DH is defined as the percentage of hydrolysed peptide bonds over the total number of peptide bonds present and can be calculated from **Equation 5.1** (Adler-Nissen 1986).

$$DH(\%) = V_b \cdot N_b \cdot \frac{1}{\alpha} \cdot \frac{1}{m_p} \cdot \frac{1}{h_{tot}} \times 100 \quad (5.1)$$

In which V_b represents the volume of base added in mL, N_b the normality of the base, α the average degree of dissociation of the amino group (1/α = 1.31 at 37 °C and pH 8) (Adler-Nissen 1986), m_p the mass of protein in g, and h_{tot} the total number of peptide bonds in mmol per gram protein substrate that was determined from the amino acid composition of the insect extracts.

Equation 1 is incorrect for hydrolysis at low pH (1-3.5) and **Equation 5.2** should be used to determine the DH during pepsin hydrolysis (Diermayr & Dehne 1990).

$$DH(\%) = \frac{V_a \cdot N_a}{m_p} \cdot \frac{1}{h_{tot}} \cdot F_{pH} \times 100 \quad (5.2)$$

In which V_a represents the volume of added acid in mL, N_a the normality of the acid, m_p the mass of protein in g, h_{tot} the total number of peptide bonds per gram protein substrate determined from the amino acid composition, and F_{pH} the correction factor for the average degree of dissociation of the carboxyl group (α) (1/1-α = 1.8 between 25 and 50 °C, pH 3) (Diermayr & Dehne 1990). The DH was directly estimated from the amount of 0.1 M HCl added to keep pH 3 during enzymatic hydrolysis at 37 °C for 2 h.

5.2.7. Statistical analysis

To test for significance between treatments and species, the data was statistically evaluated by analysis of variance (ANOVA) followed by LSD Alpha post hoc analysis using the SPSS 23 program.

5.3. RESULTS AND DISCUSSION

5.3.1. Endogenous phenoloxidase activity in protein extracts

The protein extract was prepared at pH 6 to obtain the highest phenoloxidase activity and browning (Janssen et. al. 2017b). This was done for all three species, of which *T. molitor* showed the highest activity giving a brown colour, followed by *A. diaperinus* with the lightest colour. The lowest activity was found for *H. illucens* with a blackish colour (**Figure 5.1**), likely caused by the higher iron concentrations as described elsewhere (**Chapter 4**).

The untreated extracts were compared with extracts where enzymatic browning was inhibited in two different ways; using addition of excess of sulphite or blanching of the larvae before extraction. Blanching was performed for 50 s at 90 °C, as preliminary work showed that this time / temperature combination was minimally necessary to prevent browning. Such minimal treatment was preferred to maintain protein functionality as much as possible by preventing denaturation. **Figure 5.1** clearly shows a lighter colour and decreased enzyme activity in all sulphite containing and blanched insect extracts. The enzyme activity was almost completely inhibited by blanching for *T. molitor* and *H. illucens.* The activity of *A. diaperinus* was less than 10% of the initial activity after blanching based on oxygen consumption. Sulphite also inhibited the enzyme activity for *T. molitor* almost completely. Although some oxidative activity was found for *A. diaperinus* and *H. illucens* after sulphite addition, the activity was six times lower compared to the untreated extract. Inhibition of phenoloxidase was confirmed as sulphite addition lightened the colour of the extracts as shown in **Figure 5.1**. Sulphite is known to inhibit phenoloxidase irreversibly in a time-dependent manner (Kuijpers et al. 2013). Both sulphite and blanching as inhibition methods were tested in order to investigate their effect on the protein solubility and digestibility.

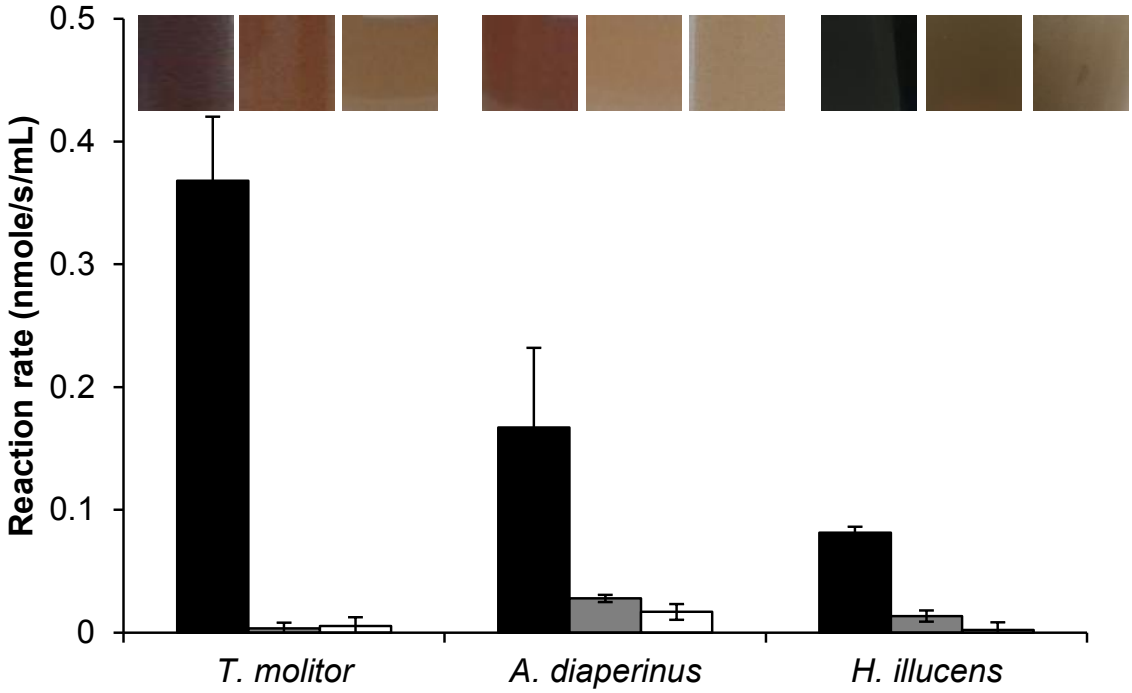


Figure 5.1. Enzymatic phenoloxidase activity of the untreated (black), sulphite treated (grey) and blanched (white) crude protein extracts from *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* based on oxygen consumption using 3 mM L-DOPA as substrate (n=3). The respective colours of the extracts are shown above the bar diagram. All extracts were obtained at pH 6.0.

5.3.2. Effect of blanching and sulphite pre-treatments on the quality of protein extracts

In **Table 5.1**, the amino acid composition of the extracts with inhibited endogenous PO enzymes were compared to the composition of untreated extracts (Janssen et al. 2017a). Inhibition using sulphite resulted in a similar amino acid composition (in g/100 g protein) and purity (based on amino acid content per 100 g dm)compared to the untreated extract for all three species (P<0.05). SDS PAGE (**Figure 5.2**) showed that this was also the case for the protein composition with and without sulphite addition for *T. molitor* and *A. diaperinus*, although differences between the two species in protein composition were observed based on the band pattern. Even though the same protein concentration based on nitrogen was applied, the bands of the sulphite-treated *Hermetia illucens* sample appeared lighter on the gel than those of the untreated extract for unknown reasons.

Blanching before extraction significantly (P<0.05) decreased the total amino acid content, indicating a lower protein purity (**Table 5.1**) compared to the untreated and sulphite containing extract. In general, the relative amount of the hydrophobic amino acids, i.e. isoleucine, leucine, tyrosine, phenylalanine and valine decreased after blanching in the

soluble extract. Likely, this decrease was due to aggregation of proteins rich in hydrophobic amino acid residues and subsequent precipitation (Aalbersberg et al. 2003). The larger proteins were mainly precipitated by blanching, as fewer high molecular weight bands for all three species were observed as shown in **Figure 5.2**.

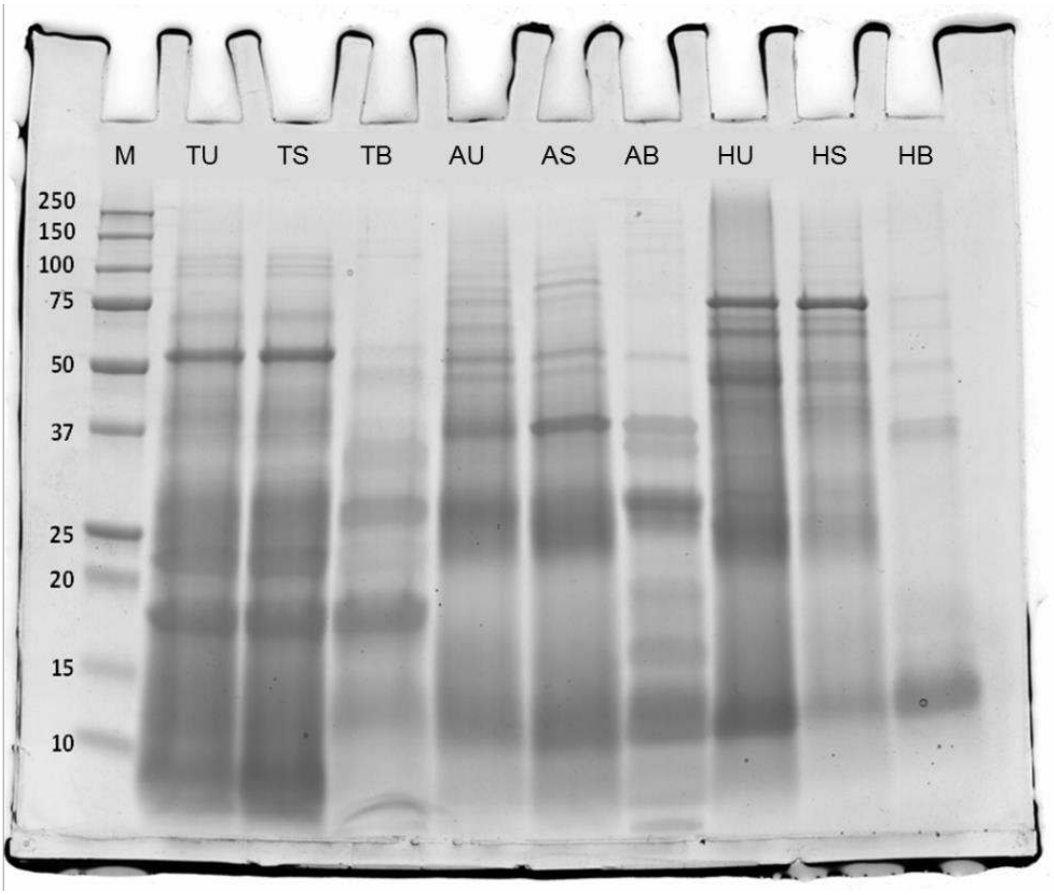


Figure 5.2. SDS-PAGE with marker in kDa (M) of different extracts from *Tenebrio molitor* (T), *Alphitobius diaperinus* (A) and *Hermetia illucens* (H) for three different extraction methods: untreated (U), sulphite (S) and blanched (B).

