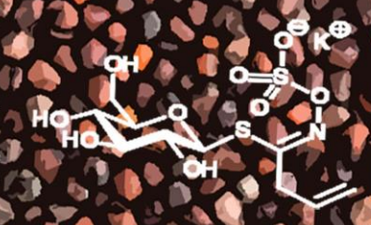
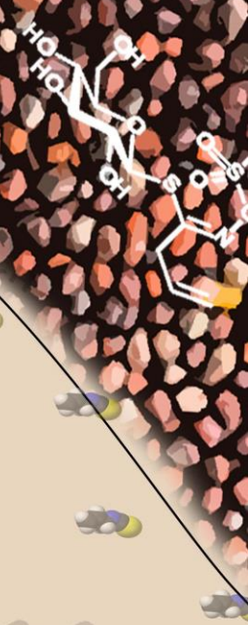
Antimicrobial Food Packaging Design

Exploring the potential of Allyl isothiocyanate release from mustard seeds





Nur Alim Bahmid

Antimicrobial food packaging design: Exploring the potential of Allyl isothiocyanate release from mustard seeds

Nur Alim Bahmid

2021

Propositions

1. Fat content is the most important factor for the design of a packaging system releasing antimicrobial compounds
   (this thesis)

2. In-situ formation of antimicrobial compounds is a very efficient way to control the release of these compounds in a packaging system
   (this thesis)

3. The rise of a political dynasty in a democracy is more likely in low-income societies (Mendoza, Beja, Venida, & Yap, 2016, Oxford Development Studies, 44(2), 189-201).

4. Sugar rushes for kids are a myth (Wolraich, Wilson, & White, 1995, Jama, 274(20), 1617-1621; Del-Ponte, *et al.,* 2019, Journal of Affective Disorders, 243, 290-296)

5. Eating rice by hand has the same image perception for western people as eating insect food

6. The impact factor of a journal is only a number, but the real impact is how the published work is implemented in real life

Propositions belonging to the thesis, entitled :

“Antimicrobial Food Packaging Design: Exploring the potential of allyl isothiocyanate release from mustard seeds.”

Nur Alim Bahmid

Wageningen, 21 April 2021

Antimicrobial Food Packaging Design:

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Nur Alim Bahmid

Thesis committee

Promotor

Prof. Dr V. Fogliano
Professor of Food Quality and Design
Wageningen University and Research

Co-promotors

Dr M. Dekker
Associate Professor, Food Quality and Design
Wageningen University and Research

Dr J.K. Heising
Associate Professor, Food Quality and Design
Wageningen University and Research

Other members

Prof. Dr J.P. Vincken, Wageningen University and Research
Prof. Dr C.G.P.H. Schroen, Wageningen University and Research
Prof. Dr rer. nat. H.C. Langowski, Technische Universität München, Germany
Dr C.S.C. Nunes, University of Aveiro, Portugal

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Antimicrobial Packaging Design:

Exploring the potential of allyl isothiocyanate release from mustard seeds

Nur Alim Bahmid

Thesis

submitted in fulfilment of the requirements for the degree of doctor

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Prof. Dr A.P.J. Mol,

in the presence of the

Thesis Committee appointed by the Academic Board

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Nur Alim Bahmid

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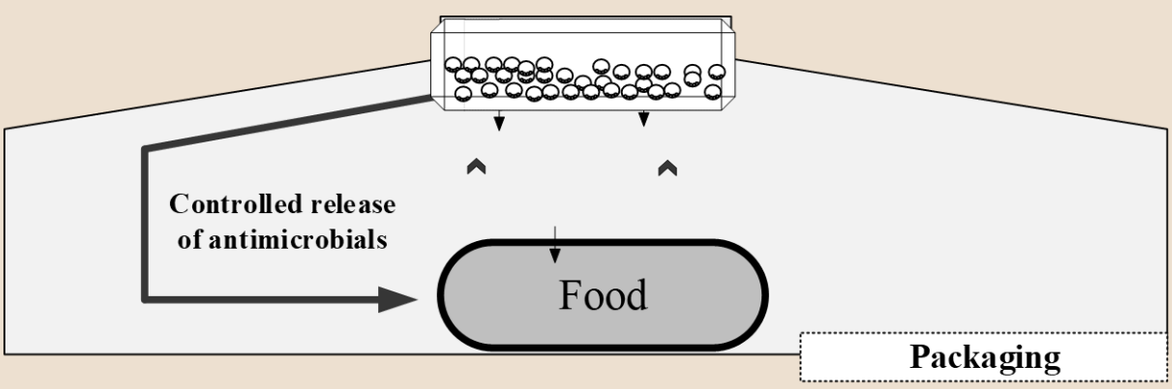
To my wife, Ayu Yustika,
and my children, Alesha Inara Bahmid and Alfaraby Oryza Bahmid

Table of Contents


Controlled release
of antimicrobials
Food
Packaging

1

General Introduction

Consumer demands for fresh and nutritious food, with an adequate shelf life, safety, and convenience, increase nowadays (Singh et al., 2011). The freshness and minimal processing make these food products prone to contamination and spoilage by microorganisms during their harvesting, processing, distribution, and (home) storage (Seo et al., 2012). Food products that are spoiled after the production chain, for example, at retail and consumer levels, are wasted. The Food and Agriculture Organization (FAO) estimated that global food waste at 1.3 billion tons annually, and at least 89 million tons of food waste are estimated for Europe. Food waste contributes to increasing greenhouse gas emissions in our environment. This gas emission is not mainly from food waste but from the resources used to produce the part of food that is wasted. Perishable products mishandling by retailers, consumer behaviour, inappropriate quality control, and overstocking (Buisman et al., 2017), and high safety expectancy by consumers (Luning et al., 2002) are known as the main factors contributing to food spoilage or contamination. Therefore, ways to reduce food spoilage and contamination is a big challenge to fulfil the consumer's needs and to reduce food waste.

Food packaging serves basic functions; containment, protection, convenience, and communications towards the consumer (Robertson, 2010). With these functions, food packaging can help customers fulfil their needs to consume fresh and nutritious products. The food packaging can keep the food "fresh" for a certain period and protect it from spoilage and any unwanted chemical and biological changes (after food production) to maintain its quality and safety and prolong its shelf life. Therefore, the food packaging in the food supply chain has a central role in preserving the food quality during the distribution and at retail and consumer levels (Guillard et al., 2018; Isshiki et al., 1992).

In terms of protection and preservation, antimicrobial packaging has been developed by adding antimicrobial compounds to the packaging system to inhibit the growth of microorganisms on and/or in the food, thereby extending the shelf life of foods (Mihindukulasuriya and Lim, 2014; Vilela et al., 2018). Compounds that have been applied in antimicrobial packaging are, for example, carvacrol (Wang et al., 2020c), lysozyme (Gemili et al., 2009), thymol (Ramos et al., 2020), allyl isothiocyanate (AITC) (Nazareth et al., 2019b), etc. AITC is a strong antimicrobial compound that is promising to be applied to inhibit spoilage and pathogenic bacteria (Delaquis and Sholberg, 1997; Isshiki et al., 1992; Kanemaru and Miyamoto, 1990; Lin et al., 2000; Luciano and Holley, 2009; Mushantaf et al., 2012; Park et al., 2012; Seo et al., 2012). Although AITC has been applied as an antimicrobial in varying packaged food products, an effective design of antimicrobial packaging is still needed to optimize its performance to extend the shelf life of the packaged food. In this introductory chapter of the thesis, general antimicrobial packaging and AITC as an antimicrobial compound are first introduced (**Sections 1.1 and 1.2**). Possible mass transfer mechanisms of the antimicrobial compounds in the food package are described (**Section 1.3**), and possible factors and effects are then explained (in **Sections 1.4 and 1.5**, respectively).

1.1. Antimicrobial Packaging

Antimicrobial packaging is defined as a type of active packaging releasing antimicrobial agents to inhibit the growth of microorganisms, e.g. moulds, fungi, and (aerobic) spoilage and pathogenic bacteria in the packaged foods (Jideani and Vogt, 2016; Vermeiren et al., 1999). The antimicrobial agents interact with the food products by direct or indirect contact (Appendini and Hotchkiss, 2002), as shown in **Figure 1.1**. For the direct contact method, nonvolatile antimicrobial agents can be incorporated into the packaging system in different ways; direct coating on the food surface or packaging, edible film, or a pad containing antimicrobial compounds in contact with the food (Ahvenainen, 2003). For the indirect contact method, volatile compounds can be blended homogenously and incorporated in the packaging material and are then released through the headspace to the food products (Mousavi Khaneghah et al., 2018). The released antimicrobial agents can reduce, suppress, or obstruct the growth of (spoilage) microorganisms present (Appendini and Hotchkiss, 2002).

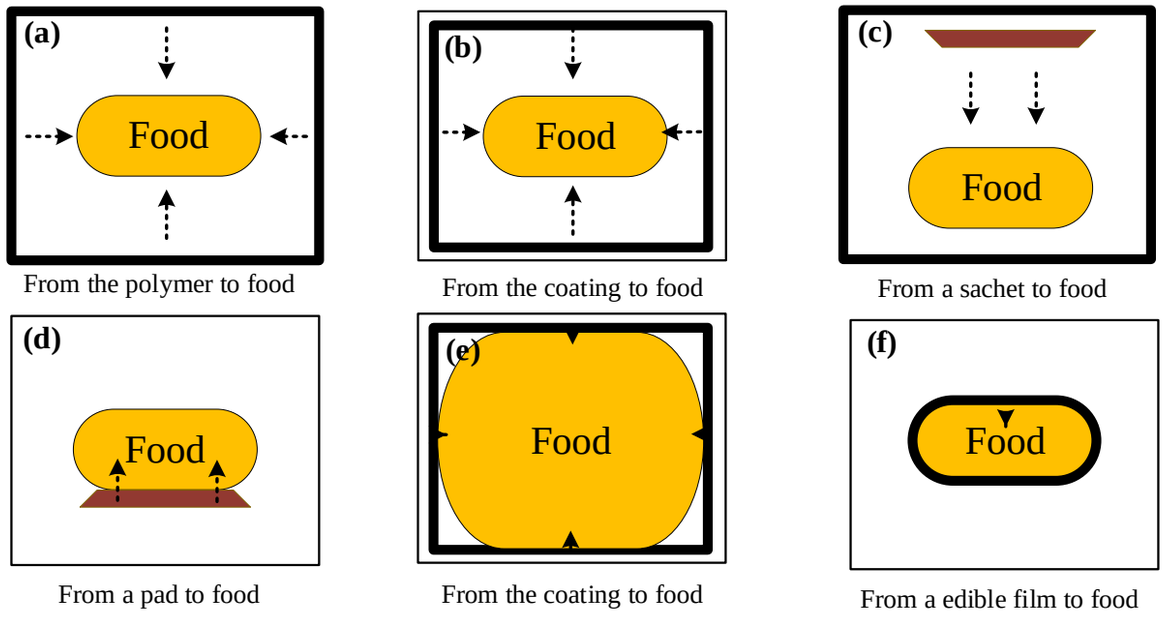


Figure 1.1. *Possible methods for releasing antimicrobial agents in active packaging;* ***(a-c)*** *indirect contact (via headspace) and* ***(d-f)*** *direct contact. The figure was adapted from Limbo and Khaneghah (2015).*

Many natural antimicrobials are derived from plant, animals, bacteria, algae, and fungi that can be applied in the antimicrobial packaging system (Gyawali and Ibrahim, 2014). The plant-based sources, e.g. essential oils, have good antimicrobial activity towards a variety of spoilage bacteria (Dorman and Deans, 2000). The antimicrobial effectiveness of the compounds depends on their characteristics and concentration (Clemente et al., 2017; Li et al., 2017) because each antimicrobial compound has a different mode of action against microorganisms. Understanding the characteristics of antimicrobials and their antimicrobial

effects against microorganisms gives the insight to determine an effective method, whether using direct or indirect contact, for the antimicrobial packaging system. The environment and the food inside the package also need to be considered to obtain optimal antimicrobial effects against bacterial growth to reach an extended shelf life of the packaged food (Mousavi Khaneghah et al., 2018).

1.2. Allyl isothiocyanate (AITC) as an antimicrobial agent

1.2.1. Mustard seeds as a source of AITC

AITC is naturally derived from the mustard plants that belong to the *Brassicaceae* family (Harvey et al., 2002). Mustard seeds are known for their high concentration of sinigrin (2-propenylglucosinolate or allylglucosinolate, $C_{10}H_{16}KNO_9S_2$). Sinigrin is a type of glucosinolate (a group of nitrogen and sulfur-containing natural pseudo-glucosides) present in the oriental mustard (*Brassica Juncea*) (Lin et al., 2000). It is a precursor of AITC formation (Rangkadilok et al., 2002). Inside the mustard seed cells, as shown in **Figure 1.2**, sinigrin is located in the S-cells' vacuoles. Besides sinigrin, the mustard seeds contain an endogenous enzyme called myrosinase, located in separate compartments from sinigrin, myrosin cells (Kissen et al., 2009; Nakano et al., 2014; Stauber et al., 2012; Torrijos et al., 2019). Myrosinase and sinigrin are the important components for the AITC formation in the mustard seeds.

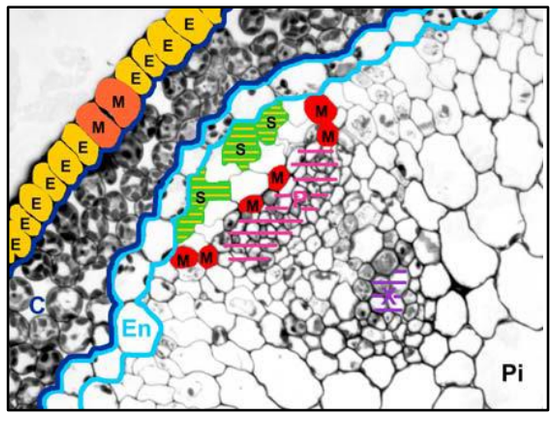


Figure 1.2. *S-cells (green) denote sinigrin, and M-cells denote phloem cells (red) and guard cells (orange), respectively, express myrosinase (Kissen et al., 2009).*

1.2.2. AITC formation

AITC is formed from the enzymatic hydrolysis of sinigrin. This formation was explained by a 'mustard oil bomb' theory. The theory was established for a subcellular organization of the glucosinolate-myrosinase system (Lüthy and Matile, 1984; Stauber et al., 2012), and then Kissen et al. (2009) assessed the theory by clarifying that the hydrolysis of glucosinolate by myrosinase occurred only in the case of cell damage. **Figure 1.3a** shows the interaction between sinigrin and myrosinase after the mustard tissues are disrupted by the physical disruption (Nakano et al., 2014).

Figure 1.3b shows the sinigrin hydrolysis is catalyzed by myrosinase in the presence of moisture by cleaving the D-glucose group from sinigrin to form an unstable intermediate aglycone. These aglycone fragments rearrange to form isothiocyanates, nitriles, epithionitriles, thiocyanates, and oxazolidine-2-thiones. The formation of those compounds depends on the type of glucosinolates, the reaction conditions (e.g. pH), the specifier proteins (e.g. epithiospecifier protein (ESP)), and the presence of additional cofactors (e.g. metal ions) (Oliviero et al., 2018; Saladino et al., 2017a). At pH< 5, the nitrile formation is favoured over isothiocyanates, while at pH >5, 90% of the formed degradation products are AITC (Shofran et al., 1998; Stoin et al., 2009). Therefore, the pH should be neutral to ensure the AITC formation in mustard seeds.

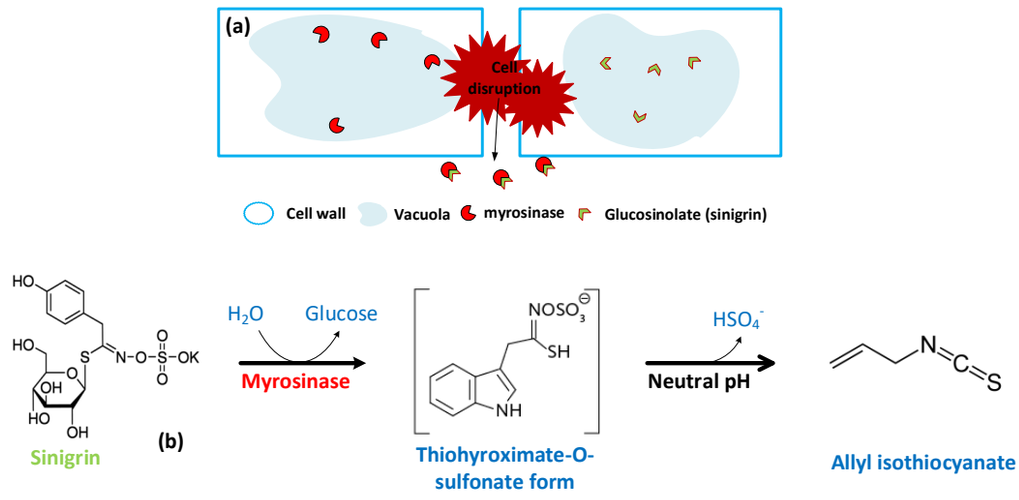


Figure 1.3. (a) *Schematic representation of allyl isothiocyanate (AITC) formation from the interaction between sinigrin and myrosinase, adapted from Nakano et al. (2014) and* ***(b)*** *schematic reaction of the AITC formation, adapted from Shofran et al. (1998).*

1.2.3. Mode of Action

AITC was demonstrated to have strong bactericidal activity in both liquid and vapour form (Kim et al., 2015; MejÍA-Garibay et al., 2015; Muscolino et al., 2016; Nazareth et al., 2016; Quiles et al., 2015; Shin et al., 2010; Tracz et al., 2018). Suhr and Nielsen (2003) and MejÍA-Garibay et al. (2015) found that the AITC vapour (indirect method) is hundreds of times more effective than AITC directly added to the food (direct addition) for inhibition of a variety of pathogenic and spoilage microorganism in an agar model or (model) food products. The advantage of volatile compounds is their capacity to penetrate the bulk food matrix (Appendini and Hotchkiss, 2002) and their effectiveness to suppress bacterial growth on the food surface. AITC inhibits the microorganisms by penetrating the microbial cells to disrupt the cytoplasmic membrane of the bacteria. The AITC penetration causes leakage of intracellular compounds and disaggregation of cell walls (Dufour et al., 2015). This cell leakage and the disaggregation of the cell wall cause loss of the integrity of the cellular structure (Dufour et al., 2015; Lin et al., 2000; Luciano and Holley, 2009). The antimicrobial effects of AITC on the vast range of pathogenic and spoilage bacteria are further discussed in the next section.

1.3. Mass transfer of antimicrobial agents in the packaging system

1.3.1. Mass transport mechanisms of antimicrobials in a packaging system

In the antimicrobial packaging system, mass transfer is an essential mechanism for the movement of the antimicrobial agents from a phase to another phase inside the packaging system. **Figure 1.4** shows that the antimicrobial compounds can be transferred to all phases (packaging materials, headspace, and (model) food) in the package/food system (**Figure 1.4a**) and package/headspace/food system (**Figure 1.4b**). The diffusion of the compounds within each phase is described by the diffusion coefficients, consisting of the diffusion coefficient within the packaging material (D_p), within the food (D_f), and within the headspace (D_g) (Brody et al., 2001). While the mass transfer between different phases is described as mass transfer coefficients, e.g. m_{t1} (from packaging material to headspace) and m_{t2} (from headspace to food products).

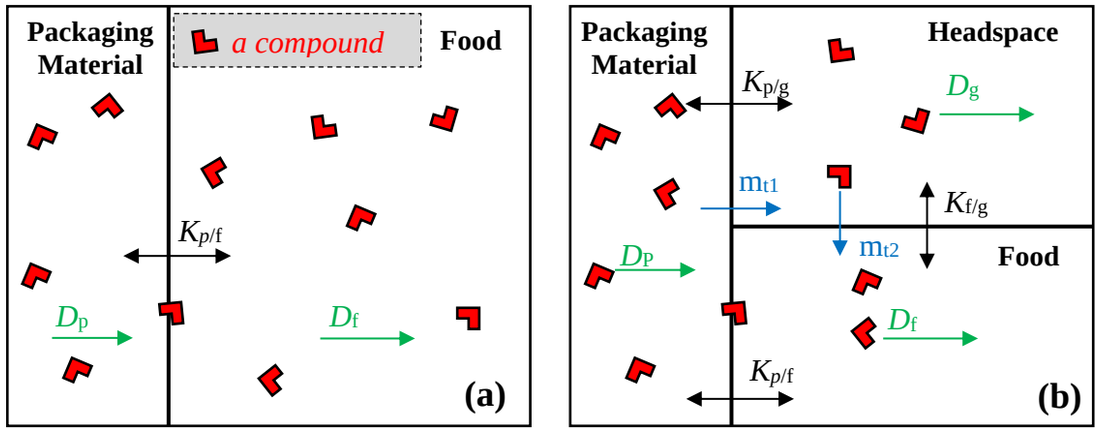


Figure 1.4. *Mass transport phenomena in* ***(a)*** *package/food system and* ***(b)*** *package/headspace/food system. The figures were adapted from (Brody et al., 2001); Jideani and Vogt (2016). D and K denote diffusion and partition coefficient, respectively, and p, f and g are symbols for packaging material, food and headspace phases. m_{t1} and m_{t2} denote the mass transfer coefficient from packaging material to headspace and from headspace to food products.*

For methods with direct contact (**Figure 1.4a**), the non-volatile compounds are transported directly from the packaging material to the surface of the foods, and the ratio of compound concentration between food and packaging material phases at equilibrium is described by the partition coefficient ($K_{p/f}$). Since the compounds are transported between the packaging material and the food, the release of compounds from the packaging materials becomes essential to reach effective bacterial inhibition.

For the indirect method, as shown in **Figure 1.4b**, the antimicrobial compounds migrate through the headspace to the (model) food (Zhou et al., 2010). The mass transfer of volatile compounds consists of a few steps; (1) the compounds diffuse across towards the packaging material interface, (2) are released into the packaging headspace, and (3) are absorbed by the (model) food, and (4) diffuse into the food (Chen et al., 2019). The equilibriums of compounds between packaging material and headspace and between food and headspace are described by the partitioning constants $K_{p/g}$ and $K_{f/g}$, respectively.

Both methods with direct or indirect contact could be applied for an antimicrobial package with AITC, but regarding the high volatility and antimicrobial effects of AITC, the indirect method was considered to be used in this thesis.

1.3.2. Mass transfer kinetics

The mass transfer of the antimicrobials can be studied by measuring the concentration of the compounds in each phase (active carrier, headspace, and food) in the packaging system. A mathematical model can be used to describe and predict changes in concentration during storage, e.g. by using the penetration theory (Harrison, Hills Brian, Bakker, & Clothier, 2006). In this theory, a phase, e.g. packaging material phase, is exposed to another phase, e.g. headspace, for a definite time interval. During this time, the compounds transfer from the packaging material across the interface and into the headspace through unsteady state molecular diffusion. This mass transfer occurs until reaching the equilibrium in concentration between the phases. The main parameters that describe this multiphase mass transfer are the mass transfer coefficients (m_t) of the antimicrobial compound in the different phases and the equilibrium partition coefficients (K) between the phases.

The mass transfer between one phase to another phase in the antimicrobial packaging system indicates the complexity of the system. The antimicrobial packaging becomes more complex with the possible reactions taking place in each phase as well, like the reaction of the sinigrin hydrolysis in ground mustard seeds as explained in the previous section. Therefore, it is important to understand and quantify the possible mass transfer and the reaction mechanisms, and the changes in the concentration of compounds can be described well by a kinetic model. Furthermore, the factors influencing the mass transfer and reactions need to be investigated. For example, the mass transfer coefficient is dependent on various conditions, like diffusion velocity, viscosity, and the size and geometry of the interfaces (Basmadjian, 2004). The factors influencing the AITC release and absorption are further discussed in the next part of the introduction.

1.4. Factors controlling the concentration of antimicrobial compounds in the packaging system

1.4.1. Intrinsic properties of the antimicrobial sources

The composition and structural properties of the antimicrobial sources play an important role in the release of the volatile antimicrobial compounds (Doi et al., 2019). Mustard seeds contain fat (~30% (w/w)), protein (~29%), carbohydrate (~19%), fibre (~10%), water (~7%), and ash (~5%) (Aljasass and Al-Jasser, 2012). The components of the antimicrobial sources can interact with and/or bind the volatile compounds (Ammari and Schroen, 2018). Mustard seeds have a high-fat content, and the AITC is expected to solubilize in the fat, and this might hinder the release of AITC from the mustard seeds (Dai and Lim, 2014) due to its

hydrophobicity and high stability in the lipid phase (Holley and Patel, 2005; Keppler et al., 2018). Also, the size of the seeds can influence the rate of AITC release from the seeds. Dai and Lim (2014) found that the unground mustard seeds released less AITC than the ground mustard seeds. The study also resulted in faster AITC release for the ground mustard seeds with lower fat contents (0 and 15-16%) and smaller particle sizes (fine and rough milled seeds), but the study did not specify the sizes of particles affecting the AITC release. The limited available literature that reports about factors influencing the AITC release from the mustard seeds shows that further research is required to get more understanding of the AITC formation and release mechanisms to control the release by the intrinsic properties of the mustard seeds.

1.4.2. Incorporation to the film packaging polymer

Most studies found in the literature related to the release of antimicrobial compounds are about how to control the release of compounds from packaging materials. The incorporation or blending of the compounds within the packaging material gives new possibilities to control the rates of the release of the compounds to maintain the antimicrobial activity against spoilage bacteria to obtain an extended shelf life (Appendini and Hotchkiss, 2002; Limbo and Khaneghah, 2015). The type and structure of used polymers might influence the release rate of the volatile antimicrobials such as AITC. For example, AITC encapsulated in calcium alginate beads were incorporated in porous HDPE (High-density Polyethylene) resins, in which the AITC release increases with a decreased film thickness (Seo et al., 2012).

Besides the material thickness, another way to control the AITC release is the incorporation of antimicrobial sources, such as ground mustard seeds, into a hydrophilic polymer, like cellulose acetate. The hydrophilic polymer is a good material to apply a new concept of a release mechanism triggered by an internal stimulus (e.g. water vapour release) in the antimicrobial packaging (Mousavi Khaneghah et al., 2018).

1.4.3. Extrinsic factors

Extrinsic (environmental) factors, such as temperature, influence the rate and amount of antimicrobials released from the antimicrobial sources. The factors also affect the antimicrobial stability/retention in the headspace, packaging material, antimicrobial source, and food system during storage (Ammari and Schroen, 2018). The temperature can either directly or indirectly affect the stability and release. AITC is more volatile and less stable at a higher temperature, so more AITC is observed in the headspace, but it is degraded faster (Liu and Yang, 2010). These effects of the temperature are observed as the direct effect, whereas the indirect effect of temperature can be seen at low temperature that strengthens AITC to bind with proteins in the food to increase its stability (Tiwari et al., 2009).

Additionally, the relative humidity (RH) could influence the controlled release of AITC since the formation of AITC requires water to trigger the sinigrin hydrolysis (Li et al., 2007).

Seo et al. (2012) found that the release rate of AIT encapsulated in calcium alginate beads increased with increased temperature, but it was not significantly affected by RH. While other studies (Dai and Lim, 2014; Lashkari et al., 2017; Wang et al., 2017b) found that the release rate and the amount of AITC released from the mustard seed powder or from AITC encapsulated in polymers, e.g. combination between β-cyclodextrin and polylactic acid, increased with increasing relative humidity and temperature. Lashkari et al. (2017) investigated adsorption-desorption of AITC from metal-organic frameworks and observed that an RH of 30-35% caused higher stability of AITC in the pores of materials and a high RH (95-100%) triggered more AITC release into the headspace. The different results of the different studies show the importance of storage conditions on the AITC release and should, therefore, be evaluated.

1.4.4. Food compositions

The food characteristics and components might affect the transport of antimicrobial compounds from the package into the food and its retention/stability. The food components, like proteins and carbohydrates, affect the characteristics of food, such as viscosity. An increased viscosity limits the transport of volatile compounds across the food matrix (Roberts et al., 1996). The proteins in foods can interact with AITC (Delaquis and Sholberg, 1997; Kawakishi and Kaneko, 1987; Luciano and Holley, 2009). The most important interaction between proteins and AITC is the covalent binding of AITC to the sulfide group of the proteins, reducing its antimicrobial activity (Keppler et al., 2017). Other main food components that can affect AITC are fat and water. AITC, known as a lipophilic compound, is naturally soluble in lipids in foods (Holley and Patel, 2005). Rabe et al. (2003) found that a large number of the lipid particles (emulsion droplets) should lead to an increased number of lipid-flavour interactions and thereby effectively maintain flavour compounds in the emulsion for a longer time. Liu and Yang (2010) concluded that the release of volatile compounds from soybean oil or medium-chain triglycerides in an emulsion system was affected by, for instance, droplet size, oil content, and oil type. The smaller oil droplets have an increased interfacial area and shorter diffusion distance, thereby being capable to accelerate the mass transfer. Furthermore, water in food can act as a nucleophile and trigger the decomposition of AITC to diallylthiourea (80%), diallyl urea (10%), and diallyl disulfide (10%) in aqueous solutions. The nucleophiles attack the electrophilic carbon atom within the functional N=C=S bond leading towards the decomposition of the molecule (Liu and Yang, 2010).

1.5. Effects of applying antimicrobial compounds in the packaging system on food quality

It is well-known that antimicrobial compounds can inhibit various microorganisms (Nazareth et al., 2020; Nazareth et al., 2019a) and prolong the shelf life (Torrijos et al., 2019).

Many volatile antimicrobials, like essential oils and AITC, are also having strong flavours, which can give consumers a bad perception of the packaged foods, and this can influence the sensory quality (Kramer et al., 2018). The concentration of antimicrobial compounds must be controlled in order not to surpass the concentration limit for sensory defects and not to decrease below the concentration limit for bacterial inhibition (**Figure 1.5**). If the concentration of the compounds surpasses the threshold concentration for aroma detection, the excessive accumulation would negatively affect the sensory attributes, such as providing an unpleasant off-flavour and thereby lowering the consumer acceptability of the food products (Dai and Lim, 2014; Dufour et al., 2015; Muscolino et al., 2016). On the other hand, a too low antimicrobial concentration in the packaging headspace reduces the antimicrobial activity of the compounds against the spoilage bacteria. Those effects of the antimicrobial compound, specifically AITC, are discussed below.

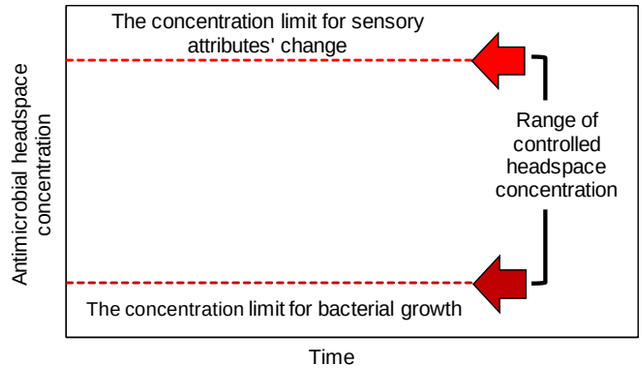


Figure 1.5. *The range of antimicrobial compound concentrations affecting the change of sensory attributes and the bacterial growth of food products*

1.5.1. Antimicrobial effects of AITC on packaged foods

For the practical applicability of the antimicrobial agent against food spoilage, the release rate of antimicrobials from the packaging material should be maintained at a minimum rate so that the surface concentration is maintained above a critical inhibitory concentration (Appendini and Hotchkiss 2002). The concentration of the compounds and their exposure duration to the food are crucial for the antimicrobial effect. Lin et al. (2000) presented an overview of known microbial inhibitory concentration (MIC) values for AITC and claimed that *E. coli O157:H7* is the most susceptible and *Listeria* for AITC*. Monocytogene* is the most resistant*.* MIC values for headspace AITC on *E. coli, S. Typhimurium,* and *S. Enteritidis* are 34, 54, and 110 μg/L, respectively, the gram-negative bacteria *Vibrio parahaemolyticus* and *Pseudomonas aeruginosa* have a MIC for AITC of 54 μg/L. MIC for the gram-positive bacteria *Bacillus subtilis, Bacillus cereus, Staphylococcus epidermidis* and *Staphylococcus aureus* ranged from 90 to 110ng/ml, indicating a stronger resistance compared to gram-negative bacteria (Isshiki et al., 1992). Moulds and yeasts show high sensitivity since 0.1 μg/L AITC in the headspace is already sufficient to inhibit moulds and yeast (Delaquis and Sholberg, 1997). Those reported values of MICs of AITC will depend on not only the type of the microorganism but also the food

composition and the environmental conditions. Therefore, all these aspects need to be considered in designing an effective food packaging system to inhibit bacteria in specific food products. **Table 1.1** shows the antimicrobial effects of AITC against various spoilage bacteria and moulds in various products.

Table 1.1. *Antimicrobial effects of allyl isothiocyanate (AITC) on a variety of microorganism in food products*

Food	AITC concentration	Organisms	Temp (°C)	Time (days)	Effect	Ref
Barley	50 µL/L	*Penicillium verrucosum*	23	1	1.9 logs CFU/g reduction	Nazareth et al. (2019b)
			23	30	completely inhibition	
Brazil nuts	2.5 µL/L	*Aspergillus parasiticus*	25	30	complete inhibition	Lopes et al. (2018)
Corn	50 µL/L	*Aspergillus parasiticus*	25	180	17 logs CFU/g reduction	Nazareth et al. (2018)
		Fusarium verticillioides			3.9 logs CFU/g reduction	
Cottage cheese	1% w/w antimicrobial sachet	yeast and moulds	5	35	fully inhibition	Conceição Gonçalves et al. (2009)
Fresh Catfish Fillet	18 µg/L	*Pseudomonas aeruginosa*	20		11 hours increasing lag phase (15 hours increasing shelf life)	Pang et al. (2013)
			8		3 hours increasing lag phase (48 hours increasing shelf life)	
Fresh sausage	0.00625-0.0125 µL/mL, combined with 0.24 mg nisin /mL	*Lactobacillus plantarum*	6	20	>3 log CFU/mL reductions	Araújo et al. (2018)
Gilthead Sea Bream (*Sparus aurata*) Fillets	3.35 and 6.70 mg/l	Specific Spoilage Organisms (SSOs)	2	7	5 logs CFU/g reduction (5-8 days increasing shelf-life)	Muscolino et al. (2016)
Iceberg lettuce and mung bean sprouts	0.81–1.41 µg/ml	*Pseudomonas spp.*	5	9	2–2.5 log CFU reductions	Kramer et al. (2018)
		Enterobacteria			up to 3 logs CFU reductions	
Kimchi	<0.1% (w/w) into kimchi	*L. plantarum* and *L. mesenteroides*	30	24 h	50% decrease	J.A. Ko (2011)
Loaf bread	5 5 µL/L	*Aspergillus parasiticus*	20	8	60% AFs reduction (3-4 days increasing shelf life)	Saladino et al. (2017b)
Maize	0.5-1 µL/L	*Aspergillus flavus*	23	30	50% inhibition	Nazareth et al. (2020)
Peanuts	0.215 ppb	*Aspergillus flavus*	25	60	4.81 logs cycles reduction	Otoni et al. (2014)
Pita Bread	33 mg/g oriental mustard flour	*Penicillium verrucosum*	23	7	3.1 logs CFU reduction (2 days increasing shelf life)	Torrijos et al. (2019)
	50 mg/g oriental mustard flour				5.7 logs CFU reduction (3 days increasing shelf life)	
Pizza crust	5 mL/L and 10 mL/L sachets AITC	*Aspergillus parasiticus*	4	30	fully inhibition	Quiles et al. (2015)
Sliced Mozzarella Cheese	2–16 µL/L	*Aspergillus parasiticus*	4	60	complete inhibition	Tracz et al. (2018)
		Penicillium digitatum				

Strawberry	15.42 µL/Lair	*Botrytis cinerea*	25	5	fully inhibition	Aguilar-González et al. (2015)
Tomatoes	0.63 mg/cm^2 in PLA film	*Fusarium oxysporum.*	4	21	3 logs CFU/g reductions	Gao et al. (2018)
Wheat flour	0.1 µL/L	*Aspergillus parasiticus* and *Fusarium poae*	23	30	6.9–23% reduction of mycotoxin production	Nazareth et al. (2016)
	10 µL/L				completely inhibition of mycotoxin production	

The shelf life extension of some packaged food, due to the presence of the ground mustard seeds or AITC, has been reported in some articles. Some studies have added mustard flour or oil to the food itself, while others use AITC in the package. Torrijos et al. (2019) found an increase in the shelf life of three days for pita bread with oriental mustard flour as an ingredient. A similar increase in the shelf life was observed in Spanish bread by inhibition of the foodborne moulds from the usage of mustard essential oil (Clemente et al., 2019). An increase in the shelf life was found for sliced mozzarella cheese (Tracz et al., 2018), hummus (Olaimat et al., 2018), grape tomato (Mukhopadhyay et al., 2018), and tomatoes (Gao et al., 2018) in the presence of pure AITC released in the package. Other perishable foods might be promising to be investigated for the effectiveness of the AITC from mustard seeds as an antimicrobial source to prolong their shelf life.

1.5.2. Sensory effects of antimicrobial on packaged foods and costumer's preferences

Since many volatile antimicrobials have a flavour, the required concentration in food packaging application needs to be low enough to not negatively impact the sensory attributes of foods, such as odour and taste (Górska et al., 2017; Kim et al., 2018). Although AITC is generally regarded as a safe compound for food application, the sensory effects are the most challenging issue on the usage of AITC in the packaging system due to its high volatility and pungent flavour. A sensory test with AITC has been done with Brazil nuts (Lopes et al., 2018), demonstrating that the highest acceptable dose for the consumer is around 2.5 µl per litre headspace. Sensory evaluation of ground beef treated with 5% and 10% mustard flour in the beef shows no difference in the consumers' acceptability (Nadarajah et al., 2005). However, the acceptable dose for AITC is highly product dependent. Products with more fat are likely to be less affected by sensory defects since most AITC will be trapped in the oil. Therefore, the acceptable concentrations of AITC as determined by consumer research will give an insight for optimizing the development of antimicrobial packages.

1.6. Objectives and Outline of this thesis

The extended shelf life of food and the maintained food quality can be reached with an antimicrobial packaging system. Understanding the complexity and mechanism of the

reactions and mass transfer occurring in the packaging system is crucial for designing antimicrobial packaging. The research described in the thesis aims to explore the potential of AITC release from the mustard seeds in antimicrobial food packaging by investigating the intrinsic and extrinsic effects and the effect of the food composition on the AITC concentration in the packaging system and (model) food. The framework of this thesis is schematically shown in **Figure 1.6. Chapter 2 and 6** aim to determine the importance of the intrinsic properties of the antimicrobial sources in a food packaging system. **Chapters 4, 5, and 6** aim to determine the role of environmental factors and food compositions on AITC partitioning and antimicrobial effects of AITC release in the food packaging system. By systematically varying those factors affecting the AITC release and by applying a kinetic modelling approach for understanding the packaging system (**Chapter 3 and 4)**, this study provides a new insight into the potential of mustard seeds as an antimicrobial source in an antimicrobial packaging system to prolong the shelf life of the packaged foods.

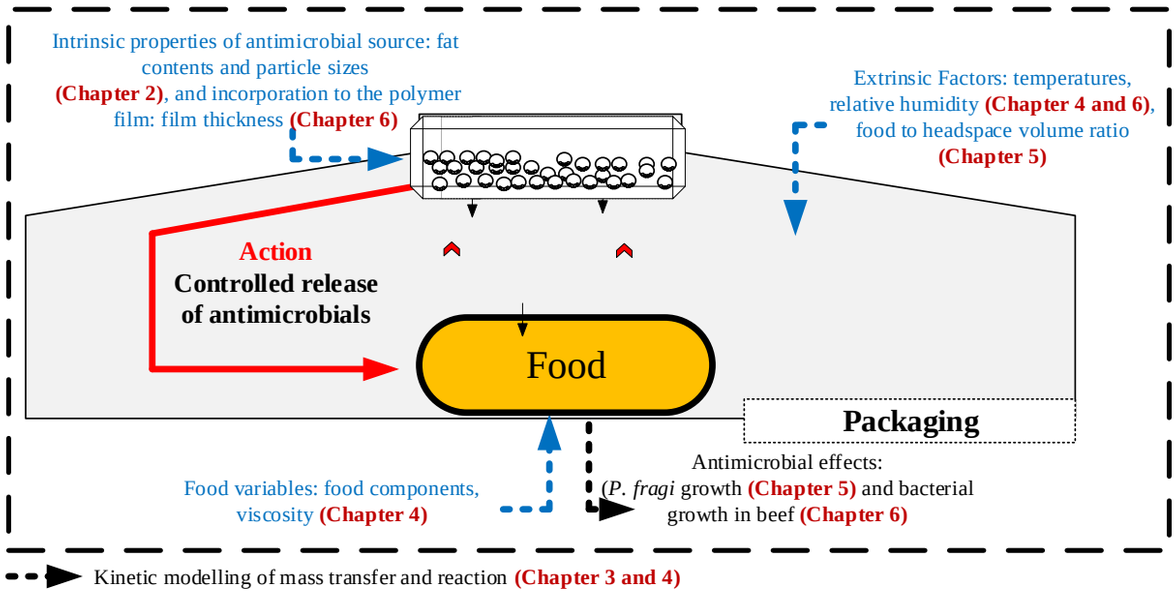


***Figure 1.6**. Schematic representation of the framework of this thesis. The (solid) red line shows an action directed to the food products; the (dash) blue line shows the factors affecting the action; the (dash) black line shows the effects of the action on the packaged food system and kinetic modelling covering for the whole food packaging system*

The first study aimed to investigate the effect of fat content and particle sizes of ground mustard seeds on the AITC release (**Chapter 2**). The cell damage of the ground mustard seeds was observed to relate to the sinigrin degradation in the ground seeds. The AITC concentration in the ground mustard seeds and headspace were measured to understand the influence of fat and particle sizes of mustard seeds on AITC release in the packaging headspace. In **Chapter 3**, the mechanisms of AITC formation in the mustard seeds and release and

degradation of AITC in the headspace by using multiresponse kinetic modelling were described.

As food contains macronutrients and has a physical structure that might affect the AITC stability in the food packaging system, in **Chapter 4**, the effect of food components and temperature on the AITC concentration is described, and its partition in the food packaging system using kinetic modelling is discussed. **Chapter 5** deals with the effect of food to headspace ratio and the amount of ground mustard seeds on the released AITC concentration and its antimicrobial effect on the growth of *P. fragi*, *a* dominant spoilage bacteria found in a variety of foods. The effects are important to understand how much mustard seeds need to be used to have an effective antimicrobial activity for a design of antimicrobial packaging using ground mustard seeds.

In **Chapter 6,** the development of a moisture-activated antimicrobial film containing ground mustard seeds and its application on meat are described. The effects of the polymer film thickness on moisture absorption, AITC formation, and release were investigated. For its application of the film on real foods, the effects of food composition, e.g. fat content on AITC concentration, and antimicrobial effect of AITC were also investigated. The shelf life extension of ground beef containing different fat contents was also predicted by modelling microbial growth.

Finally, **Chapter 7** provides the main findings and an integrated discussion of all studies in the thesis, as well as their implications for the future application of antimicrobial food packaging systems. Recommendations for future research are also provided.

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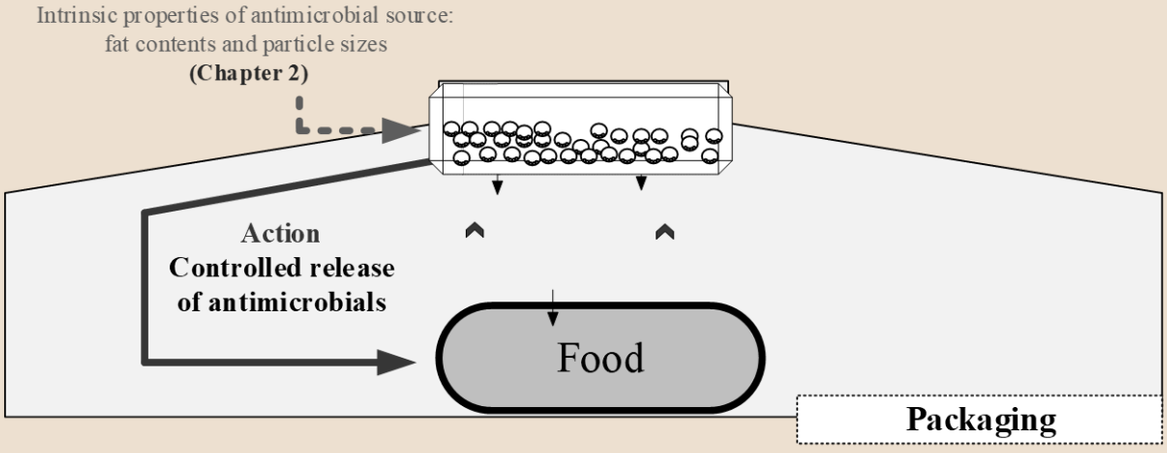
Intrinsic properties of antimicrobial source:
fat contents and particle sizes
(Chapter 2)
Action
Controlled release
of antimicrobials
Food
Packaging

2

Using particle size and fat content to control the release of Allyl isothiocyanate from ground mustard seeds for its application in antimicrobial packaging

This Chapter has been published as

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Abstract

In this study an active antimicrobial packaging based on the controlled release of allyl isothiocyanate (AITC) from mustard seed was designed. The effect of fat content and particle size of ground mustard seeds on formation and release of AITC was investigated, and the underlying mechanisms were highlighted. A smaller size of mustard particles resulted in more sinigrin conversion to AITC and a higher release of AITC in the headspace. The fat content has an important role in AITC release, a decreased fat content decreased AITC levels in the particles and increased the amount of AITC in the headspace. Based on the results of the sinigrin hydrolysis, the AITC surface exchange rate and the AITC fat solubility, an overall picture of the factors influencing the AITC release from the particles is proposed, which describes the formation of AITC and its partitioning between the compartments of the particles and the headspace.

Keywords: Allyl isothiocyanate, Controlled Release, Sinigrin, Mustard Seeds, Antimicrobial Packaging.

2.1. Introduction

Consumer demand for fresh and safe food products is increasing (Tilman and Clark, 2014; Wier et al., 2008). However, fresh food products create perfect circumstances for the growth of a variety of microorganisms that can spoil the food during storage (Buisman et al., 2017). The food spoilage often starts at the food surface, and active packaging that releases volatile antimicrobials into the headspace can effectively inhibit the growth of spoilage organisms at the food surface (Jin, 2017). These spoilage bacteria cause food products to be unacceptable for consumers from a sensory point of view and finally wasted. The control of spoilage bacteria by volatile antimicrobials is therefore commercially and sustainably interesting to retain the product quality for a longer time. The extended shelf life will help to reduce food waste which is a huge global problem in terms of sustainability and food security (Garnett, 2013).

Allyl isothiocyanate (AITC) has strong antimicrobial activity against a wide variety of spoilage and pathogenic microorganism at low concentration (Kurek et al., 2017). The presence of AITC in a vapour phase (indirect contact) is more effective than their direct addition to food product (Lin et al., 2000). At 2.5 ppm in the headspace, AITC vapour completely inhibited the growth of filamentous fungi, such as *Aspergillus parasiticus* (Lopes et al., 2018). AITC vaporised from pure mustard (3-10 µl) essential oils fully inhibits the growth of nine foodborne bacteria strains, including *S. enterica, S. aureus, B. cereus* and *E. coli OI57:H7* (Clemente et al., 2016) and ten mould strains*, A. flavus, B. fuckeliana, P. roqueforti,* etc., had been completely killed by 10 µl AITC (Clemente et al., 2019). AITC (25-50 µl/g) coated on acidified k-carrageenan/chitosan also reduced the growth of lactic acid bacteria (LAB) by 1.6 $\log_{10}$ CFU/g on chicken slices (Olaimat and Holley, 2016). Another way of application has been studied by using a sachet containing AITC (0.215 ppb), which was sufficient to reduce *A. flavus* (Otoni et al., 2014). However, the use of pure AITC is restricted in food packaging due to the extreme pungency and high volatility, which causes the AITC to quickly release into the food, interacting with food components and providing unpleasant off flavour (Dufour et al., 2015).

Mustard seeds can be used as a natural source to slowly release AITC into the headspace (Dai and Lim, 2014). This seed contains a high concentration of glucosinolates, which are the nitrogen and sulphur-containing metabolites acting as AITC precursors. Glucosinolates are present in plants of the Brassica family, like cabbage, broccoli, horseradish and wasabi (Dekker et al., 2009). The main glucosinolate compound found in mustard seeds is sinigrin, and AITC is enzymatically formed when sinigrin is hydrolysed by the endogenous plant enzyme myrosinase in a humid environment. Myrosinase is located in separate cells and can come into contact with sinigrin upon cell damage, e.g. during milling (Hanschen et al., 2018). Mustard seeds contain around 39-42% fat (Antova et al., 2017; Olgun et al., 2017), and the AITC released into the

headspace from fatty mustard seeds has been described to be lower than that from defatted seeds (Dai and Lim, 2014). The particle size of ground mustard seeds is expected to affect both the cell damage and the surface area that is available for AITC release. We, therefore, hypothesized that manipulation of the fat content and particles size is a way to optimise the release of AITC in the headspace of a mustard-based active antimicrobial packaging.

The release rate of AITC in a food packaging headspace will have consequences for the microbial growth inhibition in packed food (Gao et al., 2018). When the concentration of released AITC drops below the minimum inhibitory concentration (MIC), the inhibitory action of microbial growth will stop; consequently, the spoilage on the food surface starts and the shelf life is no longer extended (Appendini and Hotchkiss, 2002). In principle, shelf life can be extended by gradually releasing an antimicrobial compound and maintaining it at concentrations above the MIC value for a sufficiently long time. In this study, the controlled release of AITC in a food packaging environment was monitored by measuring the concentration of AITC released from ground mustard seeds into the headspace. We investigated the effect of particle sizes and fat content of the mustard seeds on the AITC release kinetics in order to understand the mechanism of the release of AITC in a food packaging headspace.

2.2. Materials and Methods

2.2.1. Materials

All chemicals used in this study were analytical grade and purchased from Sigma Aldrich or Merck. The mustard seeds (*Brassica juncea*) were purchased from natuurproduct.com, *Jacob Hooy Brown Mustard seeds.*

2.2.2. Sample Preparation

The fresh mustard seeds were weighted and then freeze-dried for 5 days (Christ Alpha 1-4 LD plus). The freeze-dried seeds were placed in desiccators for 15 minutes and then stored in the flask at -20^{0}C till usage. Afterwards, the seeds were milled in liquid nitrogen by using a milling machine (Fritsch; Pulverisette 14) at 12.000 rpm at the temperature as low as possible. All collected samples were <1 mm. The samples were collected and divided into four size ranges (50-100, 200-315, 400-500 and 630-800 µm) by using different sieves (Hosokawa Alpine Air Jet sieve 200 Ls). The seeds were defatted by the Soxhlet extraction method. Diethyl ether (boiling point of 35°C) was used as a solvent to avoid inactivation of myrosinase above 40°C (Okunade et al., 2015). Different extraction times and number of cycles were used to reach different fat contents; 29.1% (full-fat), 17.1% (partly-defatted), 2.8% (nearly-defatted), and 0.0% (fully-defatted). To determine the fat content of the samples, the flasks were weighted to determine the amount of extracted oil, thereby calculating the corresponding fat content in the ground seeds.

2.2.3. Determination of Sinigrin concentration

The method of determining the sinigrin content in mustard seeds with hot methanol as a solvent was modified from Oliviero et al. (2012). Around 0.1 g of mustard seeds were added to a 15 ml grainer centrifuge tube, and at the same time, methanol with a concentration of 70% and 100 % were incubated at 75^{o}C in a water bath. A 2.4 ml of the 100% preheated methanol was added to each sample, then followed by 0.2 ml of 3 mM glucotropaeolin as internal standard (IS). The tubes were incubated at 75^{o}C for 20 minutes while being vortexed every 5-minutes. Afterwards, the tubes were centrifuged (Thermo scientific; Heraeus Multifuge X3 R centrifuge) at 2500 rpm for 10 minutes at 4^{o}C.

After centrifugation, the supernatant was taken into a new tube, while the precipitate was re-extracted twice with 2 ml of 70 % preheated methanol, and those supernatants were combined with the previous supernatant. Around 6 ml of supernatant in total were stored at -20^{o}C until desulphation. In the desulphation, diethyl aminoethyl (DEAE) Sephadex columns were prepared firstly by dispersing 10 gr DEAE into 80 ml 2 M acetic acid and then incubating them overnight. Glass wool was added into a 2 ml syringe with a height of 1 cm, and then followed with 1 ml of the DEAE Sephadex ion exchanger solution and 1 ml the swollen DEAE. The DEAE column was washed twice with 1 ml of saturated water, and then 2 ml of the desulphated supernatants were added. To determine the retention time of sinigrin and glucotropaeolin, a solution of sinigrin (Sinigrin hydrate ≥99%) and glucotropaeolin (KVL Denmark,95%) was made and then added into another DEAE column. The columns of sample and reference were washed twice with 1 ml of sodium acetate 0.02 M. Afterwards, the syringes were transferred into new glass tubes. 75 µl of sulphatase enzyme solution was finally added into the syringe, which was then covered by parafilm. Those tubes were incubated overnight at room temperature.

The desulphated sinigrin was eluted from the columns by adding 0.5 ml Milli-Q water three times through a 25 mm syringe filter (phenomenex®; PHENEX.™-RC 45 syringe filter) into amber HPLC (High-Performance Liquid Chromatography) vials. The eluates were analysed by reversed-phase HPLC (Thermo Scientific; UHPLC+ focused Dionex Ulti-Mate 3000). The HPLC column used was A Lichrocart 125-4 column (Merck) at a flow rate of 1 ml/min with an injection volume of 20 µl. 100% Milli-Q water and 100% acetonitrile were used with as eluent A and B, respectively. UV-vis detector was set at 229 nm. Finally, the total running time was 26 minutes with a gradient elution as follows: 100% A and 0% B for 1 minute, then in 20 minutes to 0% A and 100% B, and in 5 minutes back to 100% A and 0% B. The result was then processed with Chromeleon 7. Sinigrin and IS were identified by comparing the retention time and the spectra with the reference, sinigrin and glucotropaeolin. Quantification of both was done with the help of the IS and the relative response factor (RRF) of sinigrin (1.053).

2.2.4. Determination of Allyl-isothiocyanate (AITC)

AITC was determined by the method of Oliviero et al. (2014) with minor adjustments. Around 100 mg of samples were weighed into a 15 ml Greiner centrifuge, then followed by 5 ml of dichloromethane (DCM) and 150 µl of internal standard (100 mM butyl isothiocyanate in MeOH). The samples were then centrifuged (Thermo Scientific; Heraeus Multifuge X3 R Centrifuge) at 3000 rpm for 5 minutes. The supernatant was collected by a Pasteur Pipette and then filtered into Klimax tubes through SPE columns with the help of a vacuum manifold fitted with a pump. The SPE columns (Strata® C18-E (55 µm, 70A), 3 ml SPE Tubes (0.5g)) were then washed with 1 ml of DCM. At the same time, the reagent was daily fresh prepared by adding 110 µl of 200 mM butanethiol and 14 µl of 20 mM triethylamine to 5 ml of DCM. 500 µl of the eluate and 25 µl of the reagent were transferred into the HPLC vials and then vortexed. Those HPLC vials were incubated for an hour at a heating block at 30^0C while being vortexed every 15 minutes. After incubation, the solvent was evaporated by using a Nitrogen (N_2) stream. The samples were finally re-dissolved in 750 µl of ACN and MilliQ 1:9. To quantify the concentration of AITC, calibration standards were then prepared using a 95% pure AITC standard solution dissolved in DCM. The range of used concentrations was 1 µM - 10000 µM. The DCM in the calibration standards was also evaporated with the N_2 stream and redissolved in 750 µl ACN: MilliQ (1:9).

The samples were analyzed using reversed-phase HPLC (Thermo Scientific; UHPLC + focused, Dionex Ultimate 3000) with 100% MilliQ (+ 0.1% formic acid) and 100% acetonitrile (+ 0.1% formic acid) as respective eluents A and B. An RP-18 column (Xbridge shield RP18; 3.0x 100 mm, 3,5 µm Waters) was used at a flow rate of 0.4 ml/min and an injection volume of 10 µl. The UV-Vis detectors were set to 274 nm, 290 nm and 240 nm. The total running was also 26 minutes with a gradient elution as follows: 80% A and 20% B for 1 minute, then in 20 minutes to 5% A and 95% B, and in 5 minutes back to 80% A and 20% B. The results were then processed using Chromeleon 7.2. The amount of AITC in the samples was finally quantified by the curve of the calibration standards.