As the browning in the soluble protein extracts remained after dialysis, this indicated that the colour was part of larger soluble structures (>12-14 kDa). Apparently, quinones produced by PO reacted with proteins, or polymerized into melanins, forming larger structures, but eventually the solubility and size (based on the SDS PAGE) of the protein seemed unaffected. Reactivity of quinones towards amino acid groups has been shown before in plants and in a model systems with milk proteins (Prigent et al. 2007).

Table 5.1. Amino acid composition (g/100 g protein) and total amino acid (AA) content (w/w % dw) for sulphite treated and heat treated protein extracts of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. Values for untreated extracts of the three species are from Janssen et al. (2017). Asx: no separate analysis of Asp/Asn. Glx: no separate analysis of Glu/Gln (mean ±S.D., n=2).

	T. molitor **Untreated**	***T. molitor*** **Sulphite**	***T. molitor*** **Blanched**	***A. diaperinus*** **Untreated**	***A. diaperinus*** **Sulphite**	***A. diaperinus*** **Blanched**	***H. illucens*** **Untreated**	***H. illucens*** **Sulphite**	***H. illucens*** **Blanched**
His	2.61 (±0.00)	2.52 (±0.03)	2.26 (±0.03)	2.98 (±0.00)	2.81 (±0.03)	3.12 (±0.00)	3.64 (±0.01)	3.42 (±0.00)	3.65 (±0.08)
Ile	5.54 (±0.01)	5.61 (±0.00)	4.65 (±0.00)	5.04 (±0.01)	5.21 (±0.00)	4.45 (±0.00)	5.18 (±0.00)	5.30 (±0.01)	4.28 (±0.00)
Leu	9.36 (±0.02)	9.65 (±0.01)	8.80 (±0.00)	8.03 (±0.00)	8.31 (±0.04)	7.73 (±0.02)	7.99 (±0.00)	8.26 (±0.02)	5.88 (±0.04)
Lys	6.12 (±0.05)	6.16 (±0.04)	7.06 (±0.02)	8.42 (±0.02)	8.72 (±0.00)	10.28 (±0.07)	9.16 (±0.00)	9.29 (±0.01)	7.54 (±0.01)
Met	1.44 (±0.01)	1.38 (±0.02)	1.08 (±0.01)	1.68 (±0.02)	1.70 (±0.00)	1.19 (±0.01)	2.53 (±0.02)	2.55 (±0.01)	0.93 (±0.01)
Cys	1.92 (±0.01)	1.85 (±0.01)	2.31 (±0.01)	1.29 (±0.00)	1.52 (±0.00)	2.23 (±0.02)	1.32 (±0.01)	1.33 (±0.01)	1.27 (±0.04)
Tyr	5.01 (±0.03)	5.00 (±0.00)	3.50 (±0.00)	6.91 (±0.02)	5.53 (±0.05)	3.68 (±0.01)	6.28 (±0.00)	5.89 (±0.03)	4.97 (±0.01)
Phe	5.10 (±0.00)	5.10 (±0.03)	3.48 (±0.01)	5.76 (±0.01)	5.41 (±0.05)	3.14 (±0.01)	7.18 (±0.01)	6.80 (±0.10)	3.85 (±0.01)
Val	6.16 (±0.00)	6.27 (±0.00)	5.50 (±0.00)	5.50 (±0.03)	5.65 (±0.01)	4.45 (±0.00)	5.61 (±0.00)	5.58 (±0.00)	5.63 (±0.00)
Trp	n.d.	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Thr	5.85 (±0.02)	6.00 (±0.01)	6.13 (±0.02)	5.09 (±0.00)	5.28 (±0.01)	5.28 (±0.01)	4.95 (±0.00)	5.01 (±0.01)	5.06 (±0.00)
Ser	5.06 (±0.02)	5.24 (±0.01)	5.42 (±0.03)	4.54 (±0.00)	4.67 (±0.00)	4.75 (±0.01)	3.99 (±0.01)	4.14 (±0.01)	5.26 (±0.00)
Asx	14.29 (±0.13)	14.34 (±0.00)	14.07 (±0.02)	12.78 (±0.01)	13.11 (±0.06)	12.64 (±0.01)	12.56 (±0.06)	12.26 (±0.04)	11.91 (±0.04)
Glx	13.53 (±0.14)	12.87 (±0.06)	16.41 (±0.05)	14.85 (±0.06)	14.76 (±0.15)	16.87 (±0.12)	12.13 (±0.03)	12.15 (±0.20)	17.40 (±0.03)
Gly	4.25 (±0.00)	4.30 (±0.01)	4.30 (±0.01)	3.79 (±0.00)	3.89 (±0.02)	4.63 (±0.01)	3.88 (±0.01)	3.81 (±0.00)	5.17 (±0.04)
Ala	4.89 (±0.01)	4.98 (±0.03)	5.52 (±0.00)	4.43 (±0.00)	4.58 (±0.01)	5.86 (±0.01)	4.66 (±0.02)	4.77 (±0.00)	6.93 (±0.03)
Pro	4.80 (±0.06)	4.78 (±0.14)	5.34 (±0.02)	4.58 (±0.11)	4.60 (±0.03)	4.89 (±0.00)	4.38 (±0.01)	4.47 (±0.00)	5.67 (±0.10)
Arg	4.07 (±0.03)	3.96 (±0.03)	3.78 (±0.01)	4.25 (±0.01)	4.25 (±0.03)	3.95 (±0.04)	4.57 (±0.01)	4.97 (±0.02)	4.62 (±0.03)
Sum AA	**67.91 (±1.31)**	**67.19 (±0.36)**	**42.49 (±1.21)**	**72.74 (±0.82)**	**73.55 (±0.95)**	**49.24 (±0.33)**	**67.77 (±0.60)**	**68.01 (±0.21)**	**41.62 (±0.48)**

[a] n.d. is not determined.

Table 5.2. Total nitrogen (Nt), protein nitrogen (Naa), Naa/Nt ratio and nitrogen-to-protein conversion factors (Kp) of different protein extracts from *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* with inhibition by sulphite and blanching compared to untreated extract (Janssen et al. 2017) (mean ±S.D., n=2).

	Nt	Naa Asp-Glu	Naa Asn-Gln	Naa/ Nt (%)	N-Prot factor Kp
***T. molitor* untreated**	12.15 (±0.53)	10.31 (±0.26)	12.52 (±0.29)	83 < x < 103	5.59
***T. molitor* sulphite**	11.99 (±0.15)	10.19 (±0.06)	12.33 (±0.07)	85 < x < 103	5.60
***T. molitor* blanched**	8.18 (±0.32)	6.51 (±0.23)	8.01 (±0.28)	80 < x < 98	5.20
***A. diaperinus* untreated**	13.00 (±1.09)	11.09 (±0.11)	13.43 (±0.18)	85 < x < 103	5.59
***A. diaperinus* sulphite**	13.48 (±0.08)	11.28 (±0.19)	13.67 (±0.20)	84 < x < 101	5.46
***A. diaperinus* blanched**	9.46 (±1.14)	7.77 (±0.07)	9.46 (±0.08)	82 < x < 100	5.21
***H. illucens* untreated**	12.06 (±0.13)	10.52 (±0.11)	12.48 (±0.13)	87 < x < 103	5.62
***H. illucens* sulphite**	12.46 (±0.09)	10.63 (±0.04)	12.57 (±0.04)	85 < x < 101	5.46
***H. illucens* heated**	10.56 (±0.10)	6.61 (±0.09)	8.02 (±0.11)	83 < x < 101	5.24

From the amino acid composition of the three extracts, the nitrogen-to-protein factor was determined to be able to accurately determine the soluble protein yield based on nitrogen. The total nitrogen from amino acids was compared to the total nitrogen to determine nitrogen-to-protein conversion factors (**Table 5.2**). The average nitrogen-to-protein factor of 5.60 for insect extracts by Janssen et al. (2017a) for the untreated extract was not significantly different from the determined nitrogen-to-protein factor for extracts treated with sulphite. Blanching slightly decreased this factor by 7%, indicating the presence of nitrogen impurities. As this factor was only slightly lower, the average Kp value of 4.76 for the whole larvae and 5.60 for the extracts were used to determine the soluble protein yield based on nitrogen as described before (Janssen et al. 2017a).

The soluble protein yield calculated from the nitrogen content in **Figure 5.3** was similar for the untreated and PO inhibited extracts using sulphite, indicating that the difference in phenoloxidase activity (**Figure 5.1)** did not affect solubility. The soluble protein yield decreased approximately four times after blanching, due to aggregation and precipitation as described above.

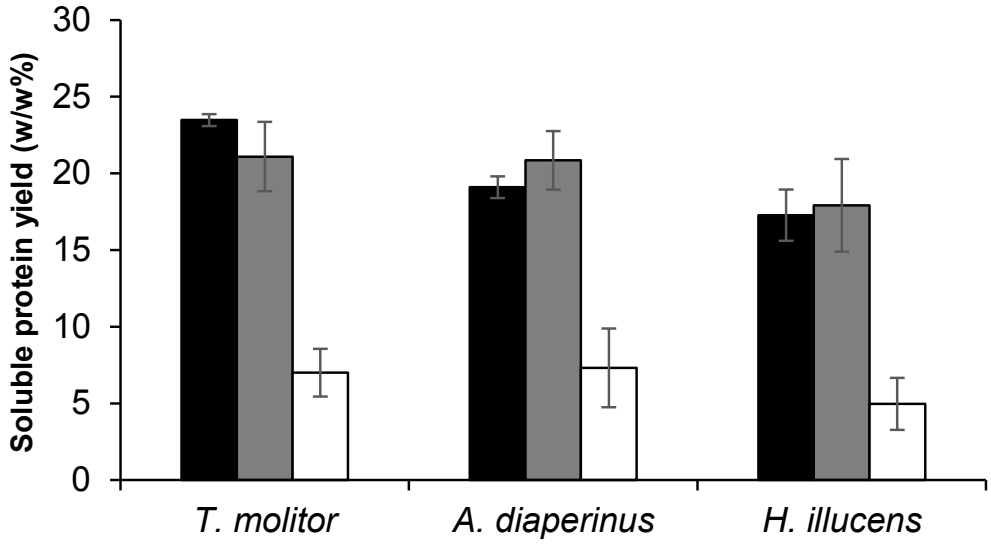


Figure 5.3. Soluble protein yield from the larvae after extraction (n=2) for *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* using 3 different extraction methods: sulphite (grey) and blanched (white) compared to untreated extract (black) (Janssen et. al. 2017a).

5.3.3. Digestibility of protein extracts from different insect species

The *in vitro* digestion in **Figure 5.4** showed the highest degree of hydrolysis for *H. illucens* compared to that of the other two species. This was mainly caused by higher susceptibility of *H. illucens* to trypsin hydrolysis and not to differences in pepsin hydrolysis. The higher digestibility for *H. illucens* compared to *T. molitor* meal was also found *in-vivo* digestion in broiler chickens (De Marco et al. 2015).