2.2.5. Determination of Headspace AITC

Headspace AITC determination was performed by using GC-FID (Thermo-Scientific, Focus GC) and autosampler (Thermo-Scientific, TriPlus Autosampler), combined with SPME (100µm polydimethylsiloxane, red fibre, 23ga). 0.16 g of samples were added in Duran bottles of 10 ml and rehydrated with 0.132 ml of Milli-Q water (ratio; 1:0,825). The AITC release in the vial headspace was automatically measured triple overtime by the GC analysis at room temperature (~20^0C). A Restek Rxi-5HT GC column (30 meters, 0.25 mm internal diameter, 0.25 µm stationary film thickness) was used with an inlet temperature of 250°C, a splitless mode for 1 minute (flow 10ml/min). The oven was set at 40°C for the first minute, with a

temperature ramp of 10°C/min increasing till 280°C. The total running time was set at 26 minutes with 1ml/min Helium gas as a carrier. The detector was set at a constant temperature of 270°C, with a flow of 350ml air and 35ml of H_2. Analysis of the AITC was performed with Xcalibur software, in which AITC is known to be detected as two separate peaks (Malabed and Noel, 2014; Marton and Lavric, 2013). The calibration was quantified using pure AITC (Allyl isothiocyanate, 97%) dissolved in Hexane, with the range of used concentrations 1ppm – 250ppm. To calculate the concentration of AITC in the headspace, the SPME values were converted by using an experimentally determined relation between the SPME and the direct injection of headspace samples and calibration with known amounts of AITC in hexane using liquid injection.

2.2.6. Confocal microscopy

This microscopy method was adopted from Zahir et al. (2018) with few adjustments. Dye Nile red (0.05 wt%) and fluorescent dyes calcofluor white (0.002 wt%) were prepared to stain the cell wall and oil body measurement in the ground seeds. The dye mixture (30 µl) was added to the homogenised ground particles (30 µl) placed in a glass slide. The prepared samples were visualized by a confocal scanning laser microscope (LSM) type 510 (Zeiss, Oberkochen, Germany) using 405 nm blue/violet diode laser for calcofluor white and Nile red with an EC Plan-Neofluar 20x/0.5 A lens. All pictures were analysed with ZEN blue edition (Carl Zeiss Microscopy).

2.2.7. Statistical analysis

Results were expressed as the mean values ± standard deviations (SD). Significant differences among samples were analyzed with analysis of variance (one-way ANOVA with Tukey post hoc test), using the statistical software R studio. The significance level of the test statistic (p) was set at $p<0.05$.

2.3. Result and Discussion

2.3.1. Morphology of ground mustard seeds

The degree of cell damage upon grinding mustard seeds is shown in **Figure 2.1** for representative mustard particles of selected sizes and fat contents. The micrographs of mustard particles with 29% fat (**Figure 2.1a** and **2.1b**) show that most cells remained intact after milling, and strong adhesion between cells was present. The damaged cells can only be seen on the surface of the ground seeds. The damaged cells at the particle surface clearly demonstrate that for the bigger particles (**Figure 2.1a**), more intact cells remain compared to the smaller particles (**Figure 2.1b**) since the small particles have a larger surface to volume ratio. The defatted particles (**Figure 2.1c** and **Figure *2.1*d**) show more ruptured cells, both internal and on the surface of the particles. Most of the cells seem to be damaged during the

fat extraction (Wang et al., 2011), which disrupts cell membranes and result in permeabilization of the cell wall (Ando et al., 2016; Hanschen et al., 2018). Interestingly, a small amount of lipids was still observed in apparently intact cells in the core of particles.

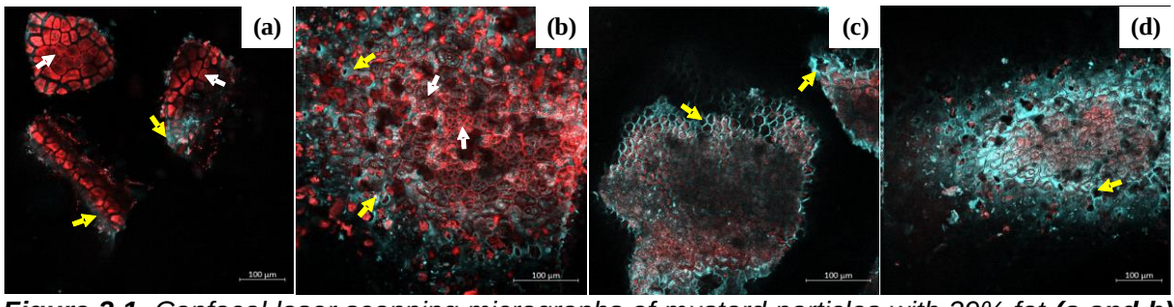


***Figure 2.1**. Confocal laser scanning micrographs of mustard particles with 29% fat **(a and b)** and 0% fat **(c and d)**. Cell wall (depicted in blue) and fat (depicted in red) were stained with calcofluor white and Nile red, respectively. Intact cells in all pictures are indicated by the white arrows, whereas cell damage was indicated by yellow arrows.*

Cell damage is connected to glucosinolate degradation: sinigrin, the substrate in the cell of mustard seeds, is located in the vacuoles of the cotyledon cells, while myrosinase can be found in separate myrosin cells (Nakano et al., 2014; Stauber et al., 2012). Upon tissue damage, myrosinase is released from the myrosin cells and can hydrolyse sinigrin into antimicrobial AITC (Nakano et al., 2014; Okunade et al., 2015).

2.3.2. The effect of particle sizes and fat content on sinigrin degradation

In **Figure 2.2,** the effects of the enzymatic degradation of sinigrin in mustard seeds having different particles and fat concentrations are shown. Results showed that the particle size significantly influenced sinigrin degradation in the full-fat particles (29.1% fat) (**Figure 2.2a**). The large particles (400-500 and 630-800 µm) showed much slower and lower sinigrin hydrolysis than the small particles (50-100 and 200-315 µm). Only around 30% of sinigrin in the large particles was degraded during 3 hours, compared with the small particles in which around 45% of sinigrin was degraded at the same time. The main effect of the particles size in the full-fat particles is the remaining sinigrin after 48 hours. Over 60% of sinigrin remained in the large particles, while only 30% of sinigrin remained in the small particles. The non-hydrolyzed sinigrin is likely due to the incomplete cell damage during milling, as supported by the micrographs shown in **Figure 2.1a** and **2.1b**. The bigger particles have less cell damage, and therefore more sinigrin is not accessible to the myrosinase action. A significant increase of glucosinolate transformation with decreasing the particle size was also reported by Sharma et al. (2012). The results are quite different in the defatted ground seeds (0% fat), as shown in **Figure 2.2b**: in this case, no significant difference in the percentage of converted sinigrin was observed in the large and small particles. After 3 hours, over half of the sinigrin in the defatted particles was already degraded for all sizes. The sinigrin concentration remained stable between 24 and 48 hours, and only 15 % of sinigrin was left after 48 hours for all particle sizes.

It can be concluded that particle sizes strongly influences the sinigrin degradation in full-fat samples due to different ratio between surface area and volume of the particles, while the small impact was found in defatted seeds due to the high cell damage that occurred during defatting.

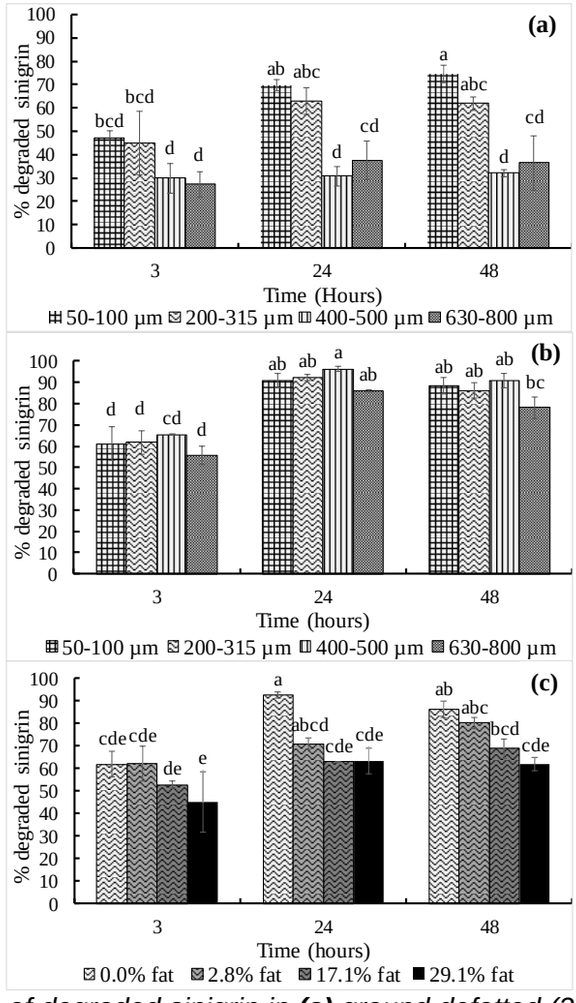


Figure 2.2. *Percentage of degraded sinigrin in* ***(a)*** *ground defatted (29.1% fat) and* ***(b)*** *full-fat mustard (0% fat) with different size ranges, and* ***(c)*** *ground mustard seeds (size 200-315 µm) with different fat contents. Different lower case letters indicate significant differences (P<0.05).*

Figure 2.2c, the effect of fat content on sinigrin degradation was shown. The fat-containing particles (2.75, 17.11, and 29.1%) show lower sinigrin degradation compared to the defatted seeds (0%). In defatted seeds, around 90% of sinigrin was enzymatically hydrolysed by myrosinase. The fat content is the most important factor influencing sinigrin degradation. The fat has induced less cell damage and has protected the intracellular cells of the ground seed from the milling process, which means only extracellular cell was damaged by the milling process as depicted **Figure 2.1b**. In **Figure 2.1c** and **2.1d**, more cell damage in fully defatted samples was caused by the solvent effect during fat extraction. Once the cell is damaged, myrosinase can easily diffuse into the cells to hydrolyse sinigrin, thereby forming AITC subsequently released in the headspace (Van Eylen et al., 2006).

2.3.3. The effect of particle sizes on AITC formation in the seeds and release into the headspace

Figure 2.3 shows the effects of particle sizes of the defatted and full-fat ground seeds on AITC concentration in the particles and headspace. In **Figure 2.3a**, the initial AITC concentration, before hydration, was in the range 30-55 µg/g in the full-fat particles, probably due to residual moisture that induces some myrosinase activity during milling (Dekker et al., 2009; Nakano et al., 2014). After 1 day, the concentration of AITC in the particles peaked at 70-93 µg/g and then decreased until day 9 where all particle sizes reached concentrations in the range of 4-7 µg/g. In the defatted particles (**Figure 2.3b**), the AITC concentration was at a low level over time. The initial AITC concentration was below 10 µg/g. This might be an indication that in the defatted seeds, almost all of the AITC is directly released into the headspace after formation, or it is lost by dissolving in the solvent during fat extraction. When the particles contain fat, the formed AITC is expected to be solubilized into the fat phase (Liu and Yang, 2010). It can be concluded that the particles size has no significant influence on the AITC concentration in the seeds.

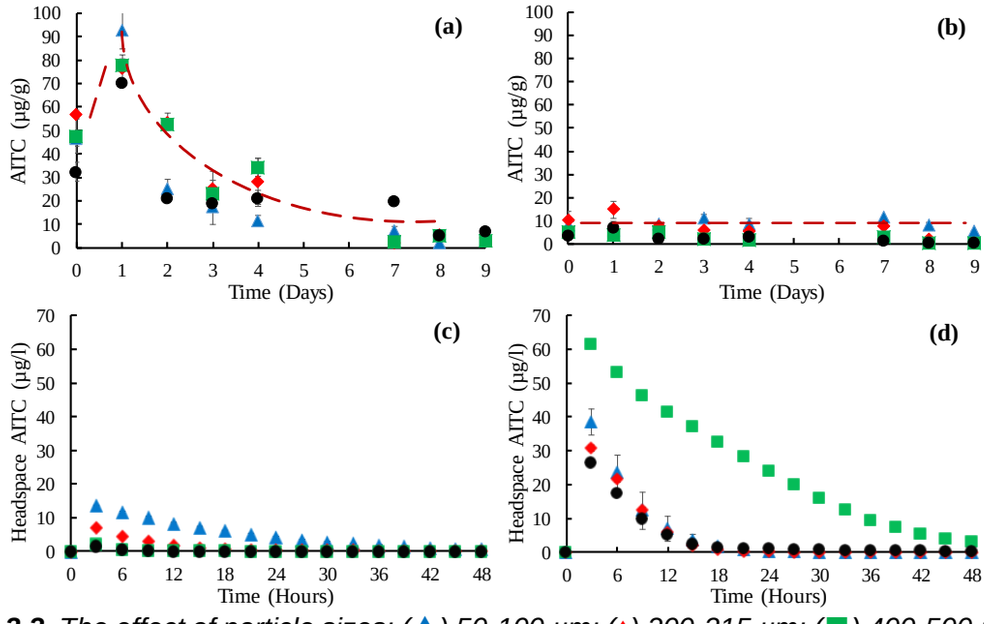


Figure 2.3. *The effect of particle sizes: (▲) 50-100 µm; (♦) 200-315 µm; (■) 400-500 µm; and (●) 630-800 µm on allyl isothiocyanate (AITC) concentration in the* ***(a)*** *full-fat seeds (29.1% fat) and* ***(b)*** *defatted seeds (0% fat) for 9 days, and headspace AITC released from* ***(c)*** *full-fat seeds (29.1% fat) and* ***(d)*** *defatted seeds (0% fat) during 48 hours.*

For the AITC release to the headspace, the particle size has a strong effect for both the full-fat (29.1% fat) and defatted particles (**Figure 2.3c** and **Figure 2.3d**). Smaller particle size resulted in higher AITC release in the headspace. Initially, no volatile AITC was detected in the headspace; however, after rehydration, the concentration of AITC in the headspace increases quickly within three hours. A preliminary experiment (data not presented) showed the concentration of AITC raised immediately after the rehydration and reached the highest levels

already between 45-90 minutes. **Figure 2.3c** shows that in three hours, around 15 and 8 µg/l of AITC was present in the headspace from the small particles (50-100 and 200-315 µm, respectively); while AITC from the bigger particles (400-500 and 630-800 µm) in the headspace was much lower around 4 and 3 µg/l, respectively. These AITC headspace concentrations were lower than those obtained from the defatted seeds (0% fat). In **Figure 2.3d**, in three hours, the concentrations of AITC in headspace reached around 28-40 µg/l (for the particle sizes: 50-100, 200-315 and 630-800 µm). The smaller particles released higher headspace AITC because of the higher surface-to-volume ratios of the seeds (Dai and Lim, 2014), which cause both fast diffusivities of moisture into the particles as well as of AITC out of the particles. This particle size effect was also reported by Dai and Lim (2014), who observed increased AITC release rates from ground mustard particles, compared to their unground counterparts. After a rapid peaking, the headspace concentration from all samples gradually dropped to 48 hours. This gradual drop can be explained by AITC degradation in the headspace due to its hydrolysis by the presence of moisture in the headspace at ~20^0C (Tsao et al., 2000). In the headspace, AITC degradation occurs due to an attack of nucleophiles, e.g. OH-, amino groups, and water, that can interact to functional N=C=S groups of AITC to generate other compounds, e.g. allylamine and carbonyl sulphide (Dias et al., 2013; Tsao et al., 2000).

The defatted particles with a size of 400-500 µm (**Figure 2.3d**) resulted in much higher headspace AITC. The much higher increase in volatile AITC is difficult to explain, it might be caused by specific optimal seed hydration due to the capillary forces being higher for this particular size, leading to faster sinigrin degradation in the sample. As shown in **Figure 2.3**, in this sample, the highest initial sinigrin degradation (almost 50%) at 3 hours was observed. After 48 hours, the percentage of degraded sinigrin was around 93%, which was higher than that of other samples. This figure suggests that an optimal release of AITC in the headspace can be obtained by regulating specific particle properties. The specific effects of particle structure and size on rehydration rate are also observed for a dried powder of fruit and vegetables (Karam et al., 2016).

2.3.4. The effect of fat content on AITC formation in the seeds and release into the headspace

Figure 2.4 shows the effects of different fat contents of the ground mustard seeds on AITC formation and release. A higher AITC concentration in the higher fat-containing particles was observed. This is likely caused by AITC migration to the fat phase in the seeds, which will improve its stability (Tsao et al., 2000). The higher remaining AITC in the fat phase (shown in Appendix 2.1). The calculation indicates that the majority of the formed AITC will be in the fat phase of the particles. It is clearly shown that the decrease of the fat content leads to a decrease of AITC concentration in the particles. **Figure 2.4a** also clearly shows the stability of AITC in the fat phase. The amount of AITC can only be maintained for 33 hours, compared to

the particles with the higher fat content that can be maintained for a longer time, around 3 mg/g for 9 days. This prolonged effect was caused by AITC is naturally oil-soluble, which means that its chemical hydrophobicity is greater than its hydrophilicity (Giroux et al., 2007).

As expected, the opposite trend was observed in the headspace. **Figure 2.4b** shows the amount of released AITC increased in the lower fat content of the particles. This is in line with Dai and Lim (2014), who also observed a lower release rate of AITC from mustard seeds meal powder (MSMP) than from defatted MSMP. Another interesting implication of this effect of fat content is that the AITC from defatted particles was no longer detected in the headspace after 36 hours, whereas the higher fat seeds maintained a certain level of AITC in the headspace (**Figure 2.4b**). This prolonged remaining level of AITC is important to prolong the inhibiting effect on the spoilage bacteria in the package. As found by Liu and Yang (2010), AITC has more stability when the fat content of the seeds increased. Otherwise, when the particles contain low fat, AITC partitions into the water phase and headspace (Giroux et al., 2007). Hence, the fat content of particles strongly influences the partitioning of AITC between the particles and the headspace.

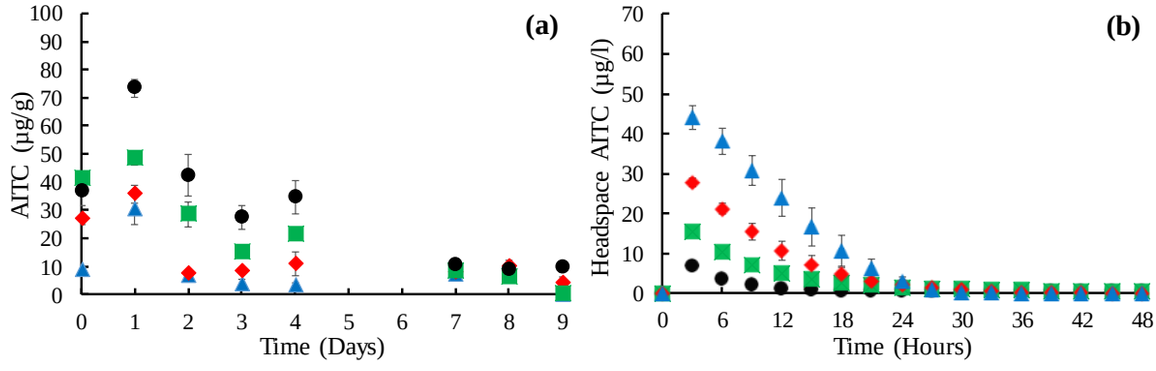


Figure 2.4. *The effect of fat contents : (▲) 0% fat; (♦) 2.8% fat ; (■) 17.1% fat; and (●) 29.1% fat in the seeds 215-300 µm on* ***(a)*** *allyl isothiocyanate (AITC) concentration in the seeds for 9 days,* ***(b)*** *headspace AITC concentration during 48 hours.*

The stability of AITC in the headspace due to the presence of fat can be optimal for microbial inhibition (Banerjee et al., 2015). In **Figure 2.4b**, the presence of fat (2.8, 17.1, 29.1% fat) still retained AITC at around 1 – 2.5 µg/l until 48 hours in the headspace. This AITC concentration is still below the MICs of AITC against the spoilage bacteria, e.g. *pseudomonas sp.* (54 µg/l), yeast (16-22 µg/l), aflatoxin-producing fungi (10 µg/ml), and moulds (16-62 µg/ml) (Isshiki et al., 1992), which means those bacteria are not sufficiently inhibited for 48 hours. To reach sufficient microbial inhibition, the amount of ground mustard seeds added in the packaging system can be increased to produce a higher AITC release, as observed by Torrijos et al. (2019), who reported that incorporation of 8 to 50 mg/g of oriental mustard flour into bioactive sauce increased the AITC concentration by 6 mg per litre packaging headspace. Otherwise, the microbial inhibition is expected to be higher initially in the absence of fat in the mustard particles

as higher AITC was released into headspace. According to those aforementioned MICs, yeast and mould might be inhibited for 15-18 hours before the bacteria start to quickly grow. From these results the usage of mustard particles without fat optimised release of AITC and the fat presence retains AITC in the headspace.

2.3.5. Correlation of AITC in the seeds with AITC in the headspace

Figure 2.5 shows the concentration of AITC in the particles versus the concentration in the headspace for all fat contents and particle sizes. An inverse proportional trend is observed: particle sizes and fat contents that result in a lower particle AITC concentration give a higher headspace AITC concentration. The effect of the fat content is larger than that of the particle size in this respect. Reducing by 29% fat in the particles diminished AITC concentration around 40 µg per gram particles and increased almost the same amount per litre headspace. Reduction sizes from 630-800 to 50-100 µm increased less than 20 µg of AITC in the headspace and particles. This correlation reveals mass transfer of AITC from the particles to the headspace due to the absence of fat in the particles.

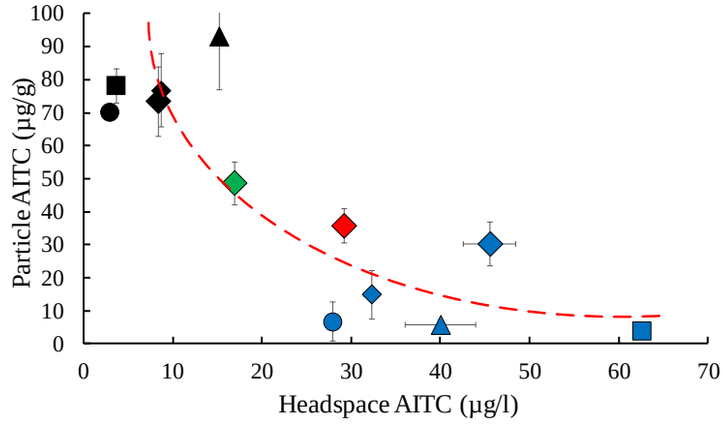


Figure 2.5. *The levels of headspace allyl isothiocyanate (AITC) (peak concentration measured after 3 hours) vs the levels of AITC in the particles (peak concentration measured after 1 day). Different shapes shows particle sizes; (▲) 50-100 µm; (♦) 200-315 µm; (■) 400-500 µm; and (●) 630-800 µm and different fat shows fat content; (♦) 0% fat; (♦) 2.8% fat; (♦) 17.1% fat; and (♦) 29.1%.*

2.3.6. The underlying mechanism of AITC formation and release

Based on the results obtained, a mechanism for the AITC release kinetics was proposed, as summarized in **Figure 2.6**. The mechanism describes the formation of AITC upon cellular damage followed by the hydration of the particles that activate the formation of AITC from sinigrin by myrosinase. This formed AITC is subsequently partitioning into either the aqueous phase, fat phase, solid phase and the headspace. As AITC is not stable, its concentration will finally decrease in all phases. The stability is highest in the fat phase, followed by the headspace and the aqueous phase.

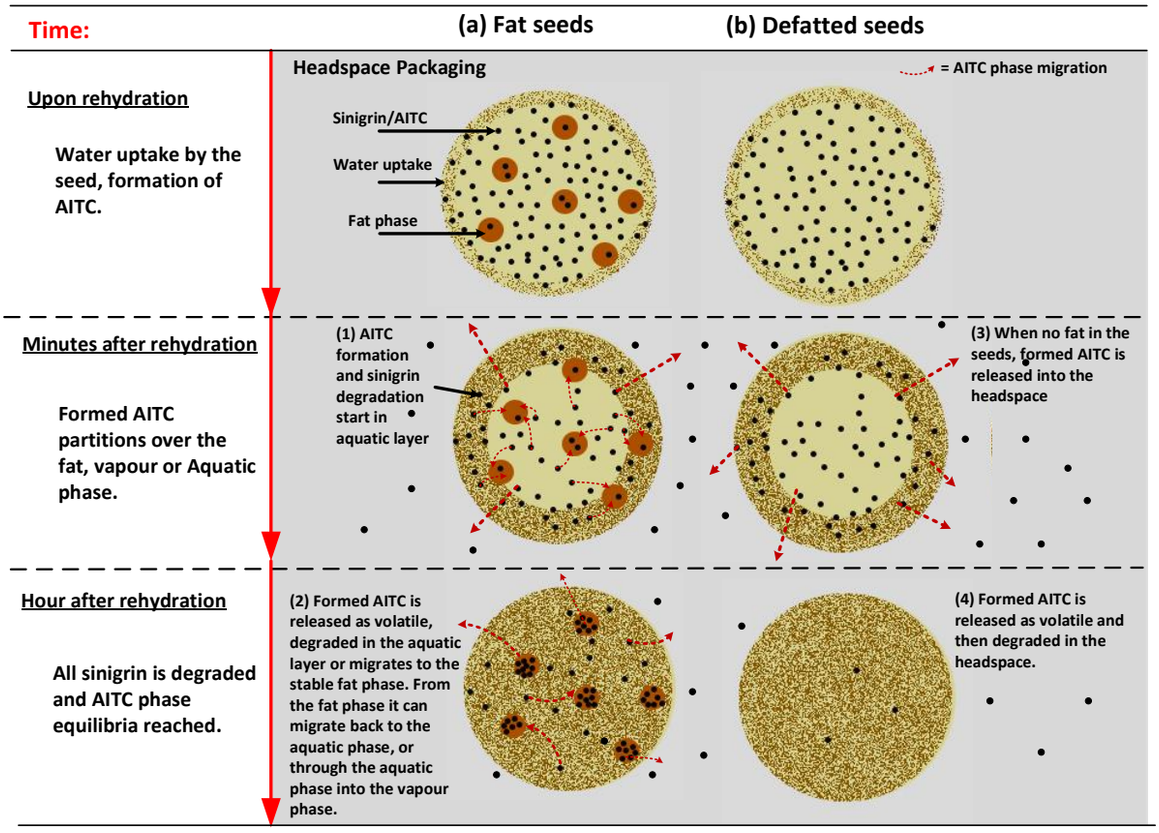


Figure 2.6. *The proposed molecular principle for allyl isothiocyanate (AITC) formation and phase migration after hydration over time;* ***(a)*** *fat and* ***(b)*** *defatted mustard seeds.*

This proposed mechanism describes the formation and partition of AITC over all phases. If fat is present, the majority of formed AITC migrates to this fat phase, thereby increasing the AITC concentration in the seeds and limiting the amount of released volatile AITC. Upon defatting, the fat phase in the seeds disappears, thereby decreasing the amount of AITC solubilized in the seeds and increasing the release of AITC to the headspace. Since the headspace concentration is relevant for microbial inhibition in packed food, the defatted ground mustard seeds induce a faster released of AITC in the headspace. However, a slow and continuous release is more desired and therefore, it is useful to keep some fat in the seeds to act as a reservoir of stable AITC that can be released at a slow rate for an extended period of time. Further research is needed to create a model that could foresee the partitioning into different food matrices, also taking into account the effect of external conditions like temperature or the nature of packaging material.

2.4. Conclusion

In this study, the effect of particle size and fat content of ground mustard seeds on sinigrin degradation, AITC formation, and AITC release into a packaging headspace was investigated. The concentration of AITC in the headspace is the result of the formation of AITC from sinigrin in the particles and the partitioning into the headspace. Sinigrin hydrolysis reaction was clearly

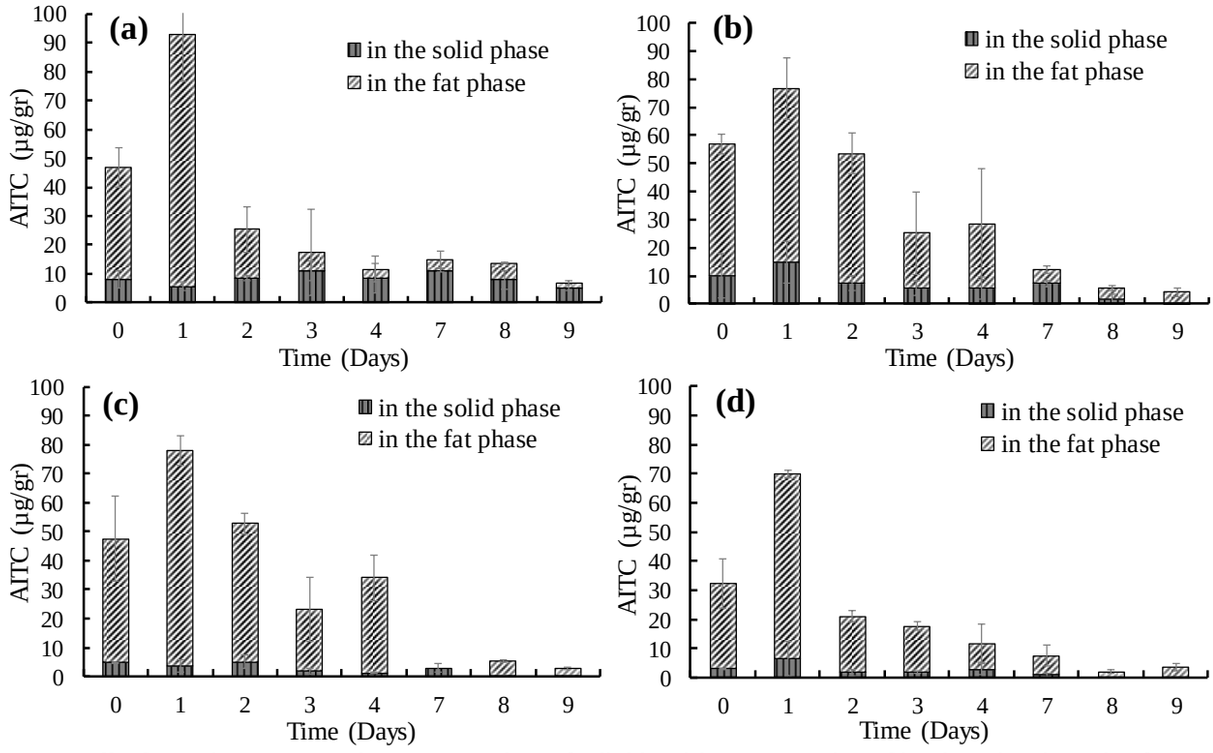
influenced by the sizes of the seeds, and a higher amount of sinigrin was degraded in the smaller ground seeds (50-100 µm). This higher degradation leads to a higher rate of released AITC in the headspace. The main factor influencing the concentration of AITC in the ground seeds was not the particle size but the fat content in the particles. This parameter, in turn, also influences the transfer of AITC into the headspace. Defatted seeds have the highest release of AITC in the headspace, while in the full fat, the AITC was solubilized into the fat phase and their release into the headspace was much slower. The higher fat content resulted in an extended-release period of the AITC from the seeds to prolong the time for its antimicrobial activity. These results support the design of an optimized active antimicrobial packaging by releasing AITC from ground mustard seeds in order to inhibit the growth of microorganism.

Acknowledgements

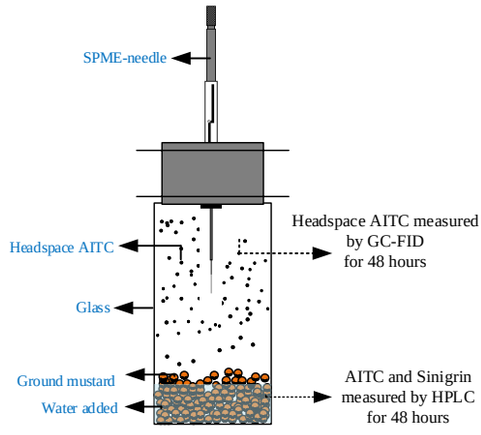
This work is supported by the Indonesia Endowment Fund for Education (LPDP). Geert Meijer is gratefully acknowledged for setting up the GC and HPLC for detecting AITC.

Appendix 2. Supplementary materials

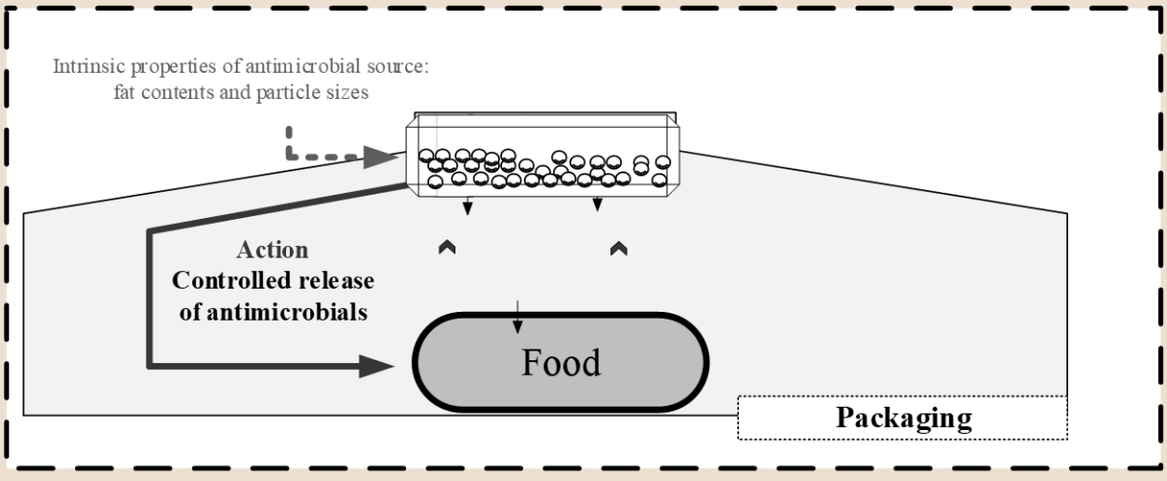
The following are the supplementary data to this article :



Appendix 2.1*. The assumed concentration of Allyl isothiocyanate (AITC) migrating into the fat phase in the full-fat seeds with different size;* ***(a)*** *50-100 µm;* ***(b)*** *200-315 µm;* ***(c)*** *400-500 µm; and* ***(d)*** *630-800 µm.*



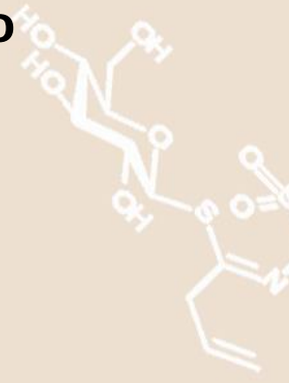
Appendix 2.2. *Model system used to measure the Allyl isothiocyanate (AITC) release in the gas phase. *GC-FID: Gas Chromatography Flame Ionization Detector, HPLC: High-Performance Liquid Chromatography, and SPME: Solid-phase Micro-extraction*



Kinetic modelling of AITC formation, release and degradation **(Chapter 3)**

3

Multiresponse kinetic modelling of the formation, release, and degradation of allyl isothiocyanate from ground mustard seeds to improve active packaging



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Abstract

This study aims to describe allyl isothiocyanates (AITC) formation from enzymatic sinigrin hydrolysis in ground mustard seeds and its release and degradation in the headspace using multiresponse kinetics modelling. The mechanistic modelling of the steps involved in the packaging system consists of a set of ordinary differential equations established from bio(chemical) reaction models combined with mass transfer models. The estimated parameters consist of the accessible sinigrin fraction, rate constants of sinigrin hydrolysis, AITC degradation in the particles and headspace, and its mass transfer coefficient. The model provides a good fit for experimental results and confirms the proposed mechanism of the AITC formation, degradation, and release inside the packaging system. Fat content has significant effects on AITC formation and release rate constants, while particle sizes significantly affect accessible sinigrin in the particles. These results give an understanding of AITC's controlled release by manipulating the mustard properties to optimize antimicrobial packaging designs.

Keywords: Allyl isothiocyanate; Mustard Seeds; Multiresponse Kinetic; Mechanistic Modelling; Mass Transfer; Antimicrobial Packaging

3.1. Introduction

Antimicrobial packages are currently being developed by the food industries for shelf life extension of food products by preventing the spoilage bacteria to grow at its food surface (Reyes-Jurado et al., 2019). Antimicrobial packages contain compounds that can be slowly released from antimicrobial carriers to food products with direct surface-food contact transfer or indirect headspace transfer (Wong et al., 2020). For indirect contact, the concentration of volatile compounds in the headspace determines the microbial growth rate in the food(Kurek et al., 2017). Therefore, controlling the release rate of the compounds to the headspace to retain sufficient concentrations has a major impact in effectively preventing bacterial growth to reach the desired product shelf life (Lorenzo et al., 2014; Quintavalla and Vicini, 2002).

Allyl isothiocyanate (AITC) is acknowledged as a strong antimicrobial agent and can be formed from the enzymatic hydrolysis of sinigrin, which is the main glucosinolate in mustard seeds (Reyes-Jurado et al., 2019). Once the cells of the mustard seeds are disrupted, e.g. during the grinding process and fat extraction, myrosinase hydrolyzes the sinigrin upon moisture exposure to yield AITC (Hanschen et al., 2018; Okunade et al., 2015). The formed AITC can be subsequently partitioned into phases with different stability; fat phase of the seeds (most stable), headspace (slightly stable), or aqueous phase (unstable). Bahmid et al. (2020b) reported that the AITC amount released from the ground mustard seeds still containing fat was lower than the AITC release from defatted ground mustard seeds (0% fat). The results indicate that the AITC prefers to move into the fat phase of the mustard particles rather than into the packaging headspace (Dai and Lim, 2014). To understand the AITC partition and stability, the kinetics of the release and degradation of AITC taking place in the seeds and headspace of the package needs to be evaluated by developing such a kinetic model.

Multiresponse modelling could be used as a powerful tool for describing physical and chemical processes using multiresponse data, such as multiple compounds involved in chemical reactions (Boekel, 2009; Verkempinck et al., 2019). This model approach is used to estimate parameters more accurately and is considered as a more comprehensive assessment of a proposed mechanistic model, compared to single response modelling (Quintas et al., 2007; Ziegel and Gorman, 1980). Some examples of studies using multiresponse modelling are on the understanding of the complex reaction mechanisms of lipid digestion (Verkempinck et al., 2019), caramelization, and Maillard reactions (Goncuoglu Tas and Gokmen, 2017; Kocadağlı and Gökmen, 2016). Applying multiresponse kinetic modelling in food packaging is still limited, whereas food packaging has a high complexity, including mass transfer/migration of compounds through the package, between the packaging material and the packaged food and reactions with food components.

A single modelling approach was applied to understand the release of compounds from a film in an active antimicrobial package by Kurek et al. (2017). The single response of the mathematical model used to describe the AITC release from the encapsulation of β-cyclodextrin (β-CD) from a polymeric matrix did not include the possible reaction in the film or the headspace, or absorption of the volatile in the packaged (model) food. Ignoring these mechanisms, which will also change the concentration in the film and headspace, will have led to less accurate predictions of the model parameters. A multiresponse kinetic model can, therefore, be a novel way to describe those changes of compound concentration in the whole packaging system, which benefits the understanding of the reactions and mass transfer involved in the packaging system in an integrated way.

The multiresponse kinetic modelling approach in this mechanistic study aims to describe the formation, release, and degradation of the AITC in the packaging system. The experimental data of sinigrin degradation, concentration AITC in mustard particles, and headspace investigated from our previous study (Bahmid et al., 2020b) were used. The fits of the developed kinetic model to the measured data of the sinigrin and AITC concentration in each phase of the package were evaluated to determine whether the established model was able to accurately describe the degradation and release of AITC. A better understanding of the reactions and mass transfer in the packaging system could help to optimize the mustard seeds properties and packaging design to effectively control the AITC release in antimicrobial packaging.

3.2. Theoretical Considerations

Sinigrin degradation is initiated upon tissue and cell damage in the mustard seeds (Okunade et al., 2015). Sinigrin is located in protein bodies or vacuoles of S-cells, and the hydrolyzing enzyme myrosinase is located in different compartments, called myrosin cells (Kissen et al., 2009; Nakano et al., 2014; Stauber et al., 2012; Torrijos et al., 2019). Once the tissue is disrupted by physical disruption or fat extraction, myrosinase instantaneously hydrolyzes sinigrin to form AITC in the presence of water (Dekker et al., 2009; Tetteh et al., 2019; Xiao et al., 2019). This mechanism of hydrolysis of sinigrin is called the “mustard oil bomb theory”. Lüthy and Matile (1984) developed this theory for the subcellular organization of the glucosinolate-myrosinase system in horse-radish roots (*Sinapsis alba*). This theory explains the stability of the glucosinolate-myrosinase system with a different compartment of the substrate and enzyme. The theory was then critically assessed by Kissen et al. (2009) that clarified that the glucosinolate hydrolysis by myrosinase happened only in the case of tissue damages.

The accessible sinigrin is enzymatically hydrolyzed by myrosinase upon the addition of water to yield AITC in the ground mustard seeds. In the mustard particles, the formed AITC can be degraded due to the water added to the packaging system. The water attacks the

sulfhydryl groups of AITC to form other compounds (Encinas-Basurto et al., 2017; Olaimat and Holley, 2016). At the same time, the volatile AITC also partitions into the headspace. From the headspace, the AITC has an antimicrobial effect on the food product in the package. In the headspace, AITC can react with moisture to form other compounds, as occurred in the mustard particles as well. These reactions and mass transfer pathways are schematically depicted in **Figure 3.1a**.

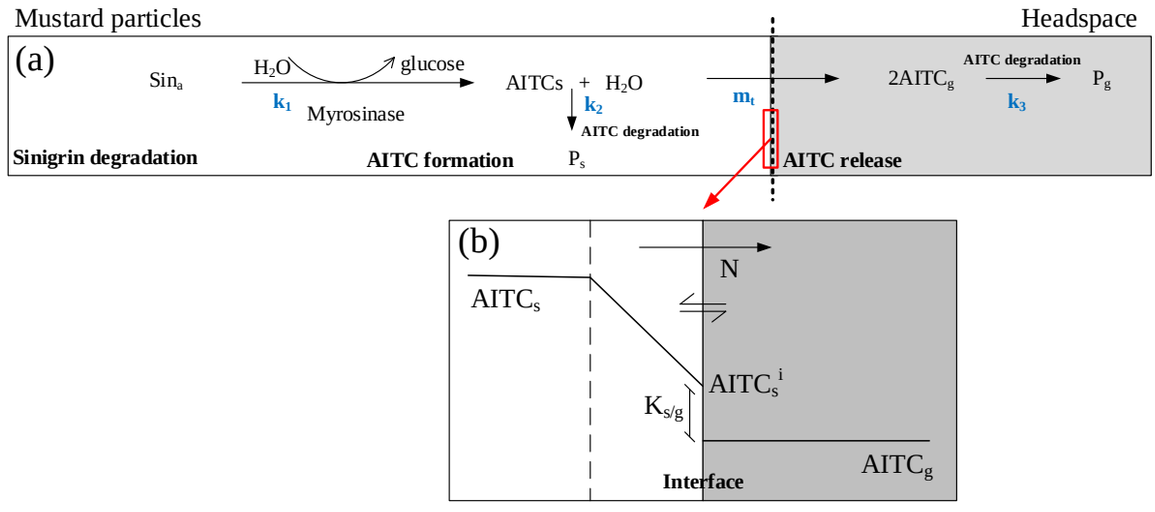

***Figure 3.1**. Schematic representation of allyl isothiocyanate (AITC) formation in mustard particles (white area) and AITC release in the headspace (grey area) **(a)** proposed reaction pathways on multiresponse modelling procedures, and **(b)** the schematic presentation of mass transfer of released AITC from the mustard seed particles into the headspace. N is molar flux, $k_{1\text{-}3}$ is the degradation rate constant of each phase, P_s and P_g are breakdown products of AITC in the solid and gas phase, respectively, $K_{s/g}$ is the partition coefficient, and m_t is interfacial mass transfer.*

Figure 3.1b shows the release of AITC from mustard particles into headspace, which can be specifically described by the theory of mass transfer from a solid to a gas (Harrison et al., 2006). In the mustard particle phase, AITC diffuses to the interface and is transferred from the ground mustard seeds, across the interface, into the headspace through unsteady state molecular diffusion. The main resistance of this transfer will be in the outer surface layer of the mustard seed particles. This mass transfer occurs until reaching the equilibrium in concentration between the ground mustard seeds and headspace.

3.3. Methods

3.3.1. Sample preparation

Mustard seeds were freeze-dried (Christ Alpha 1–4 LD plus) and then ground by using a milling machine (Fritsch; Pulverisette 14). The ground particles were sieved (Hosokawa Alpine Air Jet sieve 200 Ls) in different particle sizes (50-100, 200-315, 400-500 and 630-800 µm) and then defatted using soxhlet extraction till intended fat content (29.1%, 17.1%, 2.8%, and 0.0%) were reached. Mustard particles (0.16 g) were put inside the Duran bottles (10 mL)

and rehydrated with 0.132 mL of Milli-Q water (ratio (w/v); 1:0.825) as shown in **Figure 3.2**. The samples were stored at 20 ^{o}C for 48 hours, and sinigrin and AITC were measured at certain time intervals.

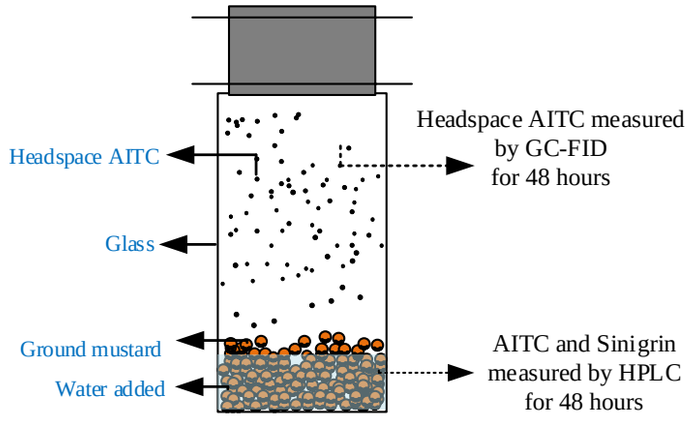

Figure 3.2. *The model system used to measure the allyl isothiocyanate (AITC) release in the gas phase. *GC-FID: Gas Chromatography Flame Ionization Detector, HPLC: High-Performance Liquid Chromatography, and SPME: Solid-phase Micro-extraction.*

3.3.2. Sinigrin and AITC measurement

Sinigrin ($C_{10}H_{16}KNO_9S_2$; 397.46 g/mol) and AITC (C_4H_5NS; 99.15 g·mol^{-1}) concentration in the mustard particles and AITC in the headspace were determined using the method described by Bahmid et al. (2020b). The analysis of sinigrin and AITC in the ground mustard seeds was done by extraction followed by High-Performance Liquid Chromatography (HPLC; Thermo Scientific; UHPLC + focused Dionex Ulti-Mate 3000) according to reported by Oliviero et al. (2012) with minor adjustments. The concentration of sinigrin in mustard particles was calculated with the help of glucotropaeolin as an internal standard, and the relative response factor (RRF) of sinigrin (1.053). The AITC concentration in the mustard particles was quantified by the curve of the calibration standards of pure AITC (Allyl isothiocyanate purchased from Sigma Aldrich Chemie GmbH, 97%).

Analysis of AITC in the headspace was performed using Gas Chromatography – Flame Ionization Detector (GC-FID; Thermo-Scientific, Focus GC) and autosampler (Thermo-Scientific, TriPlus Autosampler) for AITC in the headspace as described by Bahmid et al. (2020b). The calibration was done with pure AITC dissolved in Hexane (n-Hexane PEC grade, Actu-All chemicals), with the range of used concentrations 1ppm – 250ppm.

Data (mol compounds in litre volume of mustard particles or headspace) for modelling the sinigrin degradation and AITC release in the headspace were collected by sampling every three hours for 48 hours in mol/m^3. Data for modelling AITC remaining in the mustard particles were collected by sampling at 0, 24, and 48 hours. Each dataset was obtained using duplicate samples and expressed as the mean values ± standard deviations (SD).

3.3.3. Determination of the partition coefficient of AITC

The partition coefficient ($K_{s/g}$), the concentration ratio of AITC in the immiscible phase to the headspace as expressed in **Eq. (3.1)**, was measured using the method modified from Zhang et al. (2010)

$$K_{s/g} = \frac{AITC_s}{AITC_g} \quad \textbf{(3.1)}$$

where $AITC_s$ and $AITC_g$ are the concentrations of AITC in the immiscible phase and headspace, respectively.

To investigate the effect of fat content in mustard particles on $K_{s/g}$, emulsions with different oil (mustard oil extract) content (0, 2.8, 17.1, and 29.1%) were studied as (model) ground seeds. The oil was mixed with milli-Q and 0.5% of tween 20 as an emulsifier with an Ultra-turrax (IKA Werke GmbH & Co. KG) at 9500 rpm min^{-1} within 2 minutes. The unstable emulsion was subsequently homogenized using a homogenizer (Delta Instruments) for 10 minutes at 120 bar. Afterwards, AITC (100 mg/l) was added to the emulsions. Different volumes (1, 2, 3, 4, and 5 mL) of the emulsion were poured into 20 mL vials and immediately sealed gas-tightly. The samples were stored at 20°C for 3 hours to reach an equilibrium partition between emulsion and headspace. The method used to perform AITC measurement using GC-FID is described in a previous publication (Bahmid et al., 2020b). The partition coefficient ($K_{s/g}$) of AITC between emulsion and headspace was determined according to the phase ratio variation (PRV) method (Zhang et al., 2010), which is the relationship of $K_{s/g}$, β, and the peak area (A_e) as expressed below;

$$\frac{1}{A_e} = \frac{K_{s/g}}{f_o C_o} + \frac{1}{f_o C_o}\beta \quad \textbf{(3.2)}$$

where f_0 is a proportional factor ($A_e = f_0 \cdot C_e$), and C_o and C_e are the initial concentration of AITC in the seeds and the equilibrium AITC concentration in the emulsion, respectively; A_e is gas chromatographic peak area of AITC in the headspace at equilibrium; β is the phase volume ratio of headspace to the emulsion. The value of $K_{s/g}$ can be directly calculated from the ratio of the *y*-intercept to the slope of the regression line in the plot of $1/A_e$ versus β (Zhang et al., 2010).

3.3.4. Development of the kinetic model

The mathematical model was restricted to (bio)chemical reactions, solid-gas equilibria, and physical mass transfer processes. It consists of a combination of differential equations, equilibria, and mass balances for both the mustard particles and headspace phases. The following assumptions were stated;

1. All hydrolyzed sinigrin is converted in AITC on a molar basis.
2. The mass transfer takes place from the mustard particles phase to the gaseous phase, the mustard particles are loosely packed, and the surface area of each particle is in contact with the gaseous phase.

3. The mass transfer to the gaseous phase is described with the stagnant film model. The conditions justify that the mass transfer in the gaseous phase can be assumed not to be a limiting factor.
4. The mustard particles are considered to be one phase. No separate aqueous, solid, and fat sub-phases are considered in the model.

The AITC formation by sinigrin hydrolysis catalyzed by myrosinase can be described by a first-order reaction (Hanschen et al., 2018). Part of the sinigrin can be in inaccessible parts of the mustard seeds that were not damaged by the grinding process. Therefore, this inaccessible sinigrin needs to be included in the first-order reaction to describe well the mechanism of sinigrin hydrolysis. The initial hydrolysis of accessible sinigrin fraction leads to the following differential equation for fractional conversion first-order kinetics;

$$\frac{d[Sin_{tot}]}{dt} = -k_1\,[Sin_a] = -k_1\,[Sin_{tot} - Sin_i] \qquad \textbf{(3.3)}$$

where Sin_{tot} is the concentration of total sinigrin in particles (mol/m^3), Sin_i and Sin_a are the concentration of inaccessible and accessible sinigrin (mol/m^3) respectively, t is degradation time (h), and k_1 is the rate constant of sinigrin degradation and AITC formation (h^{-1}). The formation (f) of AITC in the mustard particles is subsequently described by:

$$\left.\frac{d[AITC_s]}{dt}\right|_f = k_1\,[Sin_a] = k_1\,[Sin_{tot} - Sin_i] \qquad \textbf{(3.4)}$$

where $AITC_s$ is the concentration of AITC volatiles (mol/m^3) within the mustard particles.

The formed AITC can either be transferred to the headspace or degrade in the mustard particle. The AITC transfer to the headspace can be calculated from the penetration theory and the equilibrium constant (Harrison et al., 2006). The transfer to the headspace is determined by the mass transfer coefficient (m_t). From the theory, the mass transfer across the interface can be described by the following equation (Backhurst and Harker, 2001);

$$N = -m_t\left[AITC_s^i - AITC_s\right] \qquad \textbf{(3.5)}$$

where N is molar flux (mol/m^{2}h), m_t is the mass transfer coefficient (m/h). $AITC_s^i$ and $AITC_s$ are the concentration of AITC volatiles at the interface (mol/m^3) and within the mustard particles (mol/m^3), respectively. As N is an unknown variable, the molar flux in **Eq. (3.5)** is integrated into the mass transfer rate (m_t) of AITC in the solid and headspace described by the following equations:

$$\left.\frac{dAITC_s}{dt}\right|_{mt} = -\frac{m_t\,A}{V_s}\left[AITC_s - AITC_g\;K_{s/g}\right] \qquad \textbf{(3.6)}$$

$$\left.\frac{dAITC_g}{dt}\right|_{mt} = \frac{m_t A}{V_g}\left[AITC_s - AITC_g\;\;K_{s/g}\right] \qquad \textbf{(3.7)}$$

where t is time (h); AITC and V are AITC concentrations and volume of each phase (m^3), respectively, and the subscripts s and g denote seed particles and gas phases, respectively.

The AITC can simultaneously be degraded in mustard particles and packaging headspace. Hydroxyl groups (e.g. from water) attack sulfhydryl groups of AITC to generate

another compound, e.g. allyl-dithiocarbamate and pentasulfide (Tsao et al., 2000; Weerawatanakorn et al., 2015). As the amount of added water is in large excess and hardly changes over time during sinigrin hydrolysis, the mechanism of the AITC degradation in mustard particles can be described with the pseudo-first-order kinetics (Jiang et al., 2006). Yet the amount of water present in mustard particles is known, so the AITC degradation can be described by the following kinetic model equation:

$$\left.\frac{d[AITC_s]}{dt}\right|_{ds} = -k_2\,[AITC_s]\,[H_2O] \qquad \textbf{(3.8)}$$

In the headspace also AITC degradation takes place due to the presence of water vapour, as shown later in the results and discussion, was best described by a second-order reaction:

$$\left.\frac{d[AITC_g]}{dt}\right|_{dg} = -k_3\left[AITC_g\right]^2 \qquad \textbf{(3.9)}$$

where k_2 and k_3 in m^3/mol.h are the rate constants of AITC degradation in the seeds and headspace, respectively, and H_2O is the amount of water per volume of the seeds (mol/m^3).

3.3.5. Multiresponse kinetic modelling

The proposed pathway (**Figure 3.1a**) consists of three responses to the experimental data; sinigrin concentration in the mustard particles, AITC concentration in mustard particles, and the headspace. Those three responses were estimated using a set of differential equations of mass transfer and reaction enumerated in **Eq. (3.10)**-**(3.12)**.

$$\left.\frac{d[Sin_{tot}]}{dt}\right|_{sum} = \frac{d[Sin_{tot}]}{dt} \qquad \textbf{(3.10)}$$

$$\left.\frac{d[AITC_s]}{dt}\right|_{sum} = \left.\frac{d[AITC_s]}{dt}\right|_{f} + \left.\frac{dAITC_s}{dt}\right|_{mt} + \left.\frac{d[AITC_s]}{dt}\right|_{ds} \qquad \textbf{(3.11)}$$

$$\left.\frac{d[AITC_g]}{dt}\right|_{sum} = \left.\frac{dAITC_g}{dt}\right|_{mt} + \left.\frac{d[AITC_g]}{dt}\right|_{dg} \qquad \textbf{(3.12)}$$

These three differential equations were simultaneously fitted to the experimental data. The estimated parameter values consist of the inaccessible sinigrin fraction (Sin_1), rate constants (k_1, k_2, and k_3), and mass transfer coefficients (m_t). These parameters were estimated by multiresponse non-linear regression with the determinant criterion (Boekel, 2009) using Athena Visual Studio software (v.14.2) (AthenaVisual Inc.). In the parameter estimation solver control panel, standard options were used; the convergence criterion (= 0.01), the maximum number of iterations (=30), estimation solver options (non-linear least-squares, gradient calculation (forward differences scheme) and relative perturbation step size (10^{-3}). In total, 41 parameters were estimated based on 1836 triplicate measurements from 17 experiments, with 36 responses giving 612 data points to fit by the model. The goodness of fit of the kinetic models was then evaluated by examining the generated size and distribution of the residuals, the R-square of predicted vs observed data, and the standard deviations and correlations of the parameters.

The degradation rate in the headspace (k_3) was assumed to be independent of the fat content and particle size. The degradation rate in the particles (k_2) was assumed only to be independent of the particle size. The dependency of the partition coefficient ($K_{s/g}$) on the fat content of the particles was taken from the experimental determination explained in **Section 3.3.3.** The initial concentrations (shown in Appendix 3.1 and 3.2) were set equal to the observed initial concentrations of sinigrin and AITC in the non-hydrated mustard particles, while the initial concentration in the headspace was set equal to zero. Parameters and constants used in the modelling with known values are given in Appendix 3.3.