Pepsin digestion in **Figure 5.4** of the sulphite treated extract of *A. diaperinus* was similar to the respective untreated extract, whereas the sulphite containing extract from *T. molitor* and *H. illucens* showed a significantly higher DH (P<0.05) compared to their untreated extract indicating a negative effect of browning on pepsin digestibility. It was shown in literature that the effect of enzymatic browning was dependent on the type of protein and phenolic (Kroll et al. 2003). Differences in pepsin digestibility between these species might be due to browning reactions, as both *T. molitor* and *H. illucens* had a darker colour compared to *A. diaperinus* (**Figure 5.1**).

Trypsin hydrolysis in **Figure 5.4** performed after pepsin hydrolysis was similar for untreated and sulphite containing extracts from *H. illucens* and slightly higher for the untreated extract than sulphite treated extract of *T. molitor* and *A. diaperinus*. This higher hydrolysis by trypsin suggested that trypsin was not affected by enzymatic browning. Some studies suggest that protein become water-insoluble after the action of phenoloxidase (Hurrell & Finot 1982), but this was not the case in this study as the proteins were soluble. It is unclear whether the product of the enzymatic browning reaction are covalently bound to proteins or melanins which can non-covalently bind to proteins. Differences in effect of enzymatic browning on trypsin and pepsin hydrolysis might be due to the specificity of the protease.

Blanching decreased the pepsin hydrolysis significantly (P<0.05) for *T. molitor* and *H. illucens* and was similar for *A. diaperinus* compared to the respective untreated extracts. Higher trypsin hydrolysis was found for *T. molitor* and *A. diaperinus* compared to the respective untreated extracts, whereas lower trypsin hydrolysis was found for *H. illucens*. Differences in extent of denaturation of the insect proteins might explain the variation in susceptibility towards hydrolysis by endogenous proteases. This is similar to hydrolysis of fish and soymeal proteins, where intermediate heating gave higher digestibility compared to unheated or extensively heated proteins (Adler-Nissen 1986).

Our research focused on the effect of enzymatic browning and prevention by sulphite and blanching. No studies have been investigating the effect of enzymatic browning and thermal treatment. Therefore, the DH% with pepsin and trypsin digestion was performed for only two h each and total digestibility was determined. In other studies, digestibility of *T. molitor*, *A. diaperinus* and *H. illucens* has been investigated in feeds for livestock such as fish (Sánchez-Muros et al. 2016) and broiler chicken (De Marco et al. 2015), but also for cat and dog feed (Bosch et al. 2014). One of these studies reported that the *in vitro* protein breakdown in *T. molitor* (91 %) and *A. diaperinus* (91 %) was comparable to soybean meal (94 %) and better compared to fish meal (85 %) and poultry meat meal (87 %) (Bosch et al. 2014). Studies reported that digestibility of soluble proteins was higher compared to insoluble proteins extracted from *T. molitor* and in presence of chitin (Sánchez-Muros et al.

2016; Bosch et al. 2014; Yi et al. 2016). As this study only focused on the soluble proteins, this was likely better digestible compared to the whole larvae including insoluble proteins and chitin. Taken together, the soluble proteins from insect larvae are highly digestible based on the presented data and literature. In this research, we showed that phenoloxidase has a negative effect on the pepsin digestion and that blanching can also play a role in this.

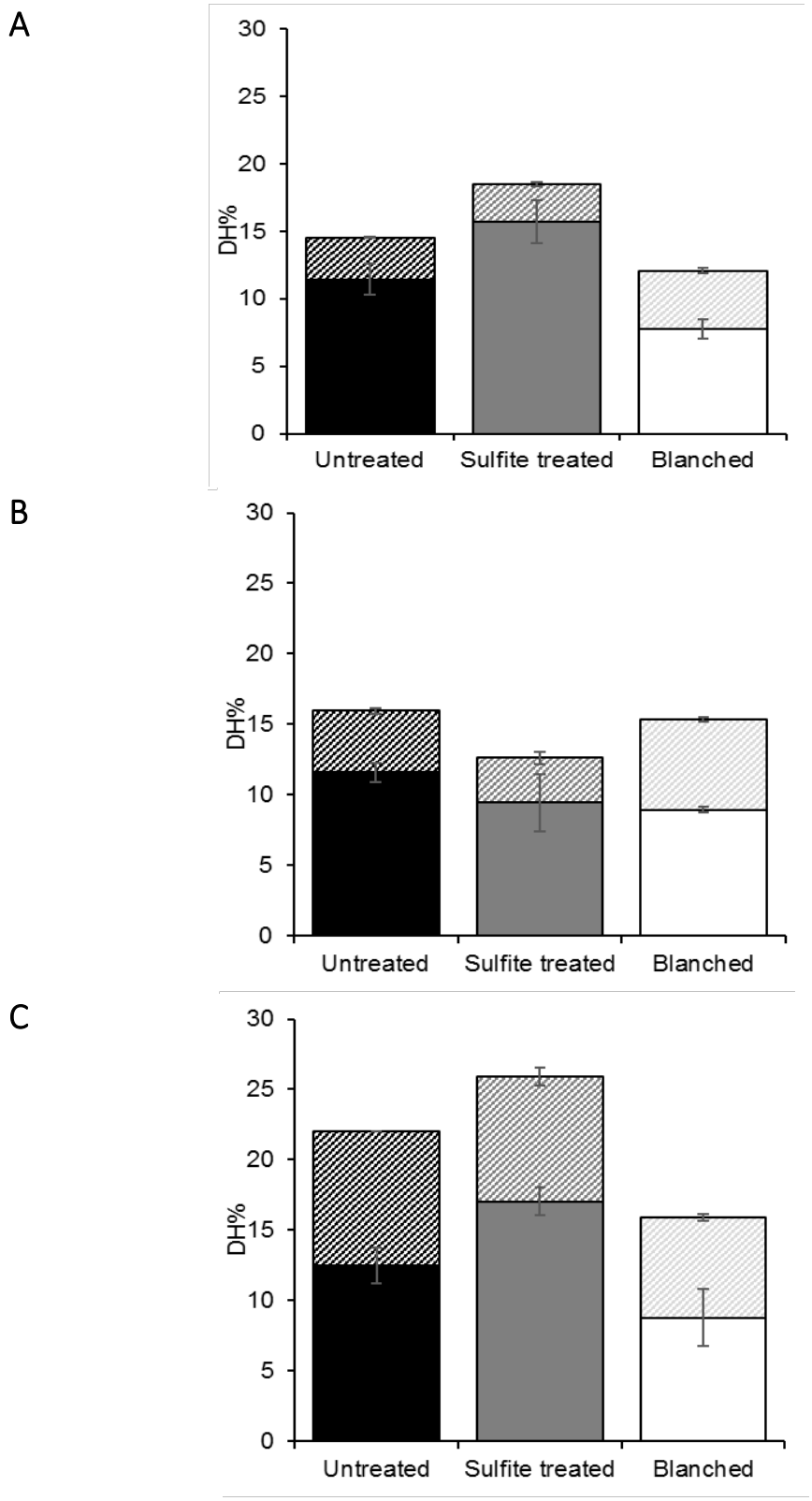


Figure 5.4. Degree of hydrolysis during pepsin (pH 3, filled) and trypsin digestion (pH 8, striped) for *Tenebrio molitor* (A), *Alphitobius diaperinus* (B) and *Hermetia illucens* (C) soluble protein extracts: untreated (black), sulphite (grey) and blanched (white).

5.3.4. Endogenous protease activity in protein extracts

When the extracts were solubilized at pH 8, endogenous protease activity appeared to be present in the solution obtained for all three species as shown by %DH (**Figure 5.5**). This protease activity is most likely explained by the insect's digestive enzymes, as the whole larvae were used during protein extraction.

Chymotrypsin and trypsin are the major proteases present in *T. molitor* (Tsybina et al. 2005) and resemble vertebrate trypsin and chymotrypsin (Terra & Ferreira 1994). Also trypsin-like proteases have been observed in *H. illucens* (Kim et al. 2011) and non-digestive proteases e.g. serine proteases are also present in the haemolymph (Kanost & Clem 2012). Trypsin-like proteases in insects are active at alkaline pH values, with pH optima 8-10, similar to insect's midgut pH (Tsybina et al. 2005). Insect trypsin and chymotrypsin differ from vertebrate trypsin and chymotrypsin in being unstable at acidic pH (Terra & Ferreira 1994). This has also been found for trypsin in *T. molitor* before (Tsybina et al. 2005). During the digestion experiments in **Figure 5.4**, the endogenous enzyme activity was not present, as endogenous trypsin-like proteases were irreversibly inactivated at acidic pH (Terra & Ferreira 1994).

Similar protease activity was found for untreated and sulphite treated extract for *T. molitor* and *H. illucens*, which was expected as sulphite is a phenoloxidase inhibitor and not a protease inhibitor (Kuijpers et al. 2012). Unexpectedly, a significant inhibition of endogenous proteases by sulphite was observed in the *A. diaperinus* extract. Furthermore, blanching decreased endogenous protease activity for *T. molitor* and *A. diaperinus*, whereas proteases were activated in *H. illucens* upon blanching. Apparently, the mild blanching conditions were not enough to inactivate proteases. Variability in protease activity was found between extractions and among different batches (data not shown).

The degree of hydrolysis in 2 h at 37 °C and pH 8 was between 0.5−4% is shown in **Figure 5.5**. Taking into account the specificity of trypsin towards arginine and lysine, the theoretical maximum DH varies between 10−13% for the different extracts. This indicates that within two hours 4−40% of the total cleavable bonds was already digested by the endogenous trypsin at pH 8. Multiple protein extractions from insects have been performed at pH above 7, because of the high protein solubility at that pH (Yi et al. 2013; Purschke et al. 2018; Bußler et al. 2016). However, these are also conditions were protease activity remains very high after protein extraction. Likely, this endogenous protease activity can degrade protein in extracts when used or extracted at high pH, thereby changing the properties of the constituent proteins.

In summary, the soluble protein yield and composition were not modified by phenoloxidase activity, whereas blanching decreased the soluble yield due to protein aggregation and

precipitation. The highest protein digestibility was found for *H. illucens* compared to *A. diaperinus* and *T. molitor*. Enzymatic browning during extraction decreased pepsin digestion for *T. molitor* and *H. illucens*, but not for *A. diaperinus*, whereas trypsin digestion was less affected by enzymatic browning. In this research, we have shown that endogenous phenoloxidase and proteases remain active after extraction. Endogenous proteases can cause a high degree of protein hydrolysis, which might significantly modify the techno-functionality of protein extracts. Moreover, differences in proteases activities between the three species suggesting that specific strategies should be developed to control the proteolytic activity depending of the insect species and on the final destination as food ingredient.

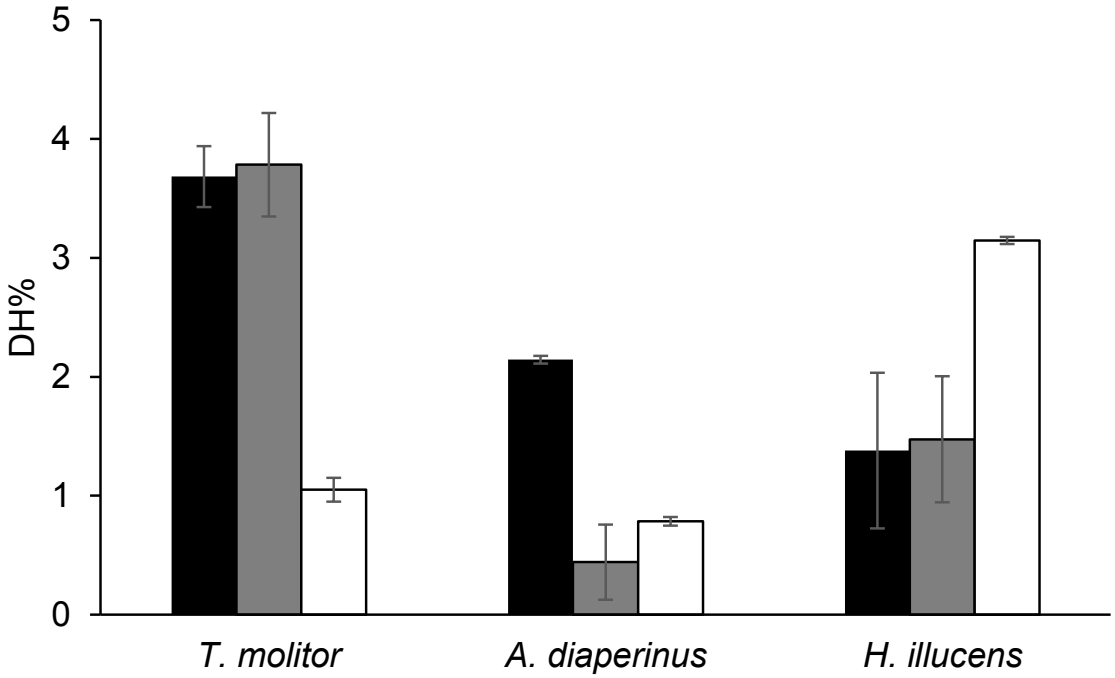


Figure 5.5. Endogenous protease activity (presented as degree of hydrolysis (DH)) in extracts from *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* for three different extraction methods: untreated (black), sulphite treated (grey) and blanched (white) at pH 8 (n=3).

6

General discussion

6. GENERAL DISCUSSION

Insects have been identified as an excellent alternative source of proteins, with protein contents of 40-75% of dry matter (Bukkens 1997). This has led to an increasing interest in insects as an alternative protein source. Most research describing the protein content of insects had focused on the crude protein content determined by the general nitrogen-to-protein conversion factor (Kp) of 6.25 (Bukkens 1997; Yi et al. 2013; Zhao et al. 2016; Rumpold & Schlüter 2013a; Finke 2013; Finke 2002), whereas specific Kp's for insect proteins still needed to be determined to calculate protein contents more accurately. It had also been shown that browning occurred upon grinding of certain insect species, which could hamper its potential as a food or feed ingredient by altering techno-functional and nutritional properties. Yet, the dark colour formation in insects during processing had not been investigated and little was understood about how it can be mitigated. Therefore, this thesis aimed to investigate the potential of insects as protein source, the mechanisms responsible for the browning or blackening of insects, and its impact on protein functionality.

In **Chapter 2**, a specific nitrogen-to-protein conversion factor for accurate protein quantification was determined based on nitrogen content and amino acid composition for *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. The enzymes responsible for browning were elucidated in insects (**Chapter 3**) and iron-phenolic complexation was identified as driver for blackening of *H. illucens* (**Chapter 4**). Their subsequent effects on protein digestibility and solubility were investigated for the larvae of the three species (**Chapter 5**). The present chapter provides a broader outlook on the potential of insects as new protein source for food and feed. The effects of the nitrogen-to-protein conversion factors and the role of endogenous enzymes together with their associated reactions are discussed in relation to a species' potential as a novel protein source.

6.1. Nitrogen-to-protein conversion factor (Kp) in insects

Insects have a high protein content, which makes them an interesting novel protein source. Yet, this is mainly based on calculations using the total nitrogen content. The total nitrogen content is a common way to determine the protein content. However, the general Kp factor of 6.25 tends to overestimate the protein content in insects due to the presence of nonprotein nitrogen. Chitin for example has glucosamine or *N*-acetylglucosamine as building blocks, both of which contain nitrogen. This nitrogen should not be accounted for upon quantification of proteins. In **Chapter 2**, the specific Kp factors of 4.76 and 5.60 were determined respectively for the whole larvae and protein extracts of the larvae from the

three insect species: *T. molitor*, *A. diaperinus* and *H. illucens*. These Kp factors enable the calculation of a more accurate protein content compared to the general factor.

6.1.1. Comparison Dumas and Kjeldahl for total nitrogen determination

Kjeldahl is routinely used for analysis of total nitrogen but the Dumas method is more desirable as measurements require less time and no chemicals. Whereas previous research suggested slightly lower total nitrogen by Kjeldahl than by Dumas depending on the food matrix analysed (Thompson et al. 2002; Jung et al. 2003), **Table 6.1** showed that total nitrogen determined by both methods was similar (P>0.05, t-test). Thus, the Kp factor determined by us is also valid for analyses done using the Kjeldahl method.

Table 6.1. Comparison of % total nitrogen between Dumas (n=3) and Kjeldahl (n=2) for whole larvae (*Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*), based on % dry matter (±S.D.).

Species	%Nt Dumas	%Nt Kjeldahl
Tenebrio molitor	9.98 ± 0.27	9.57 ± 0.27
Alphitobius diaperinus	10.47 ± 0.17	10.14 ± 0.10
Hermetia illucens	7.92 ± 0.06	7.87 ± 0.02

6.1.2. Nitrogen-to-protein conversion factor (Kp) for other insect species

The Kp factor of the three species used in this thesis was compared to literature data of the same species and extended to others. Therefore, calculations were performed based on a comparison of total nitrogen, calculated from the crude protein content, and the protein nitrogen based on the amino acid analysis as shown in **Table 6.2**.

Table 6.2. Total nitrogen (Nt), protein nitrogen (Naa), the Naa/Nt ratio and nitrogen-to-protein conversion factors (Kp) of *Tenebrio molitor*, *Alphitobius diaperinus*, *Hermetia illucens*, *Zophobas morio*, *Acheta domesticus*, *Blaptica dubia* and *Bombyx mori*. The calculations were performed on amino acid composition and crude protein from literature. The lower limit was calculated on the basis of 100% Asp/Glu and the upper level with 100% Asn/Gln. Grey shading highlights species investigated in Chapter 2.

	Naa Asp/Glu	Naa Asn/Gln	Nt (%)	Naa/Nt Asp/ Glu	Naa/Nt Asn/ Gln	Kp	% protein based on Kp	% protein based on 6.25	% difference
Larvae									
T. molitor [a]	7.20	8.31	9.41	77	88	4.75	44.8	58.8	14.0
T. molitor [b]	7.21	8.33	9.23	78	90	4.87	44.9	57.7	12.8
T. molitor [c]	6.58	7.54	7.85	84	96	5.49	41.5	49.1	7.6
T. molitor [d]	6.23	7.20	8.34	74	87	4.58	38.1	52.0	13.9
A. diaperinus [a]	7.84	9.13	10.21	77	89	4.86	48.6	63.8	15.2
A. diaperinus [b]	7.50	8.74	9.74	79	92	4.97	47.1	59.2	12.1
A. diaperinus [f]	8.00	9.21	10.86	74	85	4.60	49.9	67.9	18.0
H. illucens [a]	5.71	6.72	7.70	74	87	4.67	36.7	48.1	11.4
H. illucens [d]	6.08	7.18	8.98	68	86	4.17	37.4	56.1	18.7
Z. morio [b]	5.99	6.99	7.54	79	93	4.99	37.6	47.1	9.5
B. mori [c]	5.39	6.30	8.60	62	73	3.82	32.9	53.8	20.9
Adult stage									
A. domesticus [b]	8.52	9.83	11.25	76	87	4.61	51.9	70.3	18.4
A. domesticus [c]	8.49	9.78	10.65	80	92	4.90	52.2	66.6	14.4
A. domesticus [d]	8.27	9.58	11.30	73	85	4.42	49.9	70.6	20.7
T. molitor [c]	7.88	8.99	10.45	75	86	4.56	65.3	47.7	17.6
B. dubia [b]	6.06	7.00	9.06	67	77	4.15	37.6	56.6	19.0
Effect of feed *H. illucens* pre-pupae									
Chicken feed [f]	4.89	5.71	6.59	74	87	4.46	41.2	29.5	11.7
Digestate [f]	4.88	5.64	6.75	72	83	4.34	42.2	29.4	12.8
Vegetable waste [f]	4.71	5.51	6.38	74	86	4.45	39.9	28.5	11.4
Restaurant waste [f]	5.07	5.92	6.90	73	86	4.44	43.1	30.7	12.4

[a] Results **Chapter 2** (Janssen et al. 2017).
[b] Results based on data Yi et al. (2013).
[c] Results based on data Finke (2002).
[d] Results based on data Bosch et al. (2014), data without proline and tryptophan.
[e] Results based on data Despin & Axtell (1994).
[f] Results based on data Sprangher et. al. (2016), data was without tyrosine.

Up to 26% of the total nitrogen can be explained by nonprotein nitrogen as found in **Chapter 2** and this value can be even higher for some other species. The results of the larvae of the three species from **Chapter 2** were similar to the values based on literature (**Table 6.2**), only *T. molitor* showed a higher Kp based on data of Finke (2002). Values of Bosch et al. (2014) were slightly lower due to missing data of the amino acids proline and tryptophan. The Kp factor in **Chapter 2** has been used for *Acheta domesticus* (house crickets) by David-Birman et al. (2017) and Sipponen et al. (2017). Calculations based on the literature data for *A. domesticus* showed comparable Kp factors to the factor described for the three species. Hence, this extrapolation of our Kp towards *A. domesticus* is valid. Lower Kp's were found for species *Blaptica dubia* (cockroach) and *Bombyx mori* (silkworm) possibly due to the higher content of nonprotein nitrogen, for example from chitin. Overall, **Table 6.2** showed that all Kp factors were significantly lower ($P<0.05$) compared to the general Kp factor of 6.25 and comparable to the value of 4.76 as determined in **Chapter 2**. This lower factor does not only apply to insects in the larval stage, but also to those in the adult stage.

Spranghers et al. (2017) reared *H. illucens* pre-pupae using different substrates as feed. Based on the data from this study, the Kp factors were determined in **Table 6.2**. As the amino acid tyrosine was not determined, the Kp factor and thereby the protein content will be slightly underestimated in the table. The small variations in Kp factor indicated the limited effect of feed on the protein content of the pre-pupae. So, the Kp factor described in **Chapter 2** of this thesis gives a more accurate estimation compared to the general factor when the amino acid composition of the species is unknown.