3.3.6. Statistical analysis

Significant differences were evaluated to investigate the effects of particle sizes and fat content on parameter estimates. Statistical significance ($p<0.05$) was evaluated based on the method developed by Julious (2004) using the confidence intervals around individual means of the parameter estimates to assess statistical significance between two means.

3.4. Results and Discussion

3.4.1. Partition Coefficient ($K_{s/g}$)

The $K_{s/g}$ was thus calculated from the relationship as expressed in **Eq. (3.2)** and summarized in **Figure 3.3**. The phase ratio variation (PRV) method used to calculate $K_{s/g}$ was depicted in Appendix 3.5. The volume ratio (β) was linearly proportional with the $1/A_o$ in all different samples, which shows a good relationship between β and $1/A_o$ with R-square > 0.95.

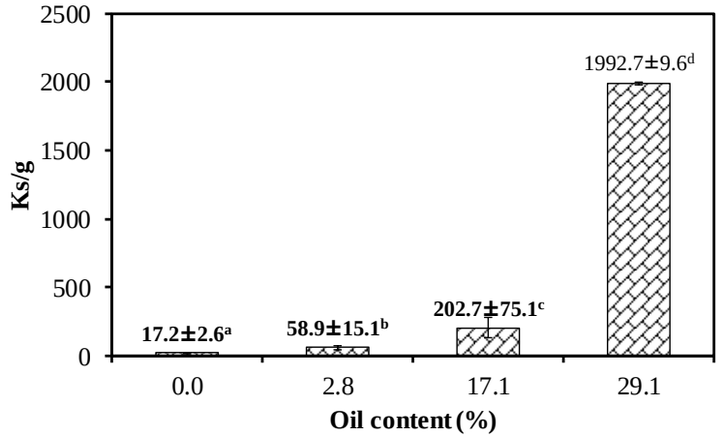

Figure 3.3. *Partition coefficient ($K_{s/g}$) of allyl isothiocyanate (AITC) with different fat content. *Means with the same lower case did not differ significantly at p <0.05.*

The calculated value of $K_{s/g}$ significantly and linearly increased in the emulsion with higher oil content, as shown in **Figure 3.3** ($p<0.05$). The emulsion containing over 17.1% fat content suddenly reduced the volatility of AITC in the headspace, in which the $K_{s/g}$ value of emulsion containing 29.1% fat was ten times higher than that of the emulsion containing 17.1% fat. It indicates that the higher fat content more effectively suppressed the volatility of the AITC

in the headspace. The $K_{s/g}$ value of emulsion with 29.1% mustard oil (1992.7 ± 9.6) is in line with the value observed by Zhang et al. (2010) using 30% canola oil, which was 1962.9 ± 502.4. Furthermore, the linear increase of $\log_{10} K_{s/g}$ value indicates that AITC has a higher affinity for the bigger volume of fat phase in an emulsion (Keppler et al., 2018), and the low value of $K_{s/g}$ for the low-fat contents indicated the high volatility of AITC in an increase in water volume in the emulsion. These results are a useful parameter for the developed multiresponse kinetic modelling.

3.4.2. Mechanistic multiresponse model

In **Figure 3.4**, the multiresponse model in **Eq. (3.10)-(3.12)** describes observed data of sinigrin and AITC concentrations in the headspace well, including AITC concentration in the mustard particles (Appendix 3.6). Linear regression of the predicted vs observed data plot provided slopes very close to 1.0 (**Figure 3.5**).

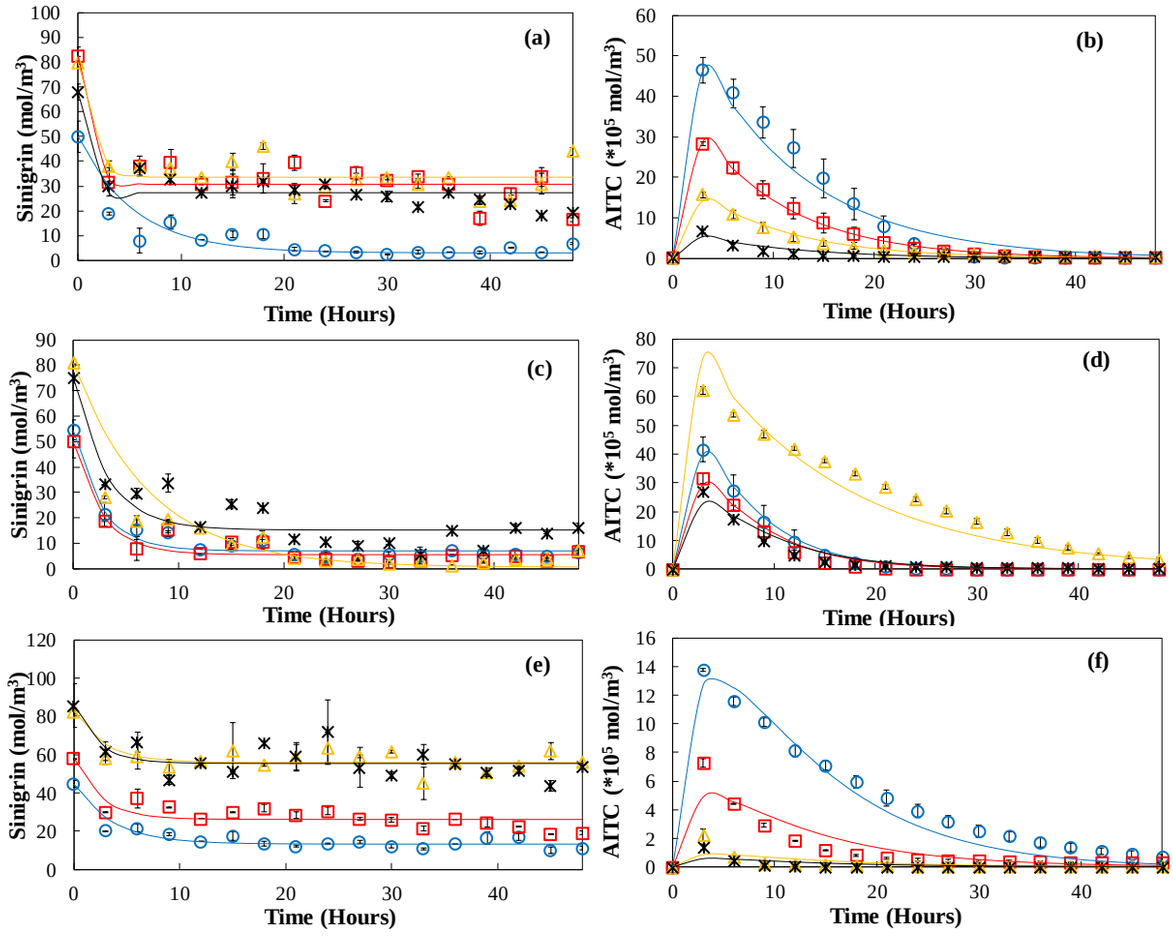

Figure 3.4. *Kinetic model fit (lines) to the experimental data (symbols) of sinigrin concentration* ***(left)*** *and allyl isothiocyanate (AITC) concentration in the headspace* ***(right)****.* ***(a, b)*** *Different fat content (○: 0, □: 2.8, △:17.1, x:29.1%), particle sizes (200-315 µm);* ***(c, d)*** *Fat content (0%), different ranges of particle sizes (○: 50-100, □: 200-315, △:400-500, x:630-800 µm);* ***(e, f)*** *Fat content (29.1%), different ranges of particle sizes (○: 50-100, □: 200-315, △:400-500, x:630-800 µm).*

There are some considerations on the assessment of the goodness of fit. **Figure 3.5a** shows several horizontal clustering of data, which indicates very similar predicted data while there is considerable fluctuation in the observed data. These predicted data were the result of the fractional conversion first-order kinetics, in which the same predicted data indicated the minimum value of sinigrin fraction accessed by myrosinase, while there is still experimental variation around these values. In **Figure 3.5b**, the predicted data of AITC in the headspace has two overestimated points at high concentration. These data points are from the observed data of the defatted particles with sizes of 400-500 µm. The results of this particle size show an unexpected and peculiar result which caused a very high standard deviation in the parameter estimates (**Table 3.2**) for this sample, compared to the other particle sizes. As discussed in our previous study (Bahmid et al., 2020b), the unexpected data shows fast hydrolysis, possibly due to the capillary forces being higher for the microstructure of this particular size, to completely degrade the sinigrin. In addition, the model did not satisfactory fit the peak value (underestimated) and the degradation rate (overestimated) of the measured data of headspace AITC concentration (**Figure 3.4f**), whereas the model did perform well on the sinigrin concentration. These deviations were observed for the bigger mustard particles (400-500 and 630-800 µm) in the 29.1%-fat particles releasing low amounts of AITC. A possible reason is that at low AITC concentration in the gaseous phase and high amounts of fat in the mustard particles, the equilibrium between headspace and mustard particles is competing with the equilibrium between the aqueous and fat phase inside the mustard particles (Keppler et al., 2018), this can result in a homogenous distribution of AITC in the mustard particles resulting in the observed deviations.

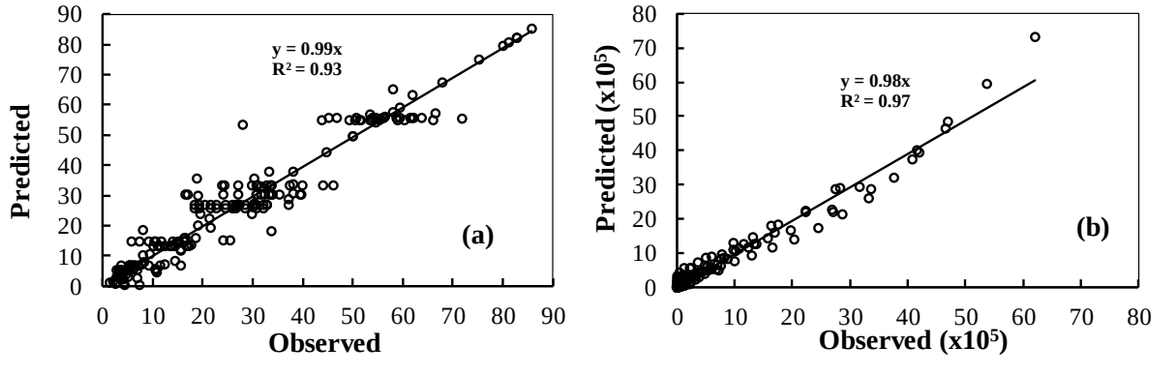

Figure 3.5. *Predicted against observed values for the established model for all batches of* ***(a)*** *sinigrin concentration,* ***(b)*** *allyl isothiocyanate (AITC) in the headspace. In all graphs, the units are mol/m³.*

Overall, the model described the observed data very well. Therefore, the established multiresponse model proposed is well applicable, and the reaction and mass transfer pathways (**Figure 3.1**) in the packaging system can describe the antimicrobial active packaging system very well.

3.4.3. Interpretation of the estimated parameters from the established multiresponse kinetic model

The kinetic parameters and their standard deviation for this multiresponse kinetic model are summarized in **Tables 3.1** and **3.2**. A low-standard deviation of the parameters (**Tables 3.1** and **3.2**) and low parameter correlation coefficients <0.8 (Appendix 3.7) were found. The effects of particle size and fat content on the estimated parameters obtained from the established kinetic model are discussed in detail below.

Table 3.1. *The estimated parameters from the multiresponse kinetic model for the effect of fat content*

Fat (%)	Sizes (µm)	Parameters				
		k_1 (h^{-1})	Sin_i (mol/m^3)	k_2 (x10^6 m^3/mol/h)	k_3 (x10^{-5} m^3/mol/h)	m_t (x10^7 m/h)
0.0	200-315	0.18±0.02^a	3.12±1.61^a	7.63±1.89^c	3.02±0.30	186.94.±74.45^c
2.8		0.90±0.37^{ab}	30.76±1.49^c	3.20±0.27^a		21.40±4.30^b
17.1		0.76±0.39^{ab}	33.60±1.56^c	3.93±0.25^b		4.25±2.21^a
29.1		1.96±0.51^b	27.33±0.88^b	3.69±0.08^{ab}		0.47±0.66^a

Values represent means ±standard deviation. Small letters indicate significant differences between particles with different fat content.

Table 3.2. *The estimated parameters from the multiresponse kinetic model for the effect of particle size of the mustard seed particles with 0 and 29.1% fat.*

Fat (%)	Sizes (µm)	Parameters				
		k_1 (h^{-1})	Sin_i (mol/ m^3)	k_2 (x10^6 m^3/mol/h)	k_3 (x10^{-5} m^3/mol/h)	m_t (x10^7 m/h)
0.0	50-100	0.37±0.03^b	7.09±1.48^b	7.63±1.89^b	3.02±0.30	20.07±8.04^a
	200-315	0.37±0.08^b	5.52±1.58^b			35.80±15.07^a
	400-500	0.14±0.01^a	0.68±1.82^a			8272.12±13048.00^a
	630-800	0.32±0.05^b	15.07±1.61^c			44.39±19.04^a
29.1	50-100	0.27±0.03^A	13.45±1.54^A	3.69±0.08^a		2.19±1.08^A
	200-315	0.39±0.06^{AB}	26.18±1.46^B			0.86±1.10^A
	400-500	0.34±0.04^{AB}	55.97±1.46^C			0.06±0.42^A
	630-800	0.43±0.07^B	55.30±1.45^C			0.04±0.40^A

Values represent means ± standard deviation. Small letters indicate significant differences between the different sizes of particles with 0% fat, while capital letters indicate significant differences between the different sizes of particles with 29.1% fat.

Sinigrin hydrolysis and AITC formation

The estimated kinetic parameters (the rate constants (k_1) and inaccessible sinigrin (Sin_i)) of the modified first-order model **Eq. (3.3)** are shown in **Tables 3.1** and **3.2**. **Table 3.1** shows that k_1 decreased significantly with defatted particles, compared to full-fat (29.1% fat) particles as presented in **Table 3.1** ($p<0.05$) and **Table 3.2** shows no significant effects of particle sizes on k_1 in general ($p>0,05$), except for the 400-500 µm-sized defatted particles. The lower k_1 in defatted particles was not our expectation; as in our previous study, it was observed that there was more cell damage in the defatted particles. A possible reason for slower k_1 is a difference in sensitivity for cell damage for different cell types. Kissen et al. (2009) have shown that

sinigrin and myrosinase are located in different compartments, where myrosinase is located inside myrosin cells and sinigrin in S-cells. The fat extraction and particle reduction might not damage the myrosin cells in a similar way as the S-cells, resulting in less myrosinase release in defatted particles.

The cell damages resulted in more sinigrin being accessible to myrosinase, and more AITCs are formed (Hanschen et al., 2018; Wang et al., 2011). In **Tables 3.1** and **3.2,** for the smallest defatted particles ($p<0.05$), indeed, less remaining sinigrin (Sin_i) after 48 hours is observed. The presence of the fat in ground seeds causes its intracellular cells to remain intact as the fat content protects the cells from the grinding process. Consequently, the higher sinigrin concentration was left in the ground seeds containing fat content. Additionally, a sudden increase in the inaccessible sinigrin for the big particles (400-500 and 630-800 µm) in **Table 3.2** is likely induced by incomplete cell damage. These results confirm the previous study that observed micrographs of the big particles containing less cell damage.

AITC release from mustard particles into packaging headspace

The mass transfer kinetic model **Eq. (3.6)** and **(3.7)** described the AITC release from mustard particles into headspace well. **Tables 3.1** and **3.2** show that the mass transfer coefficient (m_t) was significantly affected by the fat content ($p<0.05$), while no effect of particle sizes was observed ($p>0.05$). **Table 3.1** shows the m_t-values of the fat mustard particles (17.1 and 29.1% fat) were significantly lower than the m_t-values of the particles with lower fat content. This result was in line with the result found by Dai and Lim (2014); the release rate constants value of defatted mustard seed meal powder (MSMP) were faster than the normal MSMP. The fat in mustard particles reduced the release of AITC from the fat-particles, as the AITC was solubilized in the lipid phase (Tsao et al., 2000). As particle size did not significantly affect the AITC release, it indicates that the higher AITC concentrations observed in **Figures 3.4d** and **3.4f** were driven by the higher accessible sinigrin fraction available in the mustard seeds leading to more formation and then release of AITC into the headspace. This indicates that cell damage in the particle is crucial to make sinigrin accessible and get a higher AITC concentration in the headspace. These results may have important consequences for the formulation of mustard particle properties with controlled-release profiles of AITC and its application in the packaging.

AITC degradation in mustard particles and headspace

The reaction kinetics in **Eq. (3.8)** and **(3.9)** were applied to describe AITC degradation in mustard particles and headspace, respectively. The degradation rate constant in the particles (k_2) was significantly increased in particles without fat (**Table 3.1** and **3.2**) ($p<0.05$). The high solubility of AITC in the fat phase caused the lower degradation rate of AITC in the mustard particles phase with fat. In the continuous phase (aqueous phase of particles), AITC

degradation is faster, while the compounds in the dispersed phase (lipid phase) were more stable (Liu and Yang, 2010). This degradation rate of AITC in mustard particles shows that the presence of fat is the best way to stabilize the AITC concentration for a longer time.

The second-order degradation rate constant in the headspace (k_3) was estimated at around 3.02±0.30 x10^5 m^3/mol.h. As no reaction kinetics for this reaction was described in the literature, zero-order, first-order, fractional conversion first-order, and second-order kinetics were tried to fit the experimental data of the headspace AITC concentration. When fitting the model to the experimental data, only the second-order model in **Eq. (3.9)** can adequately describe the AITC degradation in the headspace without a trend in the residuals and an estimated parameter with a low standard deviation.

The effectiveness of AITC as an antimicrobial agent relies on its concentration in the packaging system (Dufour et al., 2015; Saladino et al., 2017a). Once the concentration of AITC in the packaging system is lower than its minimum inhibitory concentration (MIC) against certain bacteria, the bacteria will grow and spoil the foods. AITC degradation is accounted for its stability to maintain the capability of AITC to keep the inhibition of bacteria. The breakdown products of AITC, e.g. allyl-dithiocarbamate and pentasulfide have no antimicrobial activity (Tsao et al., 2000; Weerawatanakorn et al., 2015), so besides the controlled release of AITC in the packaging system, the rate of degradation in the packaging system needs to be hindered to maintain the concentration above MIC. Further study related to other factors reducing the AITC degradation in the headspace, like temperature (Saladino et al., 2017a), is required to retain AITC in the headspace to have a longer antimicrobial inhibition in foods.

3.5. Conclusion

The multiresponse kinetic model combining the mechanisms of (bio)chemical reactions and mass transfer of AITC inside the packaging system described the experimental observations well. This model produced a good prediction of the enzymatic hydrolysis of sinigrin, AITC formation, its release, and degradation in the mustard particle and headspace. Defatting the mustard particles has positive impacts on some parameters, e.g. increasing the accessible sinigrin and lowering the formation rate constant and increasing the mass transfer coefficient between the particles and the headspace. The particle size has a small effect on the estimated parameters, and no significant effect on the release of AITC into the headspace was found. The results also confirm our previous study that fat content has a significant effect on the partitioning of AITC in the packaging system. A better understanding of the kinetics of the concurrent reactions and mass transfer in the packaging system by this study can help to optimize the mustard seeds properties to effectively control the AITC release for antimicrobial packaging design. A similar multiresponse modelling approach, as described in this paper, can be applied for future applications, with other components used in the active packaging concept.

Acknowledgement

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Nomenclature/Abbreviation

A = the surface area of the interface mustard particles (m^2)

A_e = gas chromatographic peak area of AITC in the headspace at equilibrium

AITC = Allyl isothiocyanates

$AITC_g$ = concentration of AITC in the headspace (mol/m^3)

$AITC_s$ = concentration of AITC in the particles (mol/m^3)

$AITC_s^i$ = concentration of AITC in the interface of the particles (mol/m^3)

C_e = equilibrium AITC concentration in the emulsion

C_o = initial concentration of AITC in the seeds (mol/m^3)

d = diameter of ground mustard seeds/mustard particles (m)

f_0 = proportional factor ($A_e = f_0 \cdot C_e$)

fat = fat content in the seeds (%)

GC-FID= Gas Chromatoghraphy – Flame Ionization Detector

H_2O = concentration of added water in-ground seeds (mol/m^3)

HPLC = High-Performance Liquid Chromatography

k_1 = rate constant of sinigrin degradation and AITC formation (h^{-1})

k_2 = rate constant of AITC degradation in the mustard particles (h^{-1})

k_3 = rate constant of AITC degradation in the headspace ($m^3/mol/h$)

$K_{s/g}$ = equilibrium partition coefficient ground mustard seeds and headspace (dimensionless)

MIC = Minimum Inhibitory Concentration

m_s = mass of mustard particles (g)

m_t = mass transfer coefficient from mustard particles into headspace (m/h)

N = molar flux (mol/m^2h)

p_g = breakdown products in the headspace

p_s = breakdown products in the mustard particles

Sin_a = concentration of accessible in mustard particles sinigrin (mol/m^3)

Sin_i = concentration of inaccessible sinigrin in mustard particles (mol/m^3)

Sin_{tot} = concentration of total sinigrin in particles (mol/m^3)

t = time (h)

V_g = volume of headspace (m^3)

V_s = volume of mustard particles (m^3)

V_{vi} = volume of glass vial (m^3)

β = phase volume ratio of headspace to the emulsion

ρ_s = density of mustard particles (g/m^3)

Appendix 3. Supplementary Materials

The following are the supplementary data to this article:

Appendix 3.1. *The initial concentration of sinigrin and allyl isothiocyanate (AITC) in the mustard particles with different fat content*

Fat content	Particle size (200-315 µm)	
	Sinigrin (mol/m^3)	**AITC** (mol/m^3)
0%	50.05±6.38	0.06±0.01
2.80%	67.91±3.30	0.19±0.04
17.10%	79.96±2.50	0.30±0.06
29.10%	82.70±3.13	0.27±0.10

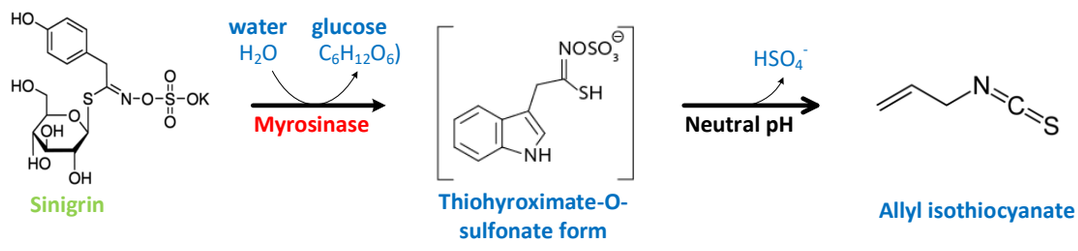
Appendix 3.2. *The initial concentration of sinigrin and allyl isothiocyanate (AITC) in the 0%-fat and 29.10%-fat mustard particles with different sizes*

Particle size	Fat content (0%)		Fat content (29.10%)	
	Sinigrin (mol/m^3)	**AITC** (mol/m^3)	**Sinigrin** (mol/m^3)	**AITC** (mol/m^3)
50-100 µm	54.55±3.97	0.06±0.03	44.68±1.75	0.34±0.07
200-315 µm	50.05±6.38	0.07±0.08	50.09±0.19	0.41±0.04
400-500 µm	81.03±1.35	0.04±0.00	82.36±1.39	0.35±0.16
630-800 µm	75.26±5.28	0.02±0.00	85.62±11.20	0.24±0.09

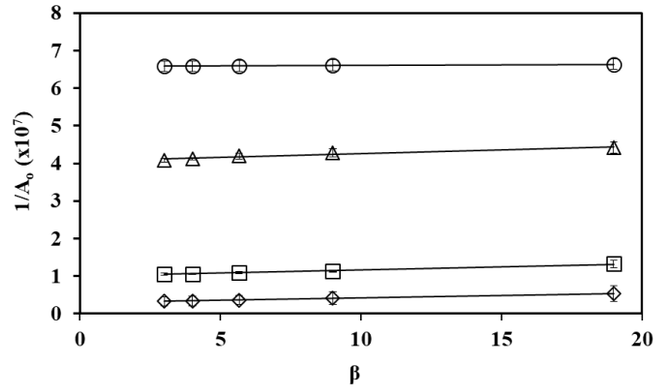
Appendix 3.3. *The known input values used in the modelling equations*

The known inputs	Abbreviation	Value
Mass of mustard particles (g)	m_s	0.16
The concentration of added water in-ground seeds (mol/m^3)	H_2O	52800
The density of mustard particles (g/m^3)	ρ_s	720000*
The volume of the glass vial (m^3)	V_{vi}	10^{-5}
The volume of mustard particles (m^3)	V_s	2.22 x10^{-7}**
The volume of headspace (m^3)	V_g	9.78x10^{-6}***
The surface area of 50-100 µm-sized particles (m^2)	A_1	0.0178****
The surface area of 200-315 µm-sized particles (m^2)	A_2	0.0052****
The surface area of 400-500 µm-sized particles (m^2)	A_3	0.0030****
The surface area of 630-800 µm-sized particles (m^2)	A_4	0.0018****

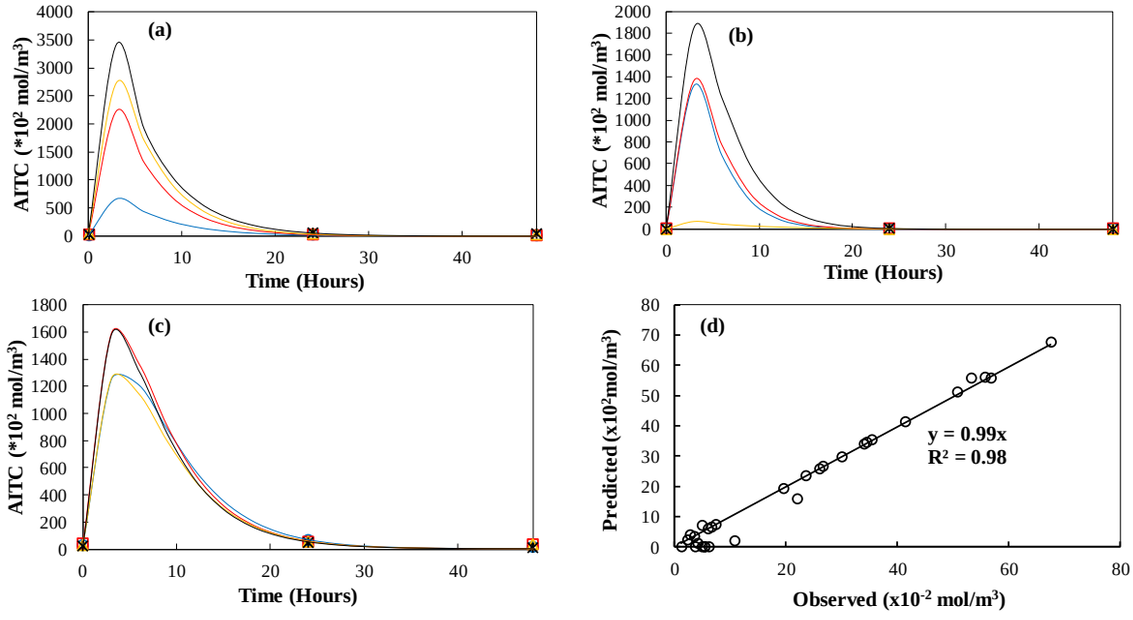
*density found from Ropelewska et al. (2018)
**volume mustard particles obtained from the total mass of mustard seeds divided density
***volume headspace obtained from the total volume of glass vial minus the volume of mustard seeds used
****surface for each particle obtained from the formula to calculate the area using the total volume of particles and average of the particle size range