6.1.3. Different nitrogen-to-protein conversion factors (Kp) for commercial insect protein extracts and flours

To lower the barriers for eating insects and increase the acceptance of consumers, multiple insect based products are commercially available as insect flour or protein powders. As shown in **Table 6.3**, the study of Churchward-Venne et al. (2017) only compared between different powders based on the crude protein content (based on 6.25) and no specific Kp factor was taken into account. The extent of overestimation of the protein content depends on the protein purification leading to removal of nonprotein nitrogen.

Table 6.3. Total nitrogen (Nt), protein nitrogen (Naa) with unknown Asp/Asn, the Naa/Nt ratio of commercially available edible insect products. From this, the nitrogen-to-protein conversion factors (Kp) and protein content based on 6.25 and Kp was determined. The calculations were done on the data of Churchward-Venne et al. (2017). The Kp and subsequently the protein content will be slightly underestimated, due to unknown asparagine/aspartic acid content.

	Naa Glu	Naa Gln	Nt (%)	Naa/Nt Gln	Naa/Nt Glu	Kp	% Protein based on 6.25	% Protein based on Kp	% Difference
Cricket protein powder 2050 (*A. domesticus*)	9.9	10.9	10.4	95	105	5.54	65.0	57.6	7.4
Cricket flour (*A. domesticus*)	9.6	10.5	9.2	105	114	6.13	57.5	56.4	1.1
Silkworm flour (*B. mori*)	8.7	9.5	8.6	101	111	6.02	53.8	51.8	2.0
Cricket flour (*G. bimaculatus*)	8.0	8.8	9.5	85	93	4.90	59.4	46.6	12.8
Locust flour (*L. migratoria*)	9.9	10.8	11.1	89	97	5.19	69.4	57.6	11.8
Mealworm protein powder 2050 (*T. molitor*)	9.0	10.0	9.3	97	107	5.79	58.1	53.9	4.2
EntoPure sports protein concentrate (*A. diaperinus*)	7.8	8.7	11.2	70	78	4.13	70.0	46.2	23.8

Insects are marketed for promoting human growth as well as for maintaining and increasing muscle mass based on their high protein content. In order to achieve this, protein dense sources are desired. By comparing different insect flours and protein powder (**Table 6.3**), it can be seen that protein content varies between type of insect product, and that likely different extents of purification of proteins affected the accuracy of the protein calculated. Minor differences in protein content were found between the general Kp factor 6.25 and specific Kp for cricket flour and silkworm flour, suggesting lesser nonprotein nitrogen. Major difference of 23.8% was found for EntoPure sports concentrate, which was made by defatting ground larvae without removing chitin (Proti-Farm 2017).

To use protein powders for nutritional purpose, only true protein content should be taken into account. The specific Kp factor excludes nonprotein nitrogen from the crude protein content, which gives a more fair comparison and accurate protein content. This can lead to different choice of protein powder.

6.1.4. Protein content of various dietary sources

Insects are considered as alternative protein source for conventional animal proteins. A comparison between the total protein content of insects as determined in **Chapter 2** and different animal sources is shown in **Table 6.4**. This was done using their specific Kp factors for different sources as determined by Sosulski & Imafidon (1990). These results show that applying the respective Kp factors gives lower protein contents in insects than meat, but still higher than in dairy products.

Table 6.4. Average nitrogen-to-protein conversion factors (Kp) of insects compared to the Kp factor of other proteins sources (Sosulski & Imafidon 1990) and its effect on the protein content based on dry matter.

	Kp	Nt (%)	% Protein based on 6.25	% Protein based on Kp
Insect larvae	4.76	6.7-9.1	48.1-63.8	36.0-49.6
Meat (beef and chicken), fish and eggs	5.6	7.8-12.9	48.4-76.5	44.4-70
Milk/dairy products	5.85	4.1-6.44	25.9-40.3	24.9-39.5

The lower total protein content decreases the potential of insects as alternative protein source, even though the protein content is still high.

An adult needs between 50-61 gram of protein a day with a mixed diet including meat (Health Council of The Netherlands 2001). The daily intake in The Netherlands was

estimated to be around 80 g per person/day (Health Council of The Netherlands 2001), so lower protein content is not an issue for the Dutch adult. Yet, this lower protein content in insects is highly relevant for developing countries, as up to 26% more insects are necessary to combat protein malnutrition.

The total protein content is a starting point for comparing sources. Using the Kp factor of 4.76 therefore provides a more accurate value of protein content based on nitrogen for whole or ground insect species when the amino acid composition is not available. This means that the total protein content is significantly lower than the reported crude protein content. After extraction, this factor changes and is dependent on the degree of purification and removal of nonprotein nitrogen.

However, when assessing the potential of insects as food and feed, also other factors should be taken into account. Insects have relatively less environmental impact as they use less land, water and emit fewer greenhouse gasses compared species producing to milk protein and meat proteins (Oonincx & de Boer 2012; Oonincx et al. 2010). Besides, other factors should be taken into account, such as essential amino acids composition, digestibility, and techno-functional properties.

6.2. Techno-functional properties of insect proteins

Research on alternative proteins sources usually focussed on the nutritional value of the proteins (Kinsella & Melachouris 1976). However, the application of insects as alternative protein source depends not only on the nutritional value of the proteins, but also on their functional properties. Endogenous enzymes are present in insects and can be active upon extraction, potentially hampering protein functionality. We showed that endogenous phenoloxidases are important for browning upon grinding (**Chapter 3**) and endogenous proteases remained active after protein extraction (**Chapter 5**). Thus, the implications of endogenous enzymes are important to consider, and the effect on functional properties will be discussed. Besides endogenous enzymes, the pH is known to largely affect protein properties, like solubility, and is therefore also taken into account.

6.2.1. Solubility of insect proteins

Solubility is an important aspect of proteins as it is necessary for many techno-functional properties, like foaming, emulsification and gelation (Boland et al. 2013; Kinsella & Melachouris 1976). The pH is known to have a large effect on protein solubility (Aalbersberg et al. 2003). The zeta potential was determined to predict the effect of pH on protein solubility as shown in **Figure 6.1A**. From this graph, the apparent pI of all proteins together was determined at zero net charge. The zeta potential for *A. diaperinus* and *H. illucens*

reached zero charge around pH 4.9, whereas for *T. molitor* the zeta potential was zero around pH 4. Similar results were found for dried defatted *T. molitor* by Sipponen et al. (2017) , whereas a zero zeta potential around pH 3.5 for soluble *T. molitor* protein was found by Azagoh et al. (2016).

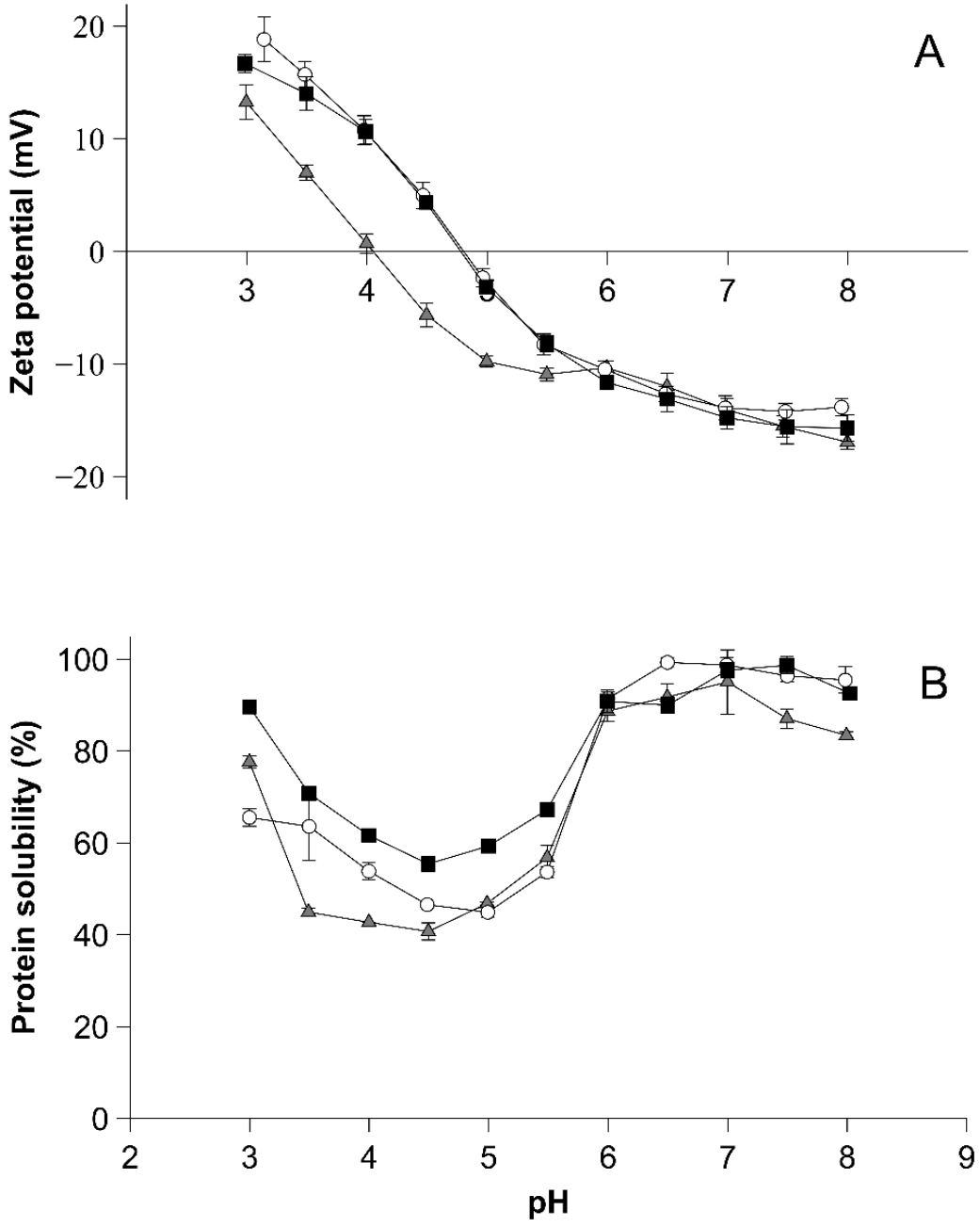


Figure 6.1. A: Effect pH on zeta potential (ζ) of *Tenebrio molitor* (triangle), *Alphitobius diaperinus* (square) and *Hermetia illucens* (circle). The point where the line crosses the x-axis represents the apparent pI of the protein solution (prepared in MilliQ water). B: Effect pH on solubility of proteins. The highest solubility was considered 100% of the respective species. The solubility test was based on Schwenzfeier et al. (2011). Error bars represent standard deviation (n=3).

Zeta-potential values (either positive or negative) at the extremities of the curve indicate in general more electrostatic repulsion, thereby increasing thereby the solubility of the proteins. On the other hand, the solubility of proteins around the apparent pI was the lowest as little repulsion occurs. This is in agreement with the solubility curve shown in **Figure 6.1B**. Minimal solubility of the proteins was found between pH 4 and 5. Highest solubility was found at lower and higher pH.

Phenoloxidases did not significantly change the solubility of the extracts (**Chapter 5**) at pH 6, where the highest enzyme activity was found (**Chapter 3**). Higher solubility was found at increasing pH values, which supports the use of alkaline pH during extraction and/ or processing. On the other hand, also endogenous proteases are active at high pH (**Chapter 5**). Hydrolysis of proteins can change the solubility. The solubility of soy proteins was for example enhanced after hydrolysis (Tsumura et al. 2005).

So, the highest solubility as desired for techno-functional properties can be achieved at alkaline conditions, whereas phenoloxidase or the origin of the proteins did not affect this property (**Chapter 5**). Proteases have an effect at alkaline pH, but whether this is positive or negative should be studied.

6.2.2. Gelation

Besides solubility, gelation is an important protein functionality that plays a role in many dairy products like yoghurt, as well as eggs, sausages and tofu. For feed, gelation is for example important in wet pet food. Preliminary experiments investigating gelation characteristics of proteins from *T. molitor*, *A. diaperinus* and *H. illucens* at different pH values showed that gelation ability at all pH values was lost after freeze-drying protein extracts (data not shown). Hence, only frozen larvae were used for gelation experiments. Different extracts were prepared from larvae blended in MilliQ water adjusted to the desired pH: (i) Mixture was used completely including for example chitin particles, (ii) Mixture was sieved through a cheesecloth to remove larger chitin particles and (iii) soluble part after centrifugation. Insect extracts with a similar protein content were heated at 86 °C for 30 minutes in a tube, then turned upside down to determine if a gel had been formed (Yi et al. 2013) as shown in **Table 6.5**.

Table 6.5. Gel formation of protein fractions from *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* at different pH values based on visual observation. Whole larvae were blended in MilliQ, and from this three extracts were prepared: (i) Whole larvae were used completely including for example chitin particles. (ii) Mixture was sieved through a cheesecloth to remove larger chitin particles, and (iii) soluble part after centrifugation. The protein concentrations were adjusted to 2.6% w/w and gelation was determined after heating for 30 min at 86 °C. G = gel, WG = weak gel and X = no gel formation.

		pH 3	pH 5	pH 7	pH 7 + HSO_3^-	pH 7 + PPO	pH 10
	Colour	Light yellow	Light brown	Dark brown	Light yellow	Dark brown	Dark brown
T. molitor	Soluble protein	G	X	X	X	X	X
	Filtrate cheesecloth	WG	n.d.	G	n.d.	n.d.	n.d.
	Whole larvae	G	X	G	G	n.d.	X
A. diaperinus	Soluble protein	G	X	X	X	G	X
	Filtrate cheesecloth	WG	n.d.	G	n.d.	n.d.	n.d.
	Whole larvae	G	X	WG	WG	n.d.	X
H. illucens	Soluble protein	X	X	X	X	n.d.	X
	Whole larvae	X	X	X	X	n.d.	X

n.d. not determined.

H. illucens did not form a gel under any of the conditions tested, also not when the protein concentration was doubled (data not shown). Gels were formed at pH 3 and pH 7 under certain conditions for *T. molitor* and *A. diaperinus*. A different effect of pH on gelation of soluble proteins was shown before, as Yi et al. (2013) found gelation at pH 7 and 10 when 30% w/v soluble fractions were used. It is hypothesized that this difference in gelation could be due to proteolytic activity upon storage. Preliminary results showed that proteolytic activity led to visually weaker gels when mixing a protein solution at pH 7 for longer time before gelation, as well as when extra trypsin was added (data not shown).

Phenoloxidase activity on the other hand seems to strengthen gels in some cases based on visual observation, likely due to crosslinking of proteins. This was shown for example by addition of PO to soluble proteins from *A. diaperinus* at pH 7.

6.3. Nutritional value of insects

Insects are mostly of interest due to their good nutritional composition, like a high protein content, source of micronutrients and a good fatty acid composition (Tzompa-Sosa et al. 2014; Rumpold & Schlüter 2013a). Digestibility of proteins (**Chapter 5**) and presence of essential amino acids are important for the nutritional value and were compared between the three species: *T. molitor*, *A. diaperinus* and *H. illucens*. Besides proteins, **Chapter 4** has indicated that iron plays role in off-colour formation. The effect of iron-phenolic complexes on the bioavailability of iron was postulated.

6.3.1. Essential amino acids and digestibility of insect proteins

The nutritional value of insect proteins depends on their digestibility, as well as on their amino acid composition. Studies on *T. molitor* reported that digestibility of soluble proteins was higher than that of insoluble proteins and that in presence of chitin (Sánchez-Muros et al. 2016; Bosch et al. 2014; Yi et al. 2016). In **Chapter 5**, we showed that the *in vitro* pepsin digestibility of soluble insect proteins was negatively affected by endogenous phenoloxidase. Thus, it would be desired to prevent this endogenous activity.

Besides digestibility, the amino acid composition is a relevant parameter for nutritional protein quality. In particular, the essential amino acids are relevant, as they cannot be synthesized in the human body and should be absorbed from food (WHO/FAO/UNU Expert Consultation 2007). The amino acid composition of the three species was determined in **Chapter 2** and the content of essential amino acids is compared to the recommended intake value for adult according to the WHO/FAO/UNU (2007) in **Table 6.6**.

Table 6.6. Essential amino acid (EAA) composition (g/100 g protein) for whole *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*, as described in Chapter 2, compared to the WHO/FAO/UNU (2007) recommendation for adults WHO/FAO/UNU.

	T. molitor	*A. diaperinus*	*H. illucens*	WHO reference
His	3.56	3.97	3.85	1.5
Ile	4.99	4.61	4.59	3.0
Leu	8.33	7.32	7.45	5.9
Lys	6.14	7.05	6.91	4.5
Met	1.52	1.59	2.00	1.6
Cys	1.13	0.96	0.97	0.6
Tyr	5.80	8.49	6.54	3.8
Phe	3.64	5.17	4.49	
Val	6.42	5.76	6.1	3.9
Trp	1.50	1.47	1.87	0.6
Sum EAA	43.03	46.39	44.77	27.7

All essential amino acids are present in sufficient quantities according to the WHO reference values. Likely, sulphur-containing amino acids, methionine and cysteine will be the first limiting amino acids. The total essential amino acid content is between 43.0-46.4 g/100 g, which is higher than the requirement of 27.7 g/100 g from the WHO (WHO/FAO/UNU Expert Consultation 2007). This indicates that all three species are a good source for essential amino acids.

6.3.2. Bioavailability of iron

Multiple insect species have been indicated as a good source of iron (Bukkens 1997; van Huis et al. 2013). Iron is an important micronutrient as iron deficiency is the most common nutritional disorder in the world (FAO & WHO 2001). Insects could thus be a source to decrease iron deficiency. However, to combat iron deficiency, iron needs to be bioavailable. Hence, it is important to know whether iron from insects is bioavailable and how processes like iron complexation (**Chapter 4**) affect this bioavailability.

The iron bioavailability *in vitro* for *T. molitor* and *A. diaperinus* was compared to that of sirloin beef (Latunde-dada et al. 2016). Iron content per 100 g dm of *T. molitor* (6.1 mg) and *A. diaperinus* (5.4 mg) as shown, in **Chapter 4**, was lower than that of sirloin beef (15.5 mg)

(Latunde-dada et al. 2016), whereas *H. illucens* had a higher iron content of 22.1 mg dry matter. The iron bioavailability of *A. diaperinus* was higher compared to that of sirloin beef or $FeSO_4$ (Latunde-dada et al. 2016).

Iron from animal source is general more bioavailable compared to plants, due to the presence of haem iron. The general range of iron bioavailability was estimated in a range between 14-18% in a mixed diet and only 5-12% for vegetarian diets (Hurrell & Egli 2010). Insects are considered of animal origin (Belluco et al. 2013), which makes it likely that the iron availability is higher compared to that in vegetables.

Nevertheless, other dietary factors play a role in iron bioavailability, like phytate, polyphenols, calcium and ascorbic acid (Hurrell & Egli 2010). Polyhenols have been shown as potent inhibitor of iron absorption in a study using beverages, as the released phenolics can form a complex with iron in the intestinal tract, making it unavailable for absorption (Hurrell et al. 1999). This study also indicated that the oxidized phenolics in black tea were a more potent inhibitor compared to non-oxidized phenolics from herb teas (Hurrell et al. 1999). Black tea is oxidized by endogenous tyrosinase, similar to PO in insects. Hence, PO oxidation might decreases the iron availability in ground insects. Besides oxidation, also the oxidative state of iron can have an effect on the bioavailability. **Chapter 4** showed that the iron was present in the ferric form in the complexes. Ferric iron has a lower bioavailability compared to the ferrous from (Habeych et al. 2016).

Prevention of discolouration in iron fortified products could be achieved by using poorly soluble iron, yet this also decreases the solubility in the gastric and thereby its bioavailability (Habeych et al. 2016). **Chapter 4** showed that no iron-phenolic complexes were present at pH 3 in the model system of L-DOPA and iron. To check the reversibility of these complexes, *H. illucens* soluble extracts were prepared at different pH values and adjusted using NaOH or HCl to mimicking conditions in the gastro-intestinal tract (**Figure 6.2**). The colour was used as indication for the reversibility. The colour formation at high pH (d) was partly reversible by decreasing the pH (b).

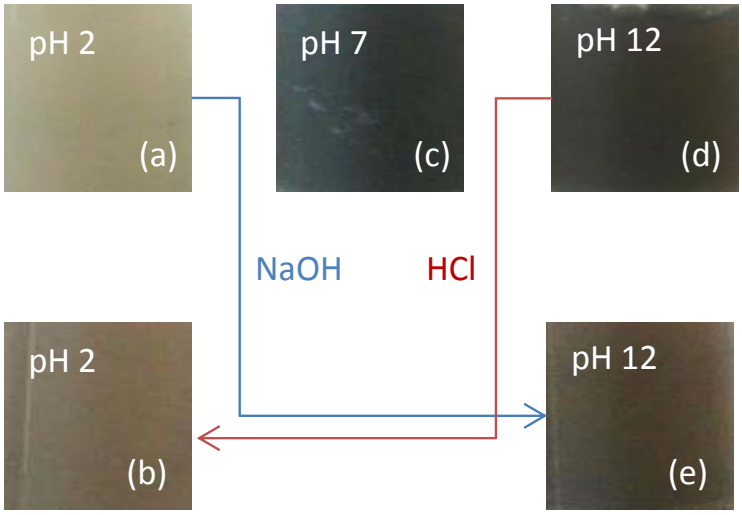


Figure 6.2. Reversible iron-phenolic complexes by pH adjustment based on colour formation of *H. illucens* extracts at pH 2 (a), pH 2 obtained by lowering from high pH (b), pH 7 (c), pH 12 (d), pH 12 obtained by increasing from low pH (e).

This leads to the hypothesis that iron in the gastric juice will be released from the complexes when the pH is decreased, as the colour lightened (**Figure 6.2**) and no complexes were found at pH 3 (**Chapter 4**). However, iron might form complexes again at the increased pH of the intestinal lumen where absorption of iron takes place (Latunde-dada & Simpson 2010). Consequently, iron absorption from the intestinal lumen might be inhibited. The solubility of iron, complex formation and phenolic oxidation can all play a role in iron absorption and should be taken into account. It is postulated that the iron in insects will be bioavailable, due to a high solubility, but this will be limited by phenolic oxidation, presence of the ferric form and complexation. To investigate the iron absorption from insects, human intervention studies are necessary using isotopic iron.