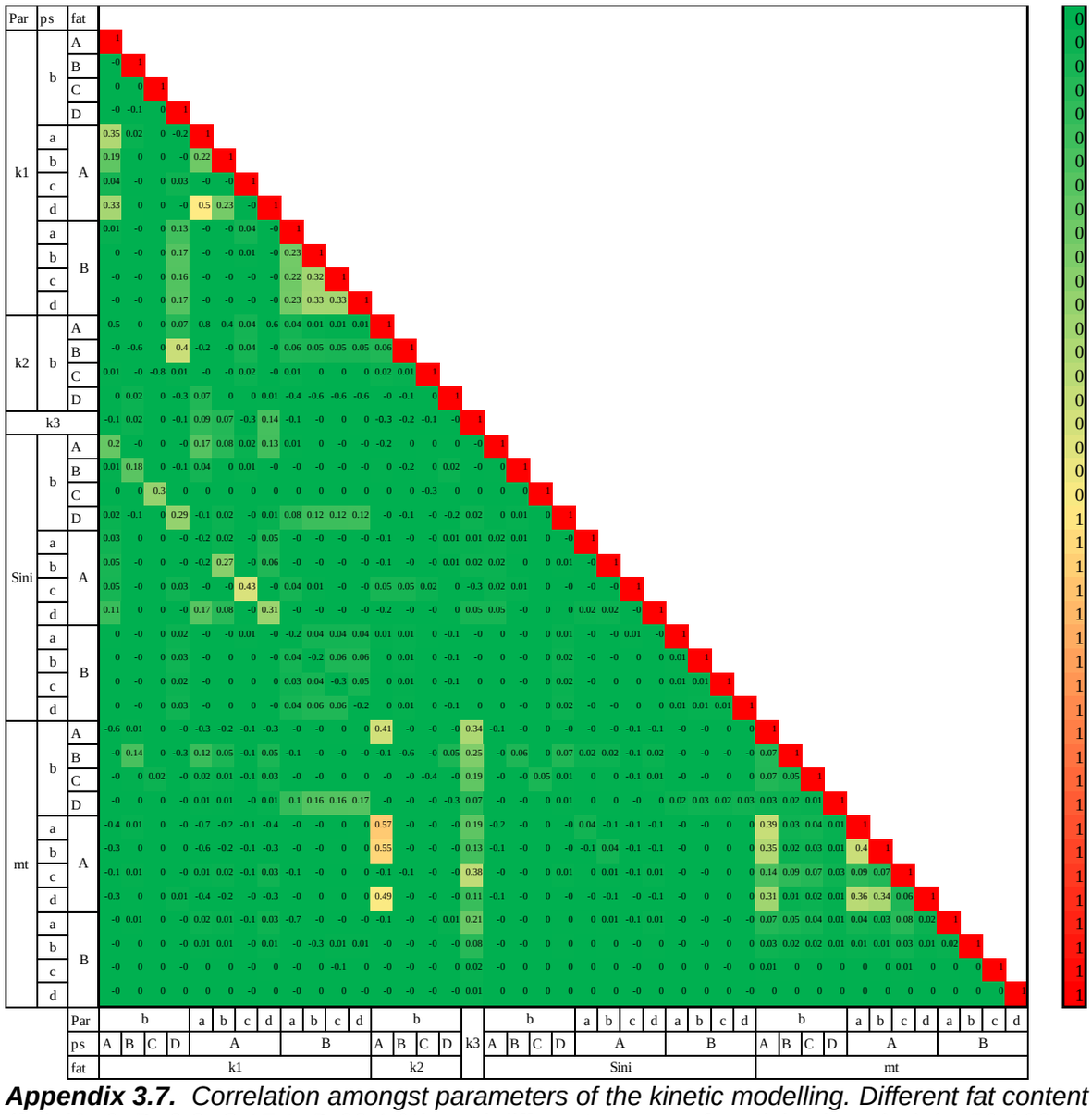
Appendix 3.4. *A schematic reaction of allyl isothiocyanate (AITC) formation adapted from Shofran et al. (1998).*



Appendix 3.5. *Reciprocal peak area (1/A$_o$) of volume ratio of headspace to emulsion containing different fat content; 0% (◊), 3% (□), 18% (△), and 30% (o);*

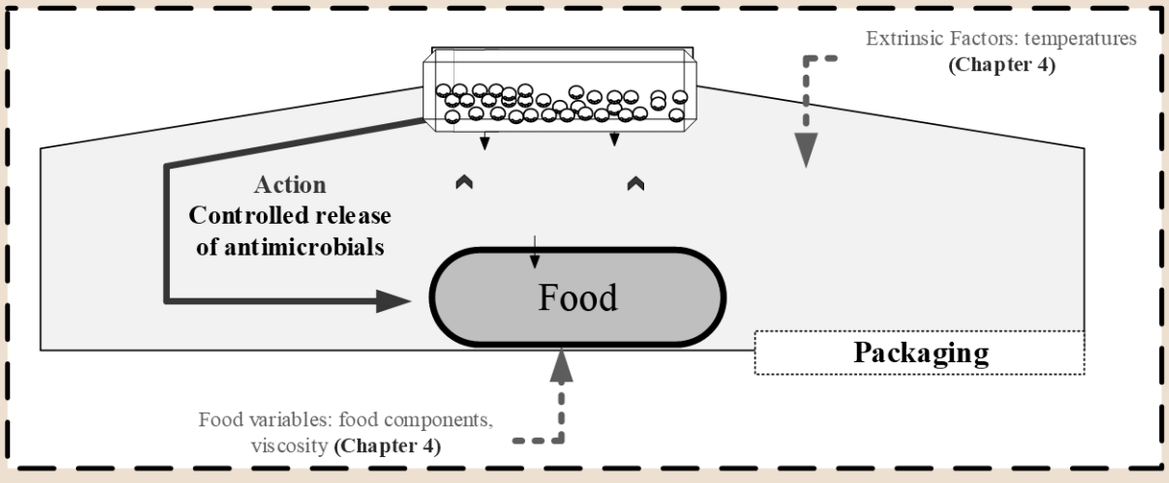


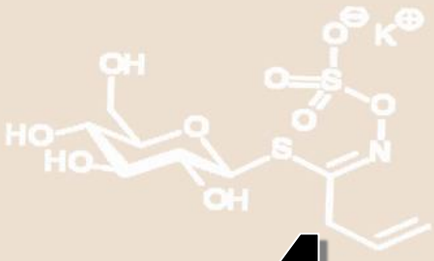
Appendix 3.6. *Kinetic model fit (lines) to the experimental data (symbols) of allyl isothiocyanate (AITC) concentration in the seed particles* ***(a)*** *Particles containing different fat content (○: 0, □: 2.8, △:17.1,* **x***:29.1 %), particle sizes (200-315 µm);* ***(b)*** *Particles containing 0% and* ***(c)*** *29.1% fat with different ranges of particle sizes (○: 50-100, □: 200-315, △:400-500,* **x***:630-800 µm));* ***(d)*** *Predicted against observed values for the established model for all batches of AITC concentration in mustard particles.*



Appendix 3.7. *Correlation amongst parameters of the kinetic modelling. Different fat content (fat) (**A**: 0, **B**: 2.8, **C**:17.1, **D**:29.1 %), and different ranges of particle sizes (ps) (**a**: 50-100, **b**: 200-315, **c**:400-500, **d**:630-800 µm). Green to red colour in the bar shows correlation coefficient from 0 to 1.0.*

3


Extrinsic Factors: temperatures
(Chapter 4)
Action
Controlled release
of antimicrobials
Food
Packaging
Food variables: food components,
viscosity (Chapter 4)
Kinetic modelling of AITC release, absorption, and degradation (Chapter 4)



4

Modelling the effect of food composition on antimicrobial compound absorption and degradation in an active packaging

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Abstract

This study aims to comprehensively describe the rate of AITC release, absorption, and degradation in the packaging system by using multiresponse kinetic modelling. The effects of food composition and temperature on allyl isothiocyanates (AITC) partitioning in an antimicrobial packaging system were investigated. A higher protein content in the food caused a higher AITC concentration in the headspace, which can be explained by a lower mass transfer coefficient of AITC transfer from the headspace into the food matrix. A higher fat content in the food matrix caused a lower AITC concentration in the headspace, which can be explained by the fat stimulating AITC partitioning into the food matrix. At a lower temperature AITC is more stable in the headspace and food matrix. The results can be used to optimize the design of a packaging system with an AITC concentration tailored to the packed food product.

Keywords: Allyl isothiocyanate; Mass Transfer; Absorption; Multiresponse Kinetic; Kinetic Modelling; Antimicrobial Packaging

4.1. Introduction

Mustard seeds can be used as a natural source of AITC. Mustard seeds contain a high content of glucosinolate, sinigrin, which is a precursor of allyl isothiocyanate (AITC) (Rangkadilok et al., 2002; Saladino et al., 2017a). AITC is formed from the enzymatic hydrolysis of sinigrin by the endogenous enzyme myrosinase (Cools and Terry, 2018; Hanschen et al., 2018). The sinigrin and myrosinase are located in distinct cells in the seeds, respectively, in S-cells and myrosin cells (Kissen et al., 2009; Nakano et al., 2014). Once the cell tissues of mustard seeds are damaged by physical disruptions or by fat extraction, for example, myrosinase is activated by moisture to hydrolyze sinigrin to generate AITC (Dekker et al., 2009). The mechanism of AITC formation in the mustard seeds shows a potential application of the mustard seeds as a natural carrier to release AITC into a packaging headspace to effectively inhibit microbial growth in foods (Bahmid et al., 2020a).

The antimicrobial effect of AITC against a variety of foodborne and spoilage bacteria depends on the concentration in the headspace (Aguilar-González et al., 2015; Clemente et al., 2016). A controlled release of AITC is important to reach a minimum headspace AITC concentration that is sufficient to inhibit bacteria for a prolonged time. In previous research, smaller particles (50-100 μm) of mustard seeds containing 0% fat content released a higher AITC content to the headspace (Bahmid et al., 2020b). However, the presence of a (model) food in the food package causes the AITC released from ground mustard seeds to partition between the headspace and the packaged (model) food (Liu and Yang, 2010). The partition might consequently reduce the AITC concentration in the headspace, where the AITC more effectively inhibits the surface bacteria than bacteria growing inside the (model) foods due to interactions with the food components.

Food is a complex system, which contains a variety of components. For the application of AITC in food packaging, food components, like fat and proteins, might chemically and/or physically interact with the AITC absorbed into the food products and then reduce the antimicrobial activity against spoilage bacteria. The AITC concentration can be affected by the presence of fat due to the solubility of AITC in the lipid phase (Keppler and Schwarz, 2017; Liu and Yang, 2010). In the presence of proteins, AITC can covalently bind amino acids, like β-lactoglobulin, from whey protein isolate (Ersöz and Dudak, 2020; Holley and Patel, 2005; Kawakishi and Kaneko, 1987; Keppler et al., 2018; Kuhn et al., 2018). AITC can also react with (di)sulfide groups in proteins and with free amino acids, like lysine, glycine, and arginine, to produce reaction products, such as allylamine and allyl thiourea (Cejpek et al., 2000; Winther and Nielsen, 2006). The possible interaction between food components and AITC is important to study to avoid a quick reduction of AITC concentration in the headspace of the packaging and to retain antimicrobial inhibition against spoilage bacteria growing in the food surface

(Kurek et al., 2017). Besides the food composition, the storage temperature that will influence AITC volatility and stability will have an effect on the effective AITC concentration in the headspace and the food (Liu and Yang, 2010).

To understand the mechanisms of the partition of AITC in the packaging, multiresponse kinetic modelling can be used as a powerful tool by fitting an established model to multiple interlinked response data (Quintas et al., 2007). Our previous study (Bahmid et al., 2021) applied the multiresponse kinetic model to describe the AITC formation in the seeds, release to the headspace, and degradation well. However, this study excluded the (model) food that can absorb AITC and cause partitioning between the headspace and the packed food. Also, the effect of temperature on the model parameters was not yet investigated. It is therefore important to describe the AITC partition between the headspace and the (model) food of different compositions and at different temperatures, so that all important steps of the mass transfer mechanism of AITC in a designed packaging system can be described.

This study aimed, therefore, to describe the mass transfer of AITC from the headspace into the food matrix and its degradation in both the headspace and the food matrix using the multiresponse kinetic model. The effects of the composition of a packed food and storage temperature on the AITC partitioning in the antimicrobial packaging system were investigated. These results provide a better understanding of the mass transfer and reactions of antimicrobial compounds in a package with a (model) food and can be used in the design of an antimicrobial package that contains the desired concentration of the AITC in the package headspace for improving microbial inhibition in different foods.

4.2. Materials and Method

4.2.1. Materials

Mustard seeds (*Brassica juncea*) were purchased from Natuurproduct.com, Jacob Hooy Brown Mustard seeds. Whey protein isolate (WPI; ≥90% (w/w)) was purchased from Davisco Foods International, and menhaden fish oil was purchased from Brevoortia Sigma-Aldrich Corporation. Other chemicals used in this study were analytical grade and purchased from Sigma Aldrich or Merck.

4.2.2. Samples Preparation

Ground defatted mustard preparation

Mustard seeds were freeze-dried for 2 days using an Alpha 2-4 LD plus freeze-dryer (Martin Christ freeze-dryers) coupled to an RZ 6 rotary vane pump (Vacuumbrand GmbH). Thereafter the seeds were stored in a desiccator to ensure full hydration. After freeze-drying, the samples were ground and milled using a freezer-mill (6875D Freezer/Mill®, SPEX SamplePrep). Particle sizes of the ground defatted mustard seeds were determined using a sieve machine (Fritsch Analysette), and seeds <100 µm were used. The samples were then

fully defatted using Soxhlet extraction for 6 hours. The ground seeds were collected and stored in a desiccator containing limps of silica gel for 1 day to remove water or solvent left in the seeds and to prevent the pre-hydrolysis of sinigrin due to moisture uptake. Samples were stored in the freezer at -20°C till usage.

Protein- and carbohydrate-based food matrix preparation

Whey protein isolates (WPI) were used to investigate the AITC absorption in the protein-based matrix. The amount of WPI was added to certain volumes of MilliQ water to reach the desired percentages of protein (0, 2.5, 5, 10, 15, and 20% w/v). The WPI was then dissolved in water by using a magnetic stirrer for 2 hours.

To confirm the effects of viscosity of the food matrix on the AITC absorption rate, a food matrix with varying viscosity was made. Methylcellulose at different concentrations (0, 2.5, and 5%) was used to vary the viscosity of the food matrix. The amounts of methylcellulose were added to MilliQ water to reach the desired concentration and then dissolved by using a magnetic stirrer for 24 hours until being well-dissolved.

Fat-based food matrix (emulsion) preparation

For the fat-based matrix, emulsions were prepared with different fat content (0, 2.5, and 5% v/v). The menhaden fish oil was mixed with Milli-Q water and 0.5% (v/v) of the emulsifier Tween 20 by using an Ultra-turrax (IKA Werke GmbH & Co. KG) at 9500 rpm min^{-1} for 2 minutes. The emulsion was subsequently homogenized using a homogenizer (Delta Instruments) for 10 minutes at 120 bar to avoid creaming.

Viscosity measurement of the fat or protein-based food matrix

Anton Paar Rheometer MCR301 (Rheology Rheometers) equipped with a double gap concentric cylinder DG26,7 (titanium) was used to measure the rheological properties of the food matrix (water, methylcellulose-, protein-based matrix) with a steady shear rate of 0.1–1000 s^{-1} at 5, 10 and 20^{o}C with a gap of 57 µm. The relationship between viscosity and shear rate under steady flow conditions was presented.

4.2.3. Determination of AITC concentration in the headspace and food matrix

Defatted mustard seeds (0.1 g) were placed inside a small self-made tea bag with a cord. Samples were rehydrated by completely submerging them in Milli-Q water for 5 s and then immediately placed in 10 mL GC vials containing 1 mL of the food matrix and then closed immediately. The samples were stored in the fridge at 5 and 10^{o}C. The AITC concentration in the headspace and food matrix was then measured at 3, 6, 24, 27, 30, 48, 51, 54, and 72 hours. The experimental setup is depicted in Appendix 4.3.

The headspace analysis of AITC was consequently performed via Gas Chromatography - Flame Ionization Detection (GC-FID) (Thermo-Scientific Focus GC), using direct injection. The method and software used for the headspace measurement in this study were according to the method described in the previous study (Bahmid et al., 2020a).

For measurement of the initial AITC concentration in ground mustard seeds and the AITC concentration in the protein- and fat-based food matrix, Gas Chromatography - Flame Ionization Detection (GC-FID) (Thermo-Scientific Focus GC) connected to an autosampler (Thermo-Scientific, TriPlus Autosampler) were used. Ground defatted mustard seeds (0.1 g) or fat- or protein-based matrices (0.1 mL) were placed in a 2 mL Eppendorf tube containing 1500 µL hexane, then centrifuged (Centrifuge 5430 R, Eppendorf) for 5 minutes at 2627 g at 20^{o}C (Marton and Lavric, 2013). Thereafter, the supernatant was filtered using a syringe filter (Phenex™-PTFE 15mm syringe filters 0.45µm) and collected in a brown vial. The filtered supernatants (1 µL) were injected in the GC apparatus set as described in the method in the previous study (Bahmid et al., 2020a). Data processing was performed using Xcalibur software (Thermo Fischer Scientific). To calculate the concentration of AITC in the headspace, ground mustard seeds, and food matrix, the calibration was quantified using pure AITC (Allyl isothiocyanate, 97%) dissolved in Hexane with the range of used concentrations 1 ppm–1000 ppm.

For modelling purposes, all AITC concentrations were expressed in mol/m^3 (mol in a volume of mustard particles or headspace or food matrix). Each dataset was obtained by analyzing samples in duplicate and expressed as the mean values ± standard deviations (SD).

4.2.4. Building up the reaction and mass transfer pathway

Possible mechanisms of AITC reactions and mass transfers in ground mustard seeds, headspace, and food matrix are given in **Figure 4.1a**. The mechanisms of AITC formation, release, and degradation in the ground mustard seeds and the headspace, which are indicated by a white and grey colour respectively in **Figure 4.1**, were built upon the previous study (Bahmid et al., 2021). With the presence of a food matrix which is indicated with dark yellow in **Figure 4.1**, AITC is subsequently absorbed in the food matrix where AITC is also degraded due to the nucleophilic attack of water on its isothiocyanate group (-N=C=S) (Luang-In and Rossiter, 2015; Ohta et al., 1995). **Figure 4.1b** shows the scheme of AITC interfacial mass transfer from ground mustard seeds into the food matrix.

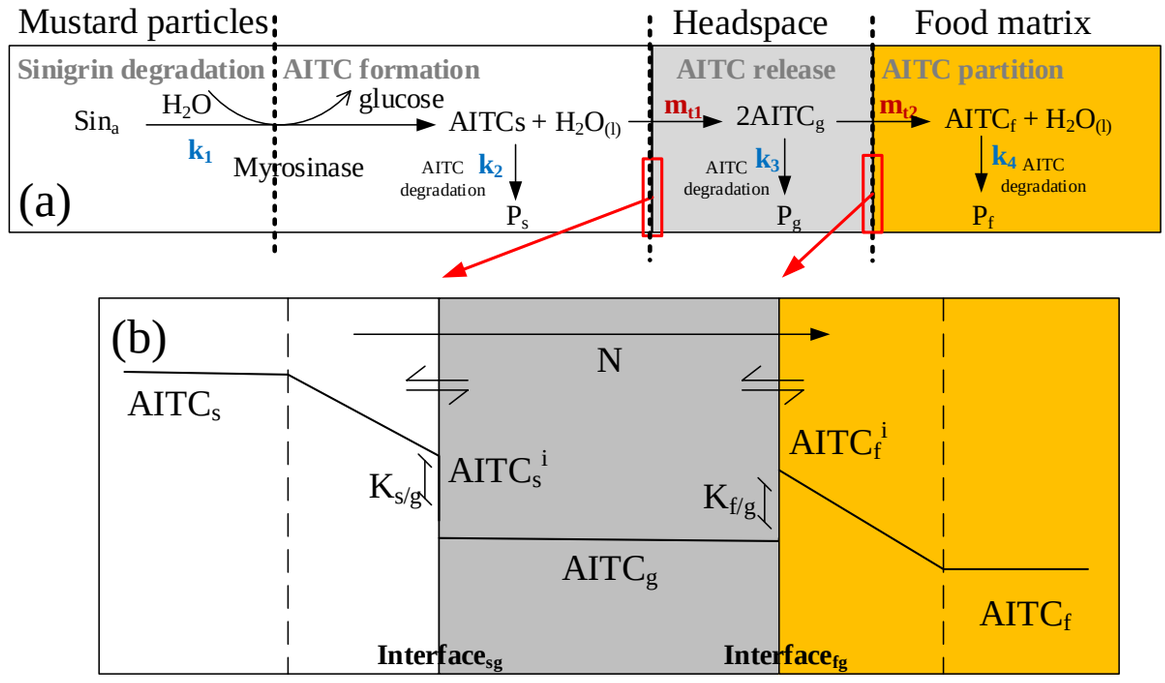

Figure 4.1. (a) *The proposed mechanism of the reaction and mass transfer of allyl isothiocyanates (AITC) in the packaging containing food matrix,* ***(b)*** *the specific mass transfer pathway in all phases; ground mustard particles (white), headspace (space grey), and food matrix (dark yellow). N is molar flux, $k_{1\text{-}4}$ are the degradation rate constants in each phase, P_s, P_g, and P_f are AITC breakdown products in each phase, $K_{s/g}$ and $K_{f/g}$ are partition coefficients between phases, and m_{t1} and m_{t2} are mass transfer coefficients.*

The multiresponse kinetic model for the proposed mechanism was an extension of the multiresponse kinetic model developed in the previous study (Bahmid et al., 2021) that described the rate of different steps of the sinigrin degradation, AITC formation, and AITC release as following

$$\frac{d[sin_{tot}]}{dt} = -k_1\,[Sin_a] = -k_1\,[Sin_{tot} - Sin_i] \quad \textbf{(4.1)}$$

$$\left.\frac{d[AITC_s]}{dt}\right|_f = k_1\,[Sin_a] = k_1\,[Sin_{tot} - Sin_i] \quad \textbf{(4.2)}$$

$$\left.\frac{d[AITC_s]}{dt}\right|_{mt1} = -\frac{m_{t1}\,A_1}{V_s}\left[AITC_s - AITC_g\,K_{s/g}\right] \quad \textbf{(4.3)}$$

$$\left.\frac{d[AITC_g]}{dt}\right|_{mt1} = \frac{m_{t1}A_1}{V_g}\left[AITC_s - AITC_g\;K_{s/g}\right] \quad \textbf{(4.4)}$$

$$\left.\frac{d[AITC_s]}{dt}\right|_{ds} = -k_2\,[AITC_s]\,[H_2O_s] \quad \textbf{(4.5)}$$

$$\left.\frac{d[AITC_g]}{dt}\right|_{dg} = -k_3\,[AITC_g]^2 \quad \textbf{(4.6)}$$

where sin_{tot}, sin_i, and sin_a are the concentration of total sinigrin, inaccessible and accessible sinigrin (mol/m^3), respectively; AITC and V are AITC concentrations (mol/m^3) and volume of each phase (m^3), respectively, and the subscripts s and g denote seed particles and gas phase, respectively; t is degradation time (h); and k_1, k_2, and k_3 are the rate constant of sinigrin degradation and AITC formation (h^{-1}) and the rate constants of AITC degradation in the seeds

and headspace, respectively (m^3/mol.h); H_2O_s is the amount of water per volume of the ground seeds (mol/m^3); $K_{s/g}$ is the partition coefficient of the partition of the concentration of AITC between the ground seeds ($AITC_s$) and headspace ($AITC_g$); m_{t1} is the mass transfer coefficient (m/h) of AITC release from ground seeds into headspace; A_1 is the surface area of the interface of mustard particles (m^2).

In this study, the development of the kinetic model focused on the AITC absorption in the food matrix. The absorption of AITC was determined by the mass transfer coefficient (m_{t2}). The mass transfer rate of AITC in the headspace and the food matrix is described by the following equations ;

$$\left.\frac{d[AITC_f]}{dt}\right|_{mt2} = \frac{m_{t2}.A_2}{V_f}\left[AITC_g\ K_{f/g} - AITC_f\right] \quad \textbf{(4.7)}$$

$$\left.\frac{d[AITC_g]}{dt}\right|_{mt2} = -\frac{m_{t2}\ A_2}{V_g}\left[AITC_g\ K_{f/g} - AITC_f\right] \quad \textbf{(4.8)}$$

where $K_{f/g}$ is the partition coefficient between the concentration of AITC in the food matrix ($AITC_f$) and the headspace ($AITC_g$); the subscript f denotes the food matrix phase; m_{t2} is mass transfer coefficient (m/h) from headspace into food matrix; A_2 is the surface area (m^2) of the interface of food matrix.

In the food matrix phase, the AITC can be degraded due to the attack of the hydroxyl groups of water on the sulfhydryl groups of AITC. The amount of water in the food matrix is considered to remain constant over time. The AITC degradation can be described with a pseudo-first-order reaction. With the amount of water (H_2O_f) is known, the equation can be described as the following:

$$\left.\frac{d[AITC_g]}{dt}\right|_{d_f} = -k_4\left[AITC_f\right]\left[H_2O_f\right] \quad \textbf{(4.9)}$$

where k_4 is the AITC degradation rate constant in the food matrix (m^3/mol.h), and H_2O_f is the amount of water per volume of the food matrix (mol/m^3).

4.2.5. Multiresponse kinetic modelling approach and software system used

The proposed pathway in **Figure 4.1a** contains four steps; sinigrin degradation, AITC formation and degradation in ground seeds, AITC release and degradation in the headspace, and AITC absorption and degradation in the food matrix. These mechanisms were described with the set of equations below, which were combined equations from **Eq. (4.1)**- **Eq. (4.9)**.

$$\left.\frac{d[Sin_{tot}]}{dt}\right|_{sum} = \frac{d[Sin_{tot}]}{dt} \quad \textbf{(4.10)}$$

$$\left.\frac{d[AITC_s]}{dt}\right|_{sum} = \left.\frac{d[AITC_s]}{dt}\right|_{f} + \left.\frac{dAITC_s}{dt}\right|_{mt1} + \left.\frac{d[AITC_s]}{dt}\right|_{ds} \quad \textbf{(4.11)}$$

$$\left.\frac{d[AITC_g]}{dt}\right|_{sum} = \left.\frac{dAITC_g}{dt}\right|_{m_{t1}} + \left.\frac{d[AITC_g]}{dt}\right|_{d_g} + \left.\frac{dAITC_g}{dt}\right|_{m_{t2}} \quad \textbf{(4.12)}$$

$$\left.\frac{d[AITC_f]}{dt}\right|_{sum} = \left.\frac{dAITC_f}{dt}\right|_{m_{t2}} + \left.\frac{d[AITC_g]}{dt}\right|_{d_f} \quad \textbf{(4.13)}$$

These differential equations were simultaneously fitted to the experimental data of AITC concentrations in the headspace and the food matrix. The estimated parameters consisted of reaction rate constants (k_1, k_2, k_3, and k_4), mass transfer coefficients (m_{t1} and m_{t2}), partition coefficients ($K_{s/g}$ and $K_{f/g}$), and initial sinigrin content in the ground seeds. The set of differential equations and the estimation of the unknown parameters were solved by using Athena Visual Studio software (v.14.2) (AthenaVisual Inc.). In the parameter estimation solver control panel, standard options were used, the convergence criterion (= 0.01), the maximum number of iterations (=30), estimation solver options (non-linear least-squares, gradient calculation (forward differences scheme), and relative perturbation step size (10^{-3}). The software estimated 58 parameters from 12 experiments and 32 responses, using 384 data points in total. The goodness of fit of the kinetic models was then evaluated by examining the R-square of parity plots of predicted vs observed data, correlations, and the standard deviations of the parameters.

The parameters k_1,k_2, k_3, m_{t1}, and initial sinigrin (Sin_0) and initial sinigrin ($AITC_0$) content are involved in mechanisms outside the food matrix and were therefore assumed to be independent of the food matrix composition. Sin_0 and $AITC_0$ were also assumed to be independent of the temperature. The initial concentrations of AITC in the headspace and food matrix were set to zero. Other parameters and constants used in the modelling with known values are given in Appendix 4.1. The assumptions corresponding to the conditions of the packaging system were given in Appendix 4.2.

2.6. Differential sensitivity analysis and principal component Analysis

A sensitivity analysis was conducted using the software Athena Visual Studio by simulating the established model for the sample of food matrix containing 2.5% protein at 5^0C. The following first-order sensitivity functions were used to assess the relative effects of the model input (Rodman and Gerogiorgis, 2020),

$$s(t;\theta) = \frac{\partial y(t)}{\partial \theta} \quad \textbf{(4.14)}$$

where s(t; θ) is the dynamic sensitivity function of parameters (θ) on the model state (y). To compare the effect of each parameter (k_1, k_2, k_3, k_4, mt_1,mt_2, $k_{f/g}$, Sin_0, and $AITC_0$) on sinigrin and AITC content in the packaging system, the mean squared summary for each parameter, δmsqr, can be used as a means analysis by the following equation where the model has been evaluated at n discrete time points (Rodman and Gerogiorgis, 2020).

$$\delta msqr = \sqrt{\frac{1}{n}\sum_{t=1}^{n} s^2(t)} \quad \textbf{(4.15)}$$

Principal components analysis (PCA) for the parameters of the model was conducted in IBM SPSS statistics 25 using factor analysis to show the effects of the estimated parameters on different factors. Some estimated parameters, like k_1, k_2, k_3, mt_1, Sin_0 and $AITC_0$, were

excluded because these were constant variables (no effects on the fat and protein). The fat content, protein content, temperature, k_4, mt_2 and $K_{f/g}$ were analyzed to investigate their dependence on fat content, protein content and temperature, and the correlation amongst the parameters.

4.3. Results and Discussion

4.3.1. Experimental Results

The AITC concentrations in the headspace and food matrix containing fat or protein in food packaging were measured. **Figures 4.2** and **4.4** show predicted and measured AITC concentration in the headspace and the protein- or fat-based matrix at 5 and 10^{o}C. The presence of a food matrix in the food packaging system results in the partition of AITC, which was released from ground seeds, between the headspace and the food matrix. In the headspace, the AITC concentration immediately peaked in the first 6 hours, while in the food matrix, the peak of AITC concentration was delayed and was reached after 24 hours. After this peak, the AITC concentration reduced gradually in the headspace and the matrix. This partition behaviour of volatile compounds in the headspace and food was similar as observed by Wang et al. (2020c), who investigated the partitioning of carvacrol in an antimicrobial package. A higher amount of carvacrol was observed in ground beef with higher fat content. The absorption of the volatile in the food depends on the composition of food products. According to the relation between the AITC concentration in the headspace and the food matrix containing fat and protein shown in Appendix 4.7, a higher fat content in the food matrix caused more AITC absorbed in the food matrix, while a higher protein content caused an increasing concentration in the headspace. These effects of fat and protein on the kinetics of AITC absorption in the food matrix are discussed further in the next sections.

4.3.2. Multiresponse Kinetic modelling and estimated parameters

To describe the partition of AITC between the headspace and the food matrix, the established model enumerated in **Eq. (4.10)** – **Eq. (4.13)** was fitted to the experimental results. The fits of the models on the experimental data are shown in **Figures 4.2** and **4.4**, and **Tables 4.1** and **4.2** show the estimated parameters resulted from the kinetic modelling, which are interpreted and discussed in later sections. **Table 4.1** shows that the most estimated parameters were observed in low standard deviations. A parameter, k_3, was estimated with an undetectable standard deviation and high correlation in **Table 4.2**. The high correlation for k_3 for both temperatures was related to the AITC release from the headspace, AITC absorption in the food matrix, and partition coefficients, so it could be difficult for the software to estimate the value. The correlation coefficients amongst the estimated parameters are shown in Appendix 4.8 and 4.9. The correlation of the parameters was simplified by the PCA analysis in Appendix 4.14b, in which the parameters k_4 and mt_2 have a high correlation. Although k_4 and

mt_2 for each sample were highly correlated (>0.90), the standard deviations of the estimations of k_4 and mt_2 for each sample were very low. The most influencing factors influencing the sinigrin and AITC content in the packaging system were analysed using the sensitivity analysis, as shown in Appendix 4.13. The k_4 and mt_2 were the main factors influencing the AITC content in the headspace, and only k_4 was influencing the AITC concentration in the food matrix. Furthermore, parity plots of observed data against predicted data, which are shown in Appendix 4.6, show a good fit of the established kinetic model fitted on the experimental data. R squares of the parity plots for the headspace concentration at 5^{o}C and 10^{o}C were 0.97 and 0.90, respectively, and for the food matrix, the plots had R^2 of 0.96 and 0.98 at 5^{o}C and 10^{o}C, respectively. The parity plots indicate that the proposed multiresponse kinetic model gave a good description of all mechanisms of mass transfer and reactions in the food packaging system.

Therefore it can be concluded that the established model is generally applicable, and the proposed mechanisms of AITC formation, release, and its partition between headspace and food matrix in the packaging system were highly acceptable for describing the experimental results.

Table 4.1. *The estimated parameters (±standard deviations) of the kinetic model*

Parameters	Temperatures	Samples							
		water	**2.5% protein**	**5% protein**	**10% protein**	**15% protein**	**20%protein**	**2.5% fat**	**5% fat**
k_4 (x10^6 m^3/mol.h)	**5ºC**	1.84±0.29	1.28±0.18	1.13±0.15	0.70±0.13	0.56±0.13	0.41±0.13	0.44±0.04	0.12±0.01
	10ºC	14.45±2.24	2.94±0.87	2.32±0.64	3.30±1.38	2.87±1.43	1.56±0.84	0.49±0.09	0.13±0.01
m_{t2} (x10^5 m/h)	**5ºC**	43.34±7.63	38.32±6.32	40.62±6.31	13.86±2.30	9.50±1.95	6.80±1.87	52.32±6.69	49.51±5.68
	10ºC	370.86±76.76	74.56±20.37	59.14±15.39	57.58±23.45	35.90±17.59	18.90±9.91	123.92±16.37	138.98±17.38
$K_{f/g}$ (dimensionless)	**5ºC**	16.21±2.38	19.75±3.48	17.90±2.87	5.37±0.40	4.04±0.38	3.04±0.46	25.52±5.82	39.23±13.93
	10ºC	4.31±1.85	8.32±1.36	7.74±1.17	3.70±0.34	3.74±0.34	3.74±0.32	14.69±4.17	25.18±12.70

Table 4.2. *The estimated parameters (±standard deviations) of the kinetic model assumed to be independent of the food matrix composition*

Temperatures	Parameters					
	k_1 (x10^2 h^{-1})	k_2 (x10^3 m^3/mol.h)	k_3 (x10^6 m^3/mol.h)	m_{t1} (x10^5 m/h)	Sin_0 (mol/m^3)	$AITC_0$ (mol/m^3)
5ºC	2.34±0.17	5.72±0.39	10.46±29.15	6.05±1.34	90.52±5.91	6.16±1.28
10ºC	2.82±0.18	14.17±0.91	12.56±UD	7.83±2.42		

*UD = undetermined standard deviations, which indicates that standard deviation could not be estimated during the fitting of the model due to high correlations

4.3.3. *Effects of the protein content of the food matrix on the kinetics of AITC absorption from the headspace*

Figure 4.2 shows that the protein content had an inverse relation with the AITC partitioning between the headspace and food matrix. When the food matrix contains high protein content, an increased AITC concentration in the headspace and a lower concentration in the food matrix was observed at 5 and 10°C. An interesting result was that increasing the protein content from 0% up to 5% resulted in increasing AITC concentrations in the food matrix, whereas further increasing the protein content (>5%) resulted in a decrease in the AITC concentration in the food matrix. **Figures 4.2c** and **4.2d** show that AITC was able to penetrate quickly to the food matrices containing low protein contents (<5%), but AITC was then degraded faster in the water at 5 and 10°C, compared to food matrix containing protein. Furthermore, **Table 4.1** shows the increased protein content in the food matrix resulted in a reduced estimated mass transfer coefficient from headspace to food matrix (m_{t2}) for temperatures 5 and 10°C. The lower AITC concentration observed for the package containing the higher-protein food matrix could be caused by the increased viscosity of the high protein food matrices slowing down the absorption into the food matrix. According to the viscosity of the protein-food matrix shown in Appendix 4.4, the higher protein caused an increase in viscosity of the matrix. This is logical because the high volatile compounds, like AITC, are hardly resistant from the gas phase, and the absorption in the liquid is thus limited (Ammari and Schroen, 2018). This effect of viscosity on volatile absorption was also described by Li et al. (2012) that the gels with higher viscosities provide stronger binding power, which limited the volatiles to diffuse into the gel (highly viscous solution).

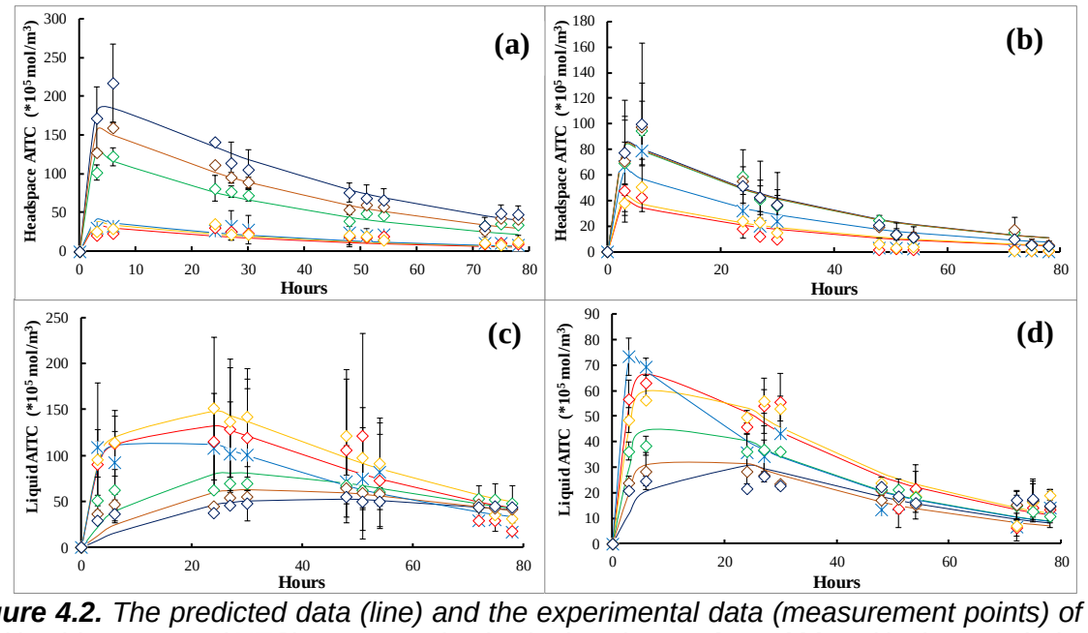

Figure 4.2. *The predicted data (line) and the experimental data (measurement points) of the allyl isothiocyanates (AITC) concentration in the headspace* ***(a and b)*** *and in the protein-based matrix* ***(c and d)*** *at 5°C* ***(left)*** *and 10°C* ***(right)****; Protein content; 0% (light blue x), 2.5% (red◇), 5% (yellow◇), 10% (green◇), 15% (brown◇), 20% (dark blue◇)*

The effect of viscosity on AITC absorption was also confirmed in Appendix 4.5. An increasing concentration of methylcellulose increased the viscosity of the matrix (Appendix 4.5a), and the presence of methylcellulose in the food matrix caused a lower AITC concentration in the food matrix and a higher concentration in the headspace (Appendix 4.5b and 4.5c). Although the viscosity of the food matrix containing 2.5% and 5% methylcellulose shows a clear difference, which was around 100 and 1000 mPa/s, respectively, the difference of AITC concentration in the headspace was not very clear. Interestingly, a lower AITC absorption was observed in the food matrix with 5% methylcellulose, which means the viscosity slowed the absorption of AITC into the food matrix. These results confirm the effect of viscosity in the food matrix on the AITC absorption, influencing the AITC partitioning between and food matrix.

Furthermore, **Table 4.1** shows that increased protein in the matrix resulted in decreased AITC degradation rate (k_4) and partition coefficient ($K_{f/g}$). The package containing the low-protein food matrix (<5%) had a higher $K_{f/g}$ than the package with only water at 5 and 10°C. This result confirmed the obtained result as shown in **Figure 4.2**, in which the package with low-protein (<5%) food matrix contained higher AITC concentration in the matrix and lower headspace concentration, compared to the package with water- or high-protein- (>5%) food matrix (**Figure 4.2**). These results could be related to the interactions between AITC and protein. When AITC was absorbed by the protein-food matrix, AITC subsequently interacts with the free water molecules and interact with the protein, as shown in **Figure 4.3a**. The effect of protein is thus twofold: It increases the AITC absorption in the food by binding and stabilizing AITC, but at the same time, it slows down the absorption rate due to the increase of the viscosity of liquid food matrices.

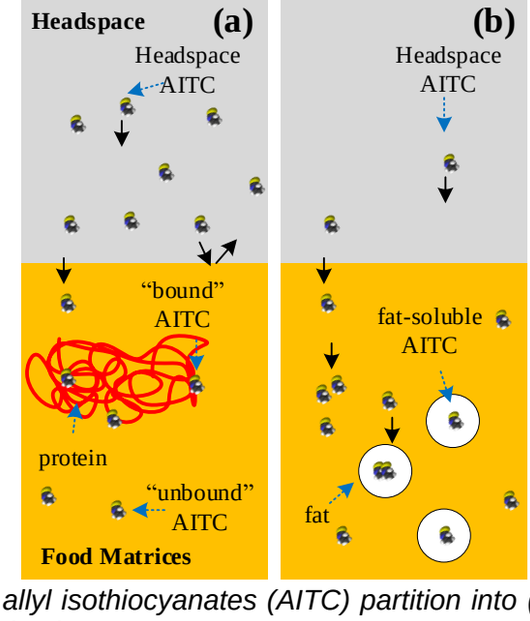

Figure 4.3. *The schematic allyl isothiocyanates (AITC) partition into* ***(a)*** *protein- and* ***(b)*** *fat-based food matrix in the packaging system*

The AITC in the solution can be present in two forms: “unbound AITC” present in the free water of the solution and “bound AITC” interacting with protein (Keppler et al., 2017). For the package containing water, AITC was only partitioned to the headspace and water phase. For the package with the protein-based matrix, the AITC absorbed in the matrix interacted with protein and the free water molecules between the protein molecules. The matrix with low protein content (<5%) will result in only weak interaction between protein molecules, so the absorbed AITC can strongly bind the high-affinity binding sites of protein and, at the same time, the free water molecule (Landy et al., 1995). The strong interaction between AITC and protein and its interaction with the free water molecule caused the $K_{f/g}$ between headspace and low-protein food matrix to be higher than $K_{f/g}$ of the packaged with water- and higher protein- food matrix. Whilst the high protein food matrix containing a strong interaction between protein molecules causes the volatile only binding the free water molecules (Landy et al., 1995; Nahon et al., 2000). Consequently, the thermodynamic equilibrium conditions were determined between the volatile-freely interacting water and headspace (Ammari and Schroen, 2018). These results were also confirmed by Keppler et al. (2017) that an increasing ratio of WPI to AITC concentration resulted in a decrease of AITC molecules bound to WPI proteins. Furthermore, the interactions between AITC and free water molecule or protein was also relevant to the AITC degradation in the protein food matrix. The AITC degraded in the food matrix was the “unbound” AITC interacted to water molecule due to its susceptibility to being attacked by nucleophiles such as H_2O, ^-OH, ^-SH, and $^-NH_2$ groups (Cejpek et al., 2000; Liu and Yang, 2010). Therefore, more free water molecules available in the food matrix caused faster AITC degradation.

Concerning the antimicrobial activity, higher AITC concentration in the headspace of packaging due to the higher viscosity of the food matrix will be beneficial to inhibit spoilage bacteria growing on the surface of the packaged foods. In the protein-based matrix, although the “unbound” AITC is less stable, this AITC is expected to potentially have growth inhibitory effects on microorganisms present in the food matrix, whereas the “bound” AITC has no antimicrobial effects (Keppler et al., 2017). Further studies are required to investigate the antimicrobial effects of AITC released from ground mustard seeds on (model) food containing different protein content.

4.3.4. Effects of fat content of the food matrix on the kinetics of AITC absorbed from the headspace

Increased fat content leads to a lower AITC concentration in the headspace and a higher concentration in the fat-based matrix (**Figure 4.4**). **Table 4.1** shows that the higher fat content strongly resulted in an increase in the estimated m_{t2} and $K_{f/g}$ at 5 and 10^0C, but a decrease in k_4. This result was related to the solubility of AITC in the fat phase. As shown in **Figure 4.3b**, when AITC was absorbed by the fat-based matrix, the AITC subsequently partitions between

the aqueous phase and lipid phase of the matrix. The fat phase contains a higher AITC concentration than the aqueous phase due to the high solubility of AITC in the fat phase (Li et al., 2015). The same result was also observed in real food. Wang et al. (2020c) found that the ground beef with higher fat content contained a higher amount of carvacrol. The solubility of AITC in the fat phase also caused better stability of AITC concentrations in the fat-based matrix. As shown in **Table 4.1**, the AITC degradation in the fat-based matrix became lower with increasing fat content. The higher fat content in the food matrix caused a higher amount of AITC to diffuse to the lipid phase, where the AITC is protected from nucleophilic attacks in the aqueous phase.

For its application in microbial inhibition, the higher fat content in the packaged food might have a negative impact on the effective AITC concentration that has antimicrobial activity against spoilage bacteria growing on the food. The higher AITC concentration in the lipid phase leads to less AITC concentration in an aqueous phase in the (model) food and headspace and thereby reduce its antimicrobial activity against a variety of microorganisms (Wang et al., 2020a). In real food, the fat and proteins can form complexes and affect the microbial inhibition in the food matrix. Therefore, it is important to further study the effectiveness of the antimicrobial compounds in real food products with different food composition.

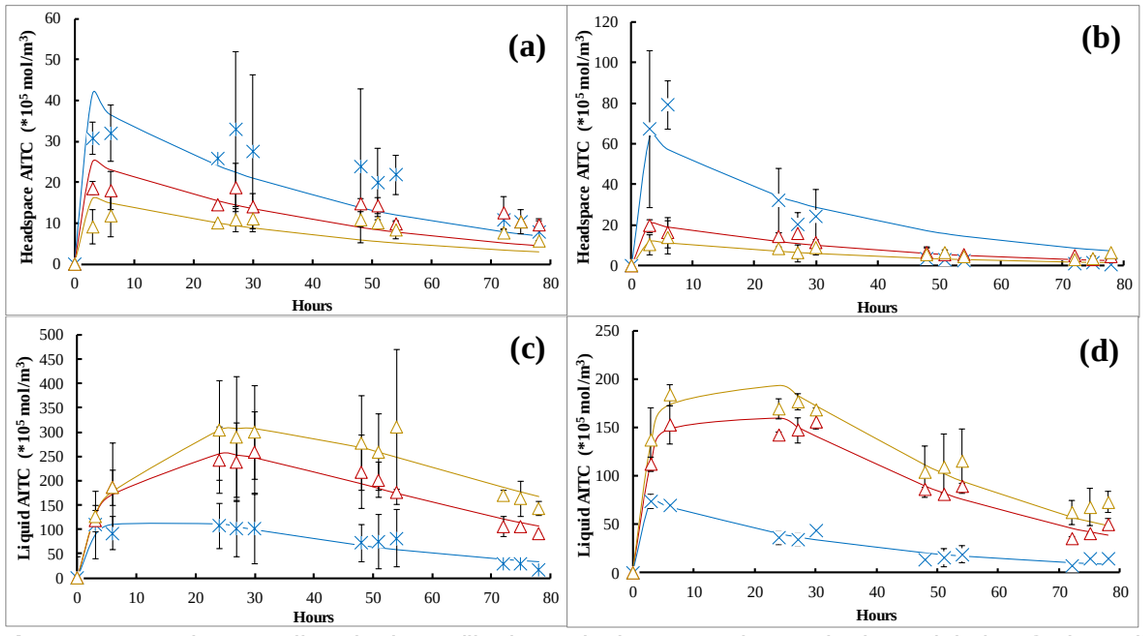

Figure 4.4. *The predicted data (line) and the experimental data (plot) of the allyl isothiocyanates (AITC) concentration in* ***(a and b)*** *the headspace and* ***(c and d)*** *the fat-based matrix at 5°C* ***(left)*** *and 10°C* ***(right)****; Fat content; 0% (light blue x), 2.5% (red Δ), and 5% (yellow Δ).*

4.3.5. Effects of temperature on the kinetics of AITC partition from headspace to food matrix

The temperature had a clear effect on all estimated parameters in this study (**Tables 4.1 and 4.2**). The higher temperature resulted in higher estimated degradation rate constants in

all phases (k_1, k_2, k_3, and k_4) and AITC absorption in the food matrix (m_{t1} and m_{t2}), and lower partition coefficient ($K_{f/g}$). A slower AITC degradation in the lower temperature was expected since, the lower temperature improves the AITC stability in the packaging system (Fares et al., 1998; Liu and Yang, 2010; Ohta et al., 1995). The reason for the lower $K_{f/g}$ was due to the high volatility of AITC in increasing temperature. This result was consistent with the result observed by Zhang et al. (2010), in which the higher temperature reduced the partition coefficient of AITC between olive or canola oil and air. Furthermore, the increasing temperatures weaken the binding sites between protein molecules and reduce the viscosity of the fat-based matrix (Hong et al., 2018), resulting in the observed higher mass transfer coefficients (**Table 4.1**), indicating the quick AITC absorption in the protein- and fat-based matrix at the higher temperature.

4.4. Conclusion

In this study, the effects of the food compositions and temperature on AITC partitioning in the food packaging system were investigated. The amount of fat and proteins present in the food matrix has a strong influence on the partitioning of AITC between the food matrix and the headspace. The AITC concentration is more stable in the higher fat-based food matrix, and a higher mass transfer coefficient from headspace to food matrix was observed. An increase in protein content increases the viscosity of the food matrix, thereby reducing the AITC penetration into the food matrix, but the complex between proteins and AITC stabilizes AITC in the food matrix. Furthermore, temperature plays an important role in the stability of AITC in the phases of food packaging. By lowering the temperatures, a higher AITC headspace concentration is obtained that can be retained for longer times. The established multiresponse kinetic model could well describe the experimental data of the release of AITC from mustard seeds into the headspace and the absorption of AITC by the water or protein- or fat-based food matrix, and AITC degradation in all phases. It is recommended to do further studies in (model) food to investigate the effect of food composition on the antimicrobial effects of AITC to extend the shelf life of packaged food. These results give a better insight into the effects of the food composition and temperature on the absorption of the compounds in different foods, helps the food industry to optimize the design of an antimicrobial package that contains the desired concentration of the antimicrobial compounds in the package headspace and in the food to inhibit the bacterial growth for an extended food shelf life.

Acknowledgement

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Nomenclature/Abbreviation

d = diameter of ground mustard seeds/mustard particles (m)

A_1 = the surface area of the interface mustard particles (m^2)

A_2 = the surface area of the interface food matrix (m^2)=

m_s = mass of mustard particles (g)

ρ_s = density of mustard particles (g/m^3)

V_{vi} = volume of glass vial (m^3)

V_g = volume of headspace (m^3)

V_s = volume of mustard particles (m^3)

V_f = volume of food matrix (m^3)

$AITC_0$ = Initial concentration of AITC in the mustard seeds particles (mol/m^3)

$AITC_s$ = concentration of AITC in the mustard seeds particles (mol/m^3)

$AITC_s^i$ = concentration of AITC at the interface of the mustard seeds particles (mol/m^3)

$AITC_g$ = concentration of AITC in the headspace (mol/m^3)

$AITC_f^i$ = concentration of AITC at the interface of the food matrix (mol/m^3)

$AITC_f$ = concentration of AITC in food matrix (mol/m^3)

$AITC_{tot}$ = total concentration of AITC in the packaging system (mol/m^3)

H_2O_s = the concentration of added water in-ground seeds (mol/m^3)

H_2O_f = the concentration of added water in-food matrix (mol/m^3)

N = molar flux (mol/m^2h)

m_{t1} = mass transfer coefficient from mustard particles into headspace (m/h)

m_{t2} = mass transfer coefficient from headspace into food matrix (m/h)

D = average diffusion coefficient of free volatile seeds (m^2)

t = time (h)

Sin_o = Initial sinigrin content in mustard particles (mol/m^3)

Sin_{tot} = total sinigrin content in the mustard particles (mol/m^3)

Sin_i = inaccessible sinigrin content in the mustard particles (mol/m^3)

Sin_a = accessible sinigrin content in mustard particles (mol/m^3)

$s(t; \theta)$ = the dynamic sensitivity function of parameters (θ) on model state (y)

k_1 = rate constant of sinigrin degradation and AITC formation (h^{-1})

k_2 = rate constant of AITC degradation in the mustard particles (h^{-1})

k_3 = rate constant of AITC degradation in the headspace ($m^3/mol.h$)

k_4 = rate constant of AITC degradation in the food matrix (h^{-1})

$K_{s/g}$ = equilibrium partition coefficient ground mustard seeds and headspace (dimensionless)

$K_{f/g}$ = equilibrium partition coefficient food matrix and headspace (dimensionless)

fat = fat content of the seeds (%)

PCA = principal components analysis

p_s = breakdown products in the mustard particles

p_g = breakdown products in the headspace

p_f = breakdown products in the food matrix

y = model state

$|_f$ = symbol of differential equation for AITC formation in the ground mustard seeds

$|_{ds}$ = symbol of differential equation for AITC degradation in the ground mustard seeds

$|_{dg}$ = symbol of differential equation for AITC degradation in the headspace

$|_{df}$ = symbol of differential equation for AITC degradation in the food matrix

$|_{mt1}$ = symbol of differential equation for AITC release into the headspace

$|_{mt2}$ = symbol of differential equation for AITC absorption by food matrix

$|_{sum}$ = symbol of sum of differential equation

θ = symbol for parameters

δmsqr = mean squared summary

Appendix 4. Supplementary Materials

The following are the supplementary data to this article:

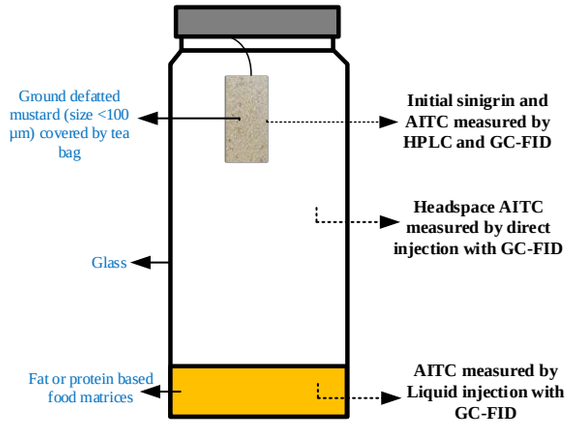
Appendix 4.1. *The known value inputs used in modelling*

The known input	Abb	Value
Mass of mustard particles (g)	m_s	0.10
Concentration of added water in ground-seeds (mol/m^3)	H_2O_s	52800
Concentration of added water in the food matrix (mol/m^3)	H_2O_f	55556
Density of mustard particles (g/m^3)	ρ_s	720000*
Volume of glass vial (m^3)	V_{vi}	10^{-5}
Volume of mustard particles (m^3)	V_s	$1.387x10^{-7}$**
Volume of headspace (m^3)	V_{ha}	$9x10^{-6}$***
Volume of the food matrix	V_f	10^{-6}
The surface area of <100 µm-sized particles (m^2)	A_1	0.011****
The surface area of the food matrix (m^2)	A_2	$7.85x10^{-5}$*****
Diameters of ground mustard particles (average 50-100 µm) (m)	d	$7.5x10^{-5}$
Inaccessible sinigrin in ground mustard seeds (mol/m^3)	Sin_i	3.12 ± 1.61******

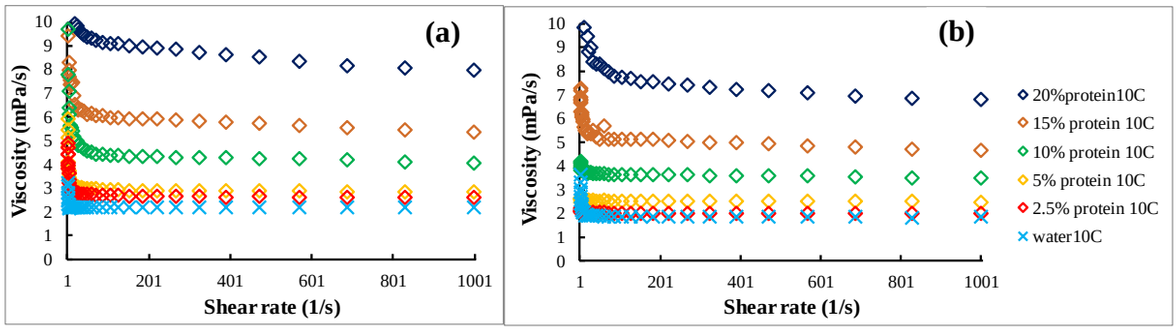
*density found from Ropelewska et al. (2018)
**volume of mustard particles obtained from the total mass of mustard seeds divided by the density
***volume of the headspace obtained from the total volume of the glass vial minus the volume of the mustard seeds used
****the surface area of the interface between mustard particles and headspace obtained from the calculation of the surface area of the mustard particles (<100 µm)
*****the surface area was calculated from the area of the glass vial (diameter 10^{-2} m)
******the value of Sin_i was taken from the previous study (Bahmid et al., 2021)

Appendix 4.2. *General assumptions corresponding to the proposed mechanism;*

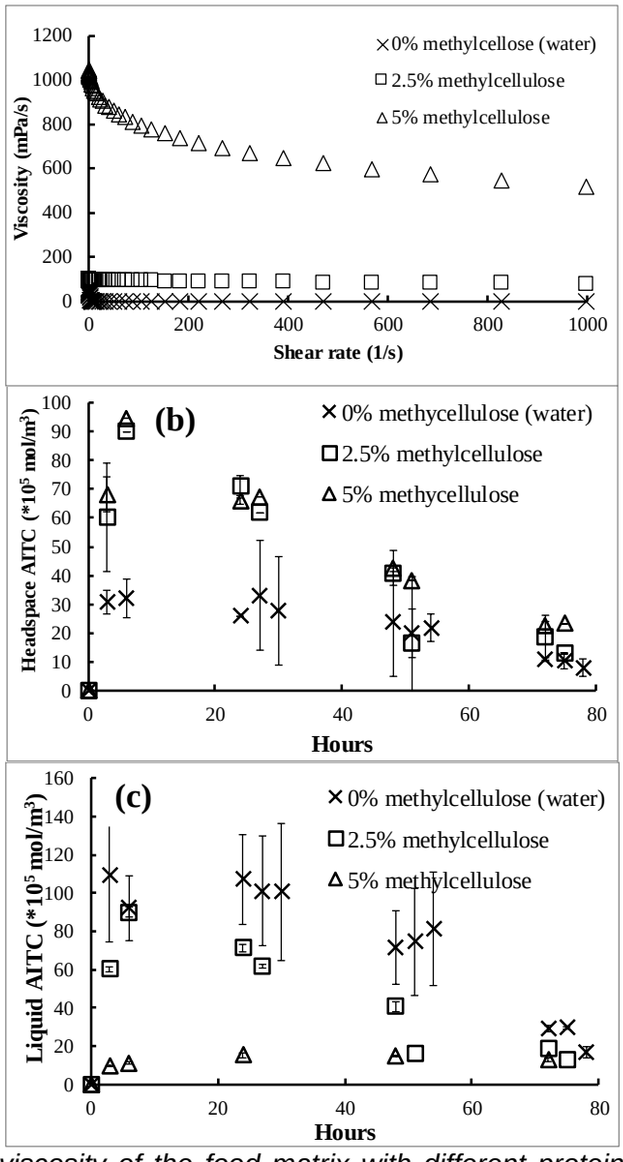
No	General assumptions
1	The mass transfer takes place from the mustard particles phase to the gaseous phase and the gaseous phase to the food matrix.
2	The mustard particles are loosely packed, and the surface area of each particle is in contact with the gaseous phase.
3	The sinigrin degradation takes place in the mustard particles, and allyl isothiocyanates (AITC) degradation takes place in the mustard particles, liquid (food matrix), and gaseous phases
4	The mustard seed particles are considered to be one phase, no separate aqueous, solid, and fat sub-phases for the seed particles are considered in the model. The same is also applied in the food matrix.
5	The emulsifier is assumed not to influence the concentration of AITC in the headspace and food matrix
6	The mass transfer through the gaseous phase to the food matrix is described with the stagnant film model. The conditions justify that the mass transfers in the gaseous phase and food matrices can be assumed not to be a limiting factor.