6.4. Prospects for insect application

Three different species, *T. molitor*, *A. diaperinus* and *H. illucens* were investigated in this thesis as they are reared and used in Europe for food and feed application. Large differences were found between the three species investigated in this thesis. The insect choice can be based on the characteristics of endogenous enzymes, techno-functional properties and nutritional value as described in this thesis. An overview of the variations between the species investigated in this thesis is shown in **Table 6.8**.

Table 6.8. Characteristics of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens* and its positive (+) or negative (-) effect on the potential of the species for food and feed.

	T. molitor	*A. diaperinus*	*H. illucens*
Protein content (%dm)	44.8	48.6	36.7
EAA (g/100 g protein)	43.03	46.39	44.77
Colour	+/-	++	-
Phenoloxidase activity	-	++	+
Protease activity	+/-	+/-	-
Digestibility soluble protein	+	+	++
Iron content (mg/kg dm)	61.0	53.5	220.7
Zero zeta potential	4.0	4.9	4.9
Gelation	+	+	-
Acceptance for food	+/-	+/-	-
Acceptance for feed	+	+	+

The lightest colour was found for *A. diaperinus* compared to the dark *T. molitor* and *H. illucens* (**Table 6.8**). Also the protein content was the highest for *A. diaperinus*. If iron content is important for application, then *H. illucens* might be a better option. *T. molitor* and *A. diaperinus* are more accepted for food (Ghaly & Alkoaik 2009), whereas *H. illucens* can also grow on manure and has therefore more potential as feed (van Huis et al. 2013; Oonincx et al. 2015). *T. molitor* and *A. diaperinus* had a higher potential compared to *H. illucens* when gelation is necessary. From a nutritional point of view, the highest essential amino acid content was found for *A. diaperinus*, whereas *H. illucens* had the highest digestibility compared to *T. molitor* and *A. diaperinus*. Based on the enzyme activity, colour formation, gelation properties and acceptance for food, *A. diaperinus* would be preferred for food purposes.

Table 6.8 shows the large variation between the species, even between *T. molitor* and *A. diaperinus*, which are from the same family. Over 2000 insect species have been identified as edible and likely every species has different characteristics. Therefore, extrapolation of characteristics from one insect species towards another should be done with care.

Overall, the results of this thesis show that the potential of insects is lower than suggested by crude protein content and the nitrogen-to-protein conversion factor determined in **Chapter 2** can be used for this. Making insects invisible may can help to make the use of insects more appropriate and increase their acceptance. Upon grinding, endogenous enzymes play an important role in this and can hamper application. This thesis indicated significant differences between species that affect protein characteristics necessary for future application. Given the differences found between species in this research, insects

cannot be considered as one novel protein source, but rather they should be studied per species. From the three species tested in this thesis, *A. diaperinus* showed the highest potential for food, as it has the highest protein content, lightest colour and the lowest endogenous enzyme activities. To increase the potential of insects, future research should focus on processing and its subsequent effect on the functionality of the proteins, and to map and make use of the characteristics of the proteins, next to its nutritional value.

R

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S

Summary

SUMMARY

Insects have been identified as excellent alternative source of proteins due to their high protein content, which is interesting from a nutritional point of view. Yet, this is often based on the crude protein content, determined from total nitrogen, using the general nitrogen-to-protein conversion (Kp) factor of 6.25. Specific Kp factors for insects have not been determined yet. The acceptance of insects as food is poor in Western countries, but increases when they are used as ingredient in an invisible manner. Hence, grinding is necessary. Off-colour formation occurs upon grinding larvae, which can hamper their potential use as ingredient for food and feed. The aim of this thesis was to investigate the potential of insects as protein source, the mechanisms responsible for browning or blackening of insects, and its impact on protein functionality. This was investigated for the larvae of three insect species: *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*.

The protein content is usually calculated from total nitrogen content using the general Kp factor of 6.25. This factor overestimates the protein content, due to the presence of nonprotein nitrogen in insects. In **Chapter 2**, a specific Kp factor of 4.76±0.09 was determined for larvae from *Tenebrio molitor*, *Alphitobius diaperinus*, and *Hermetia illucens*, using their total nitrogen content and amino acid composition. A Kp factor of 5.60±0.39 was found for protein extracts of these larvae. Thus, the currently used nitrogen-to-protein factor overestimates the protein content up to 26%, which will be avoided in the future by using the insect-specific Kp value determined here.

Chapter 3 elucidated which enzymes are responsible for browning upon grinding of insects. Activity in insect protein extracts towards L-tyrosine, L-3,4-di-hydroxy-phenylalanine (L-DOPA) and L-dopamine as substrates, based on oxygen consumption, indicated that phenoloxidase was a key player in browning. Furthermore, no reaction on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) was observed, ruling out laccase as enzyme contributing to browning. The browning reaction was most prominent at pH 6 for *T. molitor* and *A. diaperinus*, and at pH 7 for *H. illucens*. Phenoloxidase activity was confirmed for *T. molitor* and *A. diaperinus* by activity measurements after fractionation of the extracts by anion-exchange chromatography. The activity of fractions towards both L-DOPA and L-tyrosine and conversion into dopachrome after incubation with the same enzyme-enriched fractions indicated phenoloxidase activity. No DOPA-decarboxylase, tyrosine hydroxylase and peroxidase activities were observed. Active *T. molitor* protein bands were resolved using native PAGE with L-DOPA as staining-solution, and the presence of phenoloxidase was confirmed by sequencing. All together, these data confirmed that

phenoloxidase (also referred to as tyrosinase) is an important enzyme in causing enzymatic browning in *T. molitor* and likely in *A. diaperinus.*

The phenoloxidase activity of *H. illucens* was the lowest, whereas a much darker, blackish, colour was formed in the extract than in those of *T. molitor* and *A. diaperinus*. This was likely caused by a factor other than enzymatic browning. **Chapter 4** elaborates on the effect of iron, phenoloxidase and polyphenols on off-colour formation in insect larvae. The iron content in the larvae of the various species was 61±9.71, 54±1.72 and 221±6.07 mg/kg dm for *T. molitor*, *A. diaperinus* and *H. illucens*, respectively. The main phenolic compound was L-tyrosine that decreased upon browning. As only diphenolic structures can interact with iron, a model system of L-DOPA (hydroxylated form of L-tyrosine) and iron was used to elucidate the interaction of iron and L-DOPA. Their formation was evidenced by direct injection into ESI-TOF-MS, based on their charges combined with iron isotope patterns. The reversibility of the binding of iron to phenolics and thereby also the reversibility of blackening, was confirmed by EDTA addition. Besides complex formation, oxidation of L-DOPA occurred mainly by redox reactions with iron at low pH, whereas auto oxidation of L-DOPA mainly occurred at pH 10. Tyrosinase (i.e. phenoloxidase) activity did not change complex formation. The similarity in off-colour formation between the model system and ground insect larvae indicated an important role for iron-phenolic complexation in blackening of *H. illucens*.

Upon extracting soluble proteins from insects as potential food ingredient, phenoloxidase activity as determined in **Chapter 3**, was expected to negatively affect protein properties. Therefore, the effect of phenoloxidase activity on solubility and digestibility was investigated in **Chapter 5** for proteins from larvae of *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. Phenoloxidase was inhibited by blanching (50 s, 90 °C) before extraction or by extracting in presence of sulphite. Similar soluble protein yield and composition were found without and with sulphite addition, hence enzymatic browning did not change protein solubility. Blanching, on the other hand, decreased soluble protein yield. Upon *in vitro* hydrolysis by pepsin and trypsin, soluble proteins from *H. illucens* were more digestible compared to those from *T. molitor* and *A. diaperinus*. Phenoloxidase activity during processing negatively affected *in vitro* hydrolysis by pepsin, but not by trypsin. Endogenous proteases were shown to remain active at pH 8 in extracts containing sulphite and also after blanching of larvae. This stresses that protease activity needs to be carefully controlled in the design of insect based ingredients.

Chapter 6 provides a broader outlook on the potential of insect larvae as alternative protein source. The nitrogen-to-protein factors based on literature values from other insect species were comparable to the specific Kp factor determined in **Chapter 2** for *T. molitor*,

A. diaperinus and *H. illucens*, and were thus significantly lower than the general Kp factor of 6.25. Furthermore, commercial insect flours and protein powders showed different purities, indicating the necessity of using specific Kp factors. The effect of iron complexation (**Chapter 4**) on bioavailability was postulated. The effect of endogenous phenoloxidases (**Chapter 3**), proteases (**Chapter 5**) and processing conditions, like pH, on solubility and gelation properties was further elaborated. The endogenous enzyme activities and iron complexation should be taken into account for future application, as well as the specific Kp factor to prevent overestimation of the protein content of insects.

S

Samenvatting

SAMENVATTING

Insecten worden vanuit een voedingskundig perspectief vaak gezien als een excellente alternatieve eiwitbron vanwege hun hoge eiwitgehalte. Dit is vaak gebaseerd op het ruwe eiwitgehalte, bepaald op basis van totaal stikstof met behulp van de algemene-stikstof-naar-eiwitconversie (Kp-factor) van 6.25. Specifieke Kp-factoren voor de bepaling van het eiwitgehalte in insecten zijn nog niet vastgesteld. In de Westerse wereld zijn insecten als voedsel nog niet breed geaccepteerd. Acceptatie zou toenemen wanneer ze als ingrediënt worden gebruikt en niet als zodanig zichtbaar zijn. Daarom is het vermalen van de insecten noodzakelijk. Bij het vermalen van insectenlarven treedt echter bruinkleuring op, wat het potentiële gebruik als ingrediënt voor voedsel en diervoeding kan belemmeren.

Het doel van dit proefschrift was om te onderzoeken: Wat het potentieel is van insecten als eiwitbron, welke mechanismen verantwoordelijk zijn voor bruinkleuring tijdens het vermalen van insecten en wat de impact hiervan is op eiwitfunctionaliteit.

Voor dit onderzoek zijn de larven onderzocht van drie insectensoorten, te weten de *Tenebrio molitor* (meelworm), de *Alphitobius diaperinus* (buffaloworm) en de *Hermetia illucens* (zwarte soldatenvlieg).

Het eiwitgehalte wordt meestal berekend op basis van het totale stikstofgehalte vermenigvuldigd met de algemene Kp-factor van 6.25. Deze factor overschat het eiwitgehalte, omdat insecten ook niet-eiwit gerelateerd stikstof bevatten.

In **Hoofdstuk 2** werd op basis van hun totale stikstofgehalte en aminozuursamenstelling een specifieke Kp-factor van 4.76 ± 0.09 bepaald voor larven van *Tenebrio molitor*, *Alphitobius diaperinus* en *Hermetia illucens*. Voor eiwitextracten gemaakt van deze larven is een Kp-factor van 5.60 ± 0.39 gevonden. Het gebruik van de algemene Kp-factor overschat dus het eiwitgehalte met maximaal 26%. Gebruik van een specifieke, voor insecten bepaalde Kp-factor is een betere benadering.

Hoofdstuk 3 beschrijft welke enzymen verantwoordelijk zijn voor het bruin worden tijdens het vermalen van insecten. Op basis van de hoeveelheid zuurstof die wordt verbruikt op substraten: L-tyrosine, L-3,4-di-hydroxy-fenylalanine (L-DOPA) en L-dopamine is gekeken naar de enzymactiviteiten in eiwitextracten. Dit liet zien dat fenoloxidase een belangrijke rol speelt bij bruinkleuring. Verder werd geen reactie op 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonzuur) waargenomen, waardoor werd uitgesloten dat laccase als enzym de bruinkleuring veroorzaakt. De enzymactiviteit was het meest prominent aanwezig bij een pH 6 voor *T. molitor* en *A. diaperinus* en bij een pH 7 voor *H. illucens*.

Fenoloxidase-activiteit werd bevestigd voor *T. molitor* en *A. diaperinus* door meting van activiteit na fractionering van de extracten met anionenuitwisselingschromatografie. De activiteit van fracties tegen zowel L-DOPA als Ltyrosine en omzetting in dopachroom na incubatie met dezelfde met enzym verrijkte fracties duidde op fenoloxidase-activiteit. Er werden geen DOPA-decarboxylase, tyrosine- hydroxylase- en peroxidase-activiteiten waargenomen. Actieve *T. molitor* eiwitbanden werden gescheiden door PAGE onder niet-denaturerende en niet-reducerende condities met het substraat L-DOPA als kleuring voor de enzymen. De aanwezigheid van fenoloxidase werd bevestigd door sequentiebepaling. Alles bij elkaar bevestigden deze gegevens dat fenoloxidase (ook tyrosinase genoemd) een belangrijk enzym is bij het veroorzaken van de enzymatische bruinkleuring in *T. molitor* en waarschijnlijk ook in *A. diaperinus*.

H. illucens had de laagste fenoloxidase-activiteit in vergelijking met *T. molitor* en *A. diaperinus*, terwijl de donkerste, bijna zwartachtige kleur werd gevormd in het extract. Niet alle kleur kan dus verklaard worden door de enzymatische bruinkleuring en andere factoren spelen waarschijnlijk ook een rol.

Hoofdstuk 4 onderzoekt het effect van ijzer, fenoloxidase en polyfenolen op de vorming van bruin- of zwartkleuring in insecten. Het ijzergehalte in de larven van de verschillende soorten was respectievelijk 61 ± 9.71, 54 ± 1.72 en 221 ± 6.07 mg / kg droge stof voor *T. molitor*, *A. diaperinus* en *H. illucens*. De belangrijkste fenolische verbinding die tijdens bruinkleuring afnam was L-tyrosine. Alleen difenolische structuren kunnen interactie hebben met ijzer, daarom werd een modelsysteem van L-DOPA (gehydroxyleerde vorm van L-tyrosine) en ijzer gebruikt om de interactie van ijzer en L-DOPA te verklaren. De stoffen werden direct geïnjecteerd in de massaspectrometer (ESI-TOF-MS). Op basis van hun ladingen in combinatie met specifieke ijzerisotooppatronen kon complexvorming worden bewezen. De reversibiliteit van de binding van ijzer aan fenolen en daarmee ook de reversibiliteit van zwartkleuring werd bevestigd door toevoeging van EDTA. Naast complexvorming vond oxidatie van L-DOPA voornamelijk plaats door redoxreacties met ijzer bij een lage pH, terwijl auto-oxidatie van L-DOPA hoofdzakelijk plaatsvond bij pH 10. Tyrosinase (oftewel fenoloxidase)-activiteit veranderde de complexvorming niet. De overeenkomst in kleurvorming tussen het modelsysteem en gemalen insectenlarven laat een belangrijke rol zien voor ijzerfenol-complexvorming bij het zwart worden van *H. illucens*.

Bij het extraheren van oplosbare eiwitten uit insecten als potentieel ingrediënt in voedsel, kan fenoloxidase-activiteit, zoals die is bepaald in **Hoofdstuk 3**, de eiwiteigenschappen negatief zou beïnvloeden. Daarom werd in **Hoofdstuk 5** het effect onderzocht van fenoloxidase-activiteit op de oplosbaarheid en verteerbaarheid voor oplosbare eiwitten van

larven van *Tenebrio molitor*, *Alphitobius diaperinus* en *Hermetia illucens*. Fenoloxidase werd geremd door blancheren (50 s, 90 °C) voor extractie of door extraheren in aanwezigheid van sulfiet. Vergelijkbare eiwithoeveelheid en samenstelling van oplosbaar eiwit werden gevonden met en zonder toevoeging van sulfiet. Dus enzymatische bruinkleuring veranderde de eiwitoplosbaarheid niet. Blancheren daarentegen verminderde de hoeveelheid oplosbaar eiwit. Na *in vitro* hydrolyse door pepsine en trypsine waren oplosbare eiwitten van *H. illucens* beter verteerbaar dan die van *T. molitor* en *A. diaperinus*. Fenoloxidase-activiteit tijdens de extractie van eiwitten verminderde de *in vitro* hydrolyse door pepsine, maar niet door trypsine. Van endogene proteasen werd aangetoond dat ze actief bleven bij pH 8 in sulfiet-eiwitextracten en ook na het blancheren van larven. Dit benadrukt dat de proteaseactiviteit zorgvuldig moet worden gecontroleerd bij de ontwikkeling van ingrediënten op basis van insecten.

Hoofdstuk 6 biedt een bredere kijk op het potentieel van insectenlarven als alternatieve eiwitbron. Verschillende onderwerpen passeren de revue. De stikstof-tot-eiwitfactoren op basis van literatuurwaarden van andere insectensoorten waren vergelijkbaar met de specifieke Kp-factor zoals die is bepaald in **Hoofdstuk 2** voor *T. molitor*, *A. diaperinus* en *H. illucens* en waren dus significant lager dan de algemene Kp-factor van 6.25. Bovendien vertoonden commerciële insectenpoeders en eiwitpoeders verschillende zuiverheden, wat de relevantie van specifieke Kp-factoren laat zien. Verder werd er gespeculeerd over het effect van ijzercomplexvorming (**Hoofdstuk 4**) op de biobeschikbaarheid. Het effect van endogene fenoloxidasen (**Hoofdstuk 3**), proteasen (**Hoofdstuk 5**) en extractie condities, zoals pH, op oplosbaarheid en geleringseigenschappen werd verder bediscussieerd. Het verdient aanbeveling om de endogene enzymactiviteiten en ijzercomplexvorming op te nemen in de ontwikkeling van toekomstige producten, evenals als de specifieke Kp-factor om overschatting van het eiwitgehalte van insecten te voorkomen.

A

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If you want to go fast, go alone

If you want to go far, go together

(African proverb)

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A

About the author

CURRICULUM VITAE

Renske Hermine Janssen was born on June 12th 1990 in Rotterdam, The Netherlands. After graduation from secondary school (VWO, Emmaus college, Rotterdam) in 2008, she started her bachelor Food Technology at the Wageningen university. Her bachelor study was completed with a thesis on influencing the protein extractability, settling behaviour and fatty acid content of the microalgae *Tetraselmis suecica* by harvesting at different stages of the light regime within the laboratory of Food Chemistry and Bioprocess Engineering. In 2011, she continued with the master Food Technology at Wageningen University with Ingredient Functionality as specialization. She performed her master thesis at the laboratory of Food Chemistry on the inhibition of tyrosinase from different sources using natural plant extracts. After her thesis, she went for an industrial internship to Nestlé Research Centre in Lausanne, Switzerland, where she joined the Biotransformation group. Here she worked on enzymes during malt production. In 2013, she obtained her MSc degree in Food Technology and started her PhD research in the Food Quality and Design and Laboratory of Food Chemistry within the STW project In2food, under supervision of Prof. Dr Vincenzo Fogliano, Dr Jean-Paul Vincken and Dr Catriona Lakemond. The result of her PhD research are presented in this thesis. Currently, Renske is working as research/education officer at the laboratory of Food Chemistry.

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LIST OF PUBLICATIONS

Janssen RH, Vincken J-P, Arts NJG, Fogliano V, A, Lakemond CMM, Effect of the endogenous phenoloxidase on protein solubility and digestibility after processing of *Tenebrio molitor, Alphitobius diaperinus* and *Hermetia illucens (submitted)*

Janssen RH, Lakemond, Canelli G, Sanders MG, Bakx E, CMM, Fogliano V, A, Vincken J-P. Iron-polyphenol complexes cause blackening upon grinding *Hermetia illucens* (black soldier fly) larvae *(submitted)*

Janssen RH, Lakemond CMM, Fogliano V, Renzone G, Scaloni A, Vincken J-P. Involvement of phenoloxidase in browning during grinding of *Tenebrio molitor* larvae. *PloS ONE*; 2017, 12 (12)

Janssen RH, Vincken J-P, van den Broek LAM, Fogliano V, Lakemond CMM. Nitrogen-to-protein conversion factors for three edible insects: *Tenebrio molitor*, *Alphitobius diaperinus* and *Hermetia illucens*. *Journal of Agricultural and Food Chemistry*. 2017; 56 (11), pp 2275-2278

Kuijpers TFM, van Herk T, Vincken JP, **Janssen RH**, Narh DL, Van Berkel WJH, Gruppen H, Potato and mushroom polyphenol oxidase activities are differently modulated by natural plant extracts, *Journal of Agricultural and Food Chemistry*, 2014; 62, (1) pp. 214-221

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific activities

Courses

Advanced industrial food proteins	2013
Applied biocatalysis [a]	2014
Advanced food analysis [a]	2015
Biorefinery for biomolecules [a]	2015
Food and biorefinery enzymology [a]	2015

Conferences

Conference insects to feed the world (Ede, The Netherlands)	2014
Oxizymes (Vienna, Austria)	2014
Edible insect workhops (Wageningen. The Netherlands)	2015
Food Colloids (Wageningen, The Netherlands)[a]	2016
Insecta (Magdenburg, Germany)[b]	2016
Entomologendag (Ede, The Netherlands) [b]	2016
Insecta (Berlin, Germany) [b]	2017
Edible insects; the value chain (Wageningen, The Netherlands) [b]	2018

General courses

VLAG PhD week	2014
Project and Time Management	2014
Writing and presenting a scientific paper	2014
Mobilising your scientific network in 2.5h	2014
Voice matters- Voice and presentation skills training	2015
Scientific Writing	2015
Career Perspectives	2017

Optional courses and activities

Preparation of research proposal[b]	2014
Weekly group meetings FCH/FQD[b]	2014-2017
STW meetings[b]	2014-2017
PhD study tour, Thailand and Singapore [a,b]	2014
PhD study tour, Japan [a,b,c]	2016

[a] *Poster presentation*
[b] *Oral presentation*
[c] *Organizing committee*

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