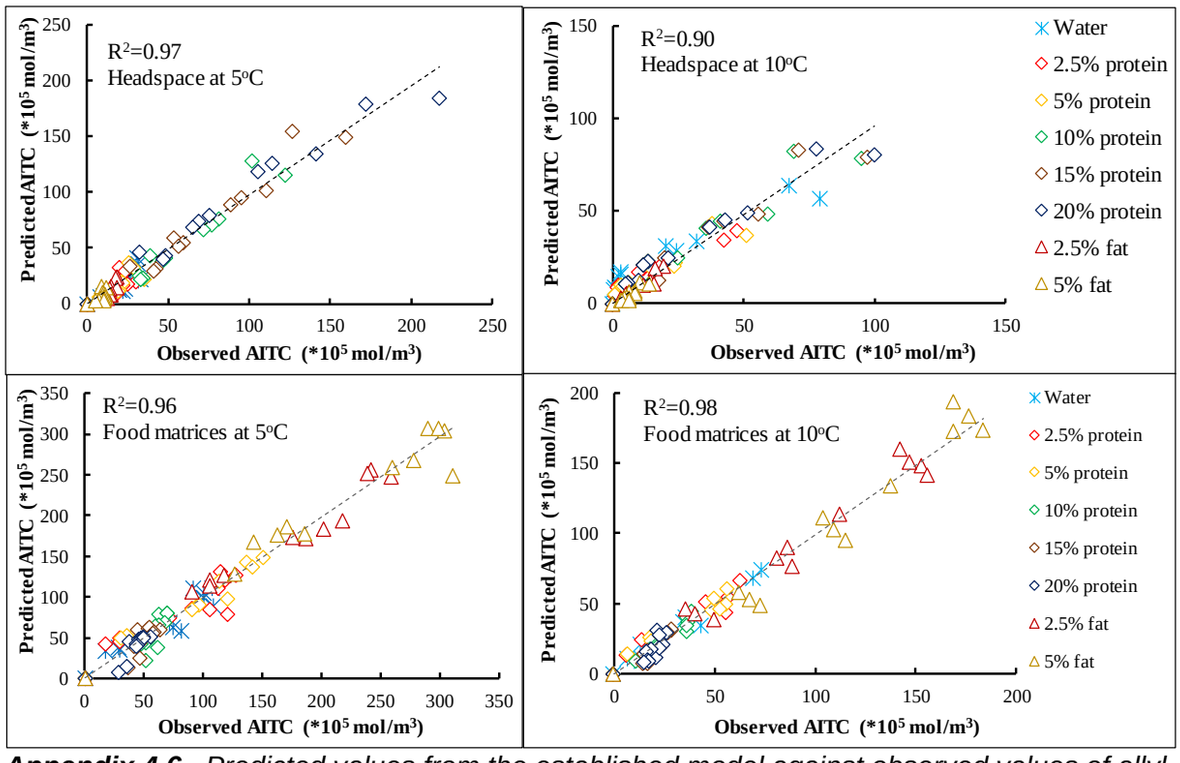
Appendix 4.3. *The set-up of an antimicrobial packaging system with a 10 ml vial containing a protein- or fat-based food matrix and a tea bag covering ground mustard seeds.*



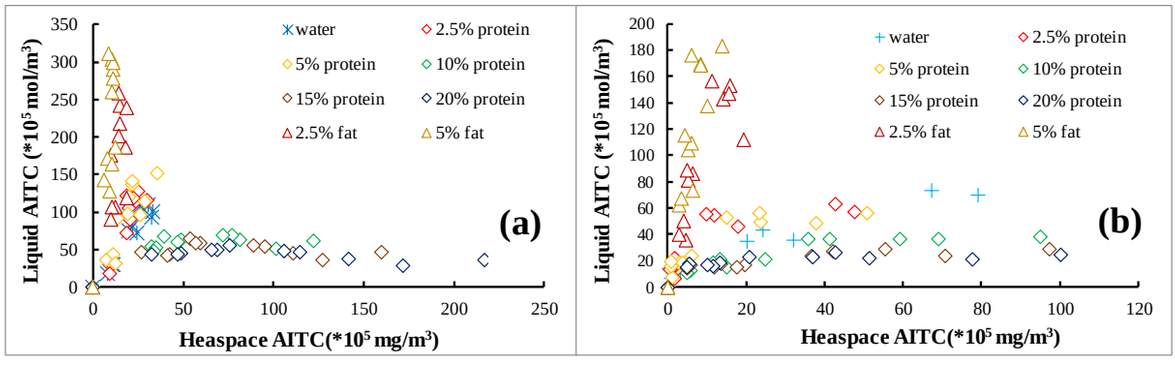
Appendix 4.4. *The viscosity of protein-based food matrix at temperatures* ***(a)*** *5°C and* ***(b)*** *10°C.*



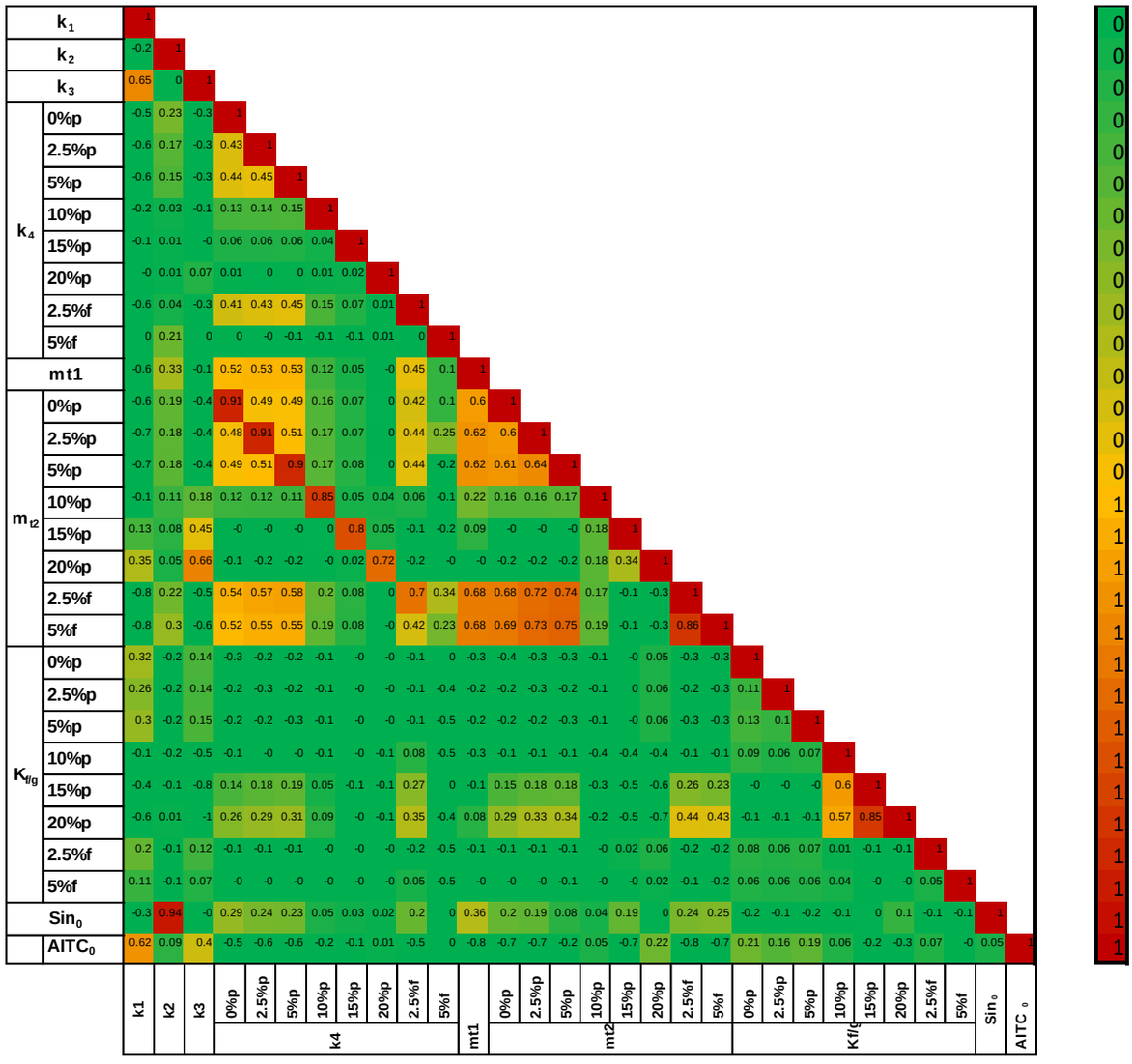
***Appendix 4.5**. **(a)** The viscosity of the food matrix with different protein and carbohydrate (methylcellulose as a thickener) content and the allyl isothiocyanates (AITC) concentration in the **(b)** headspace and **(c)** food matrix at 5ºC.*



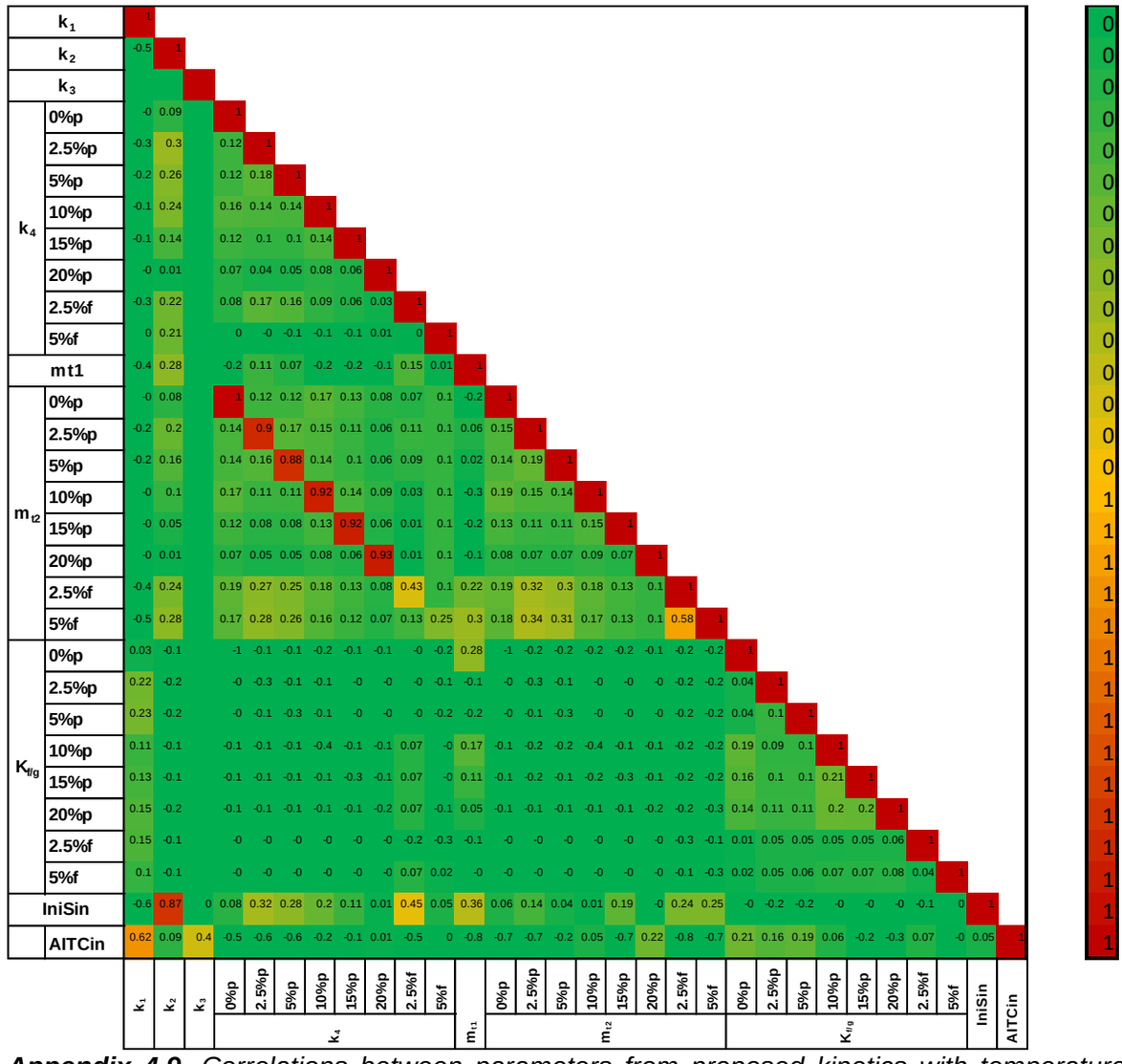
Appendix 4.6. *Predicted values from the established model against observed values of allyl isothiocyanates (AITC) concentration in the headspace at temperatures* ***(a)*** *5°C and* ***(b)*** *10°C, and in the food matrix at* ***(c)*** *5°C and* ***(d)*** *10°C.*



Appendix 4.7. *The correlation between allyl isothiocyanates (AITC) in the headspace and food matrix at temperatures* ***(a)*** *5°C and* ***(b)*** *10°C.*



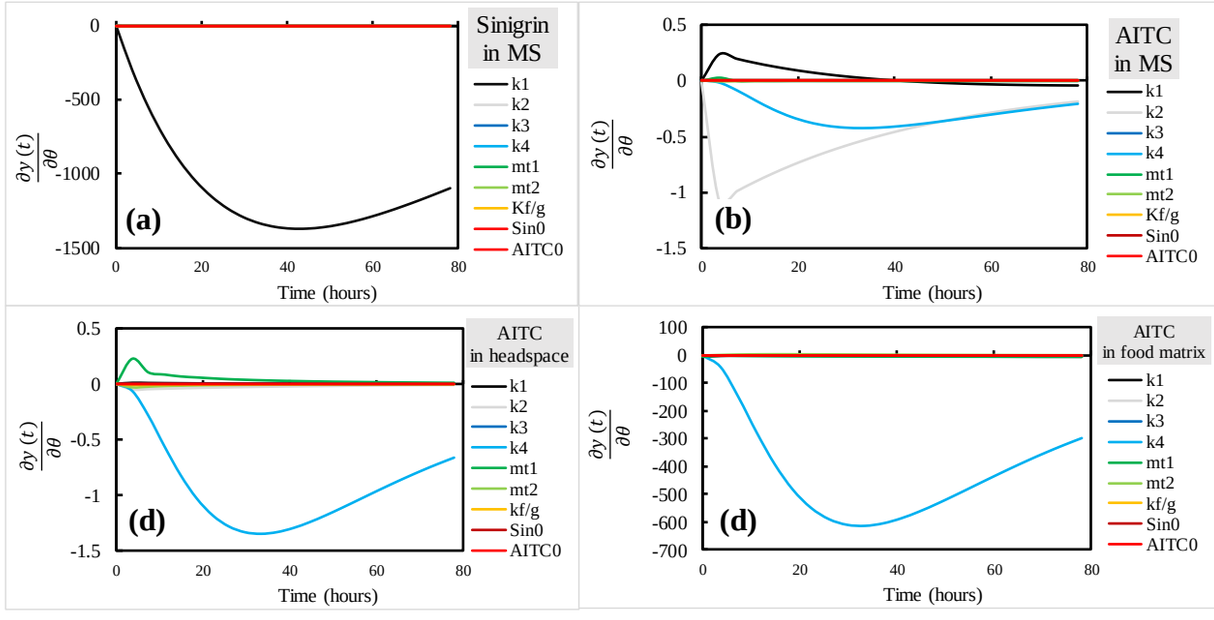
Appendix 4.8. *Correlations between parameters from proposed kinetics with temperature 5oC. Green to red colour in the bar shows the correlation coefficient from 0 to 1, and empty green columns contain correlations of the parameters with undefined standard deviations. P and f are denoted for protein and fat content of the food matrix*



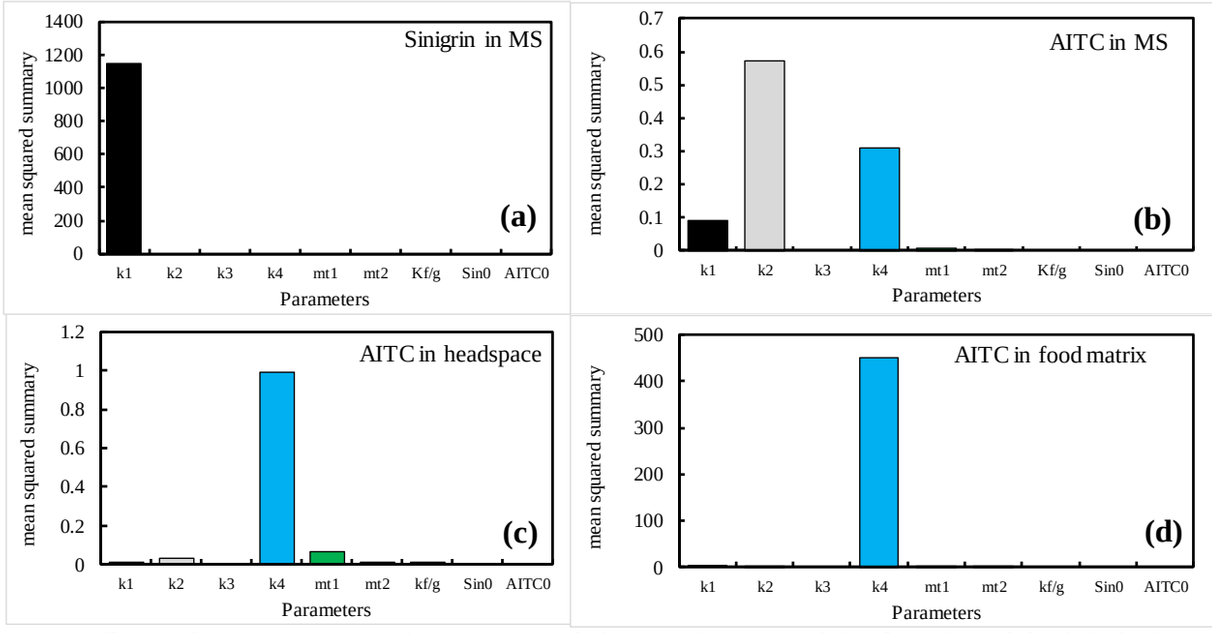
Appendix 4.9. *Correlations between parameters from proposed kinetics with temperature 10ºC. The Green to red colour in the bar shows the correlation coefficient from 0 to 1.0, and empty green columns contain correlations of the parameters with undefined standard deviations. P and f are denoted for protein and fat content of the food matrix, respectively.*

Appendix 4.10. *Differential sensitivity analysis*

Appendix 4.11 and 4.12 show the sensitivity profiles of the parameters (k_1, k_2, k_3, k_4, mt_1,mt_2, $k_{f/g}$, Sin_0, and $AITC_0$) for each kinetic model. Appendix 4.11 shows that the k_1 was negatively correlated to the sinigrin content in ground mustard seeds but was positively correlated to the AITC content in ground mustard seeds. A negative correlation for AITC in ground mustard seeds was observed for the k_2 and k_4. The k_4 has a negative correlation for AITC content in the headspace and the food matrix. The high correlations were regarded to be because of the state trajectory (Rodman and Gerogiorgis, 2020). Appendix 4.12 shows the most influential parameters for the sinigrin and AITC content in the packaging system; k_1 for the sinigrin content in mustard seeds, k_2 and k_4 for the AITC content and mustard seeds, k_4 and mt_2 for the AITC content in the headspace, and k_4 for the AITC content in the food matrix. The effects of k_4 for the AITC content in those three phases were unexpected because of the equilibrium partitioning of AITC in each phase, but interestingly, the partition coefficient did not influence the established model.



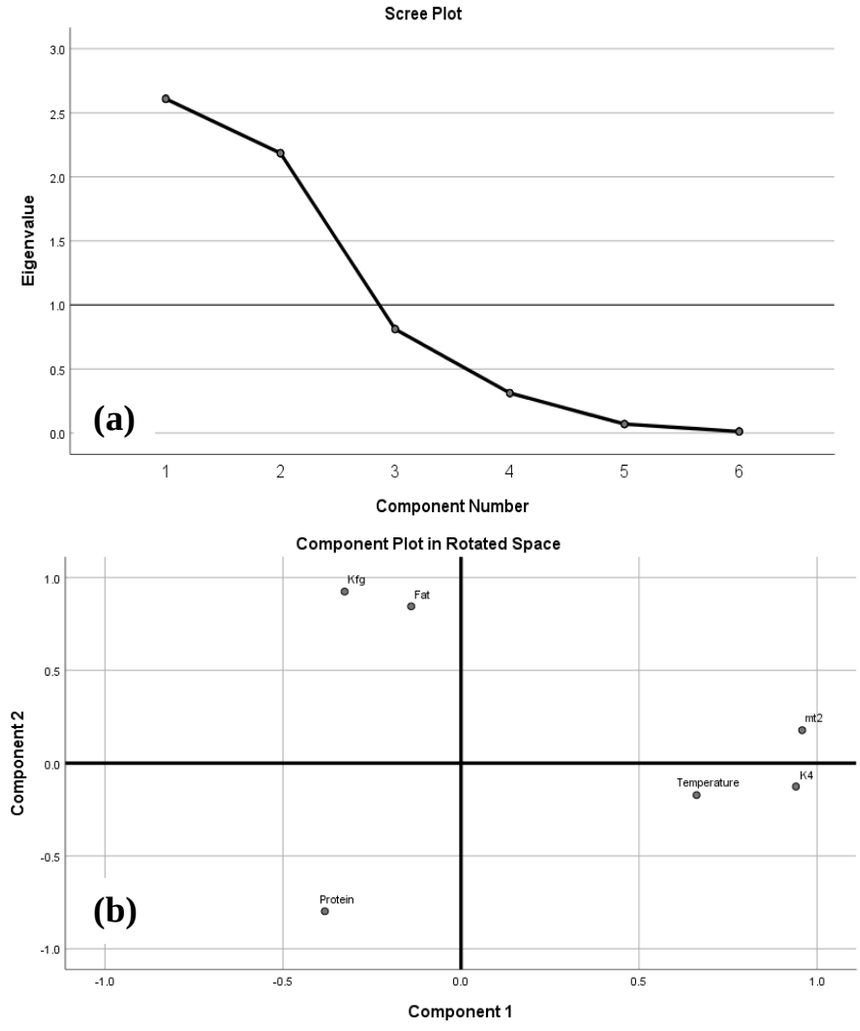
Appendix 4.11. *Parameter sensitivity profiles.* ***(a)*** *sinigrin in ground mustard seeds,* ***(b)*** *AITC in ground mustard seeds,* ***(c)*** *AITC in the headspace, and* ***(d)*** *AITC in the food matrix*



Appendix 4.12. *Mean squared summary of time-series sensitivity function.* ***(a)*** *sinigrin in ground mustard seeds,* ***(b)*** *AITC in ground mustard seeds,* ***(c)*** *AITC in the headspace, and* ***(d)*** *AITC in the food matrix*

Appendix 4.13. *Principal Components Analysis (PCA)*

Appendix 4.14a shows a scree plot used to understand the variation of each principal component captured from the data. According to the scree plot, two principal components (PC) have a higher Eigenvalue (amount of variation) than 1. It means that PC1 and PC2 were selected. Appendix 4.15 shows the variation of the variables in the PC1 and PC2. The PC1 was dominated by the estimated parameters k_4 and mt_2, while k_4 was dominating the PC2. Appendix 4.14b shows a good distribution of the variables and estimated parameters, with three clearly opposite groups. This figure simplified the correlation amongst parameters in Appendix 4.8 and 4.9. The space rotated graph in Appendix 4.14b shows the distribution of data in relation to the axes and the relationship between data and the similarities of environmental data (Gordo et al., 2015). Fat content strongly affects $K_{f/g,}$ and temperature strongly affects mt_2 and k_4. Protein content does not have an effect, which is likely to be due to the other effects depending strongly on the protein content (low protein content,<5%, has a very different effect compared to high protein content,>5%). Besides, from the component plot can also be interpreted that the protein content negatively influences the variable components in PC1 and PC2, the fat content has a positive influence for PC2, and the temperature is positive for PC1. The effects of these factors are important to be considered in the design of an active packaging with a releasing system. The PCA analysis gives insight with an easily understandable presentation on the correlation amongst the parameters.

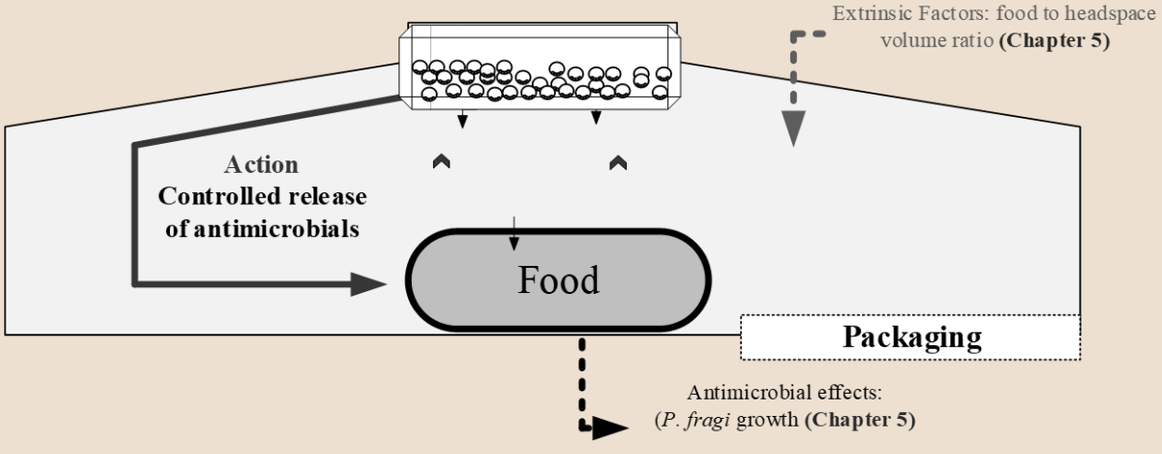


Appendix 4.14. (a) *Scree plot and* ***(b)*** *component plot of PCA Analysis in the rotate space*

Appendix 4.15*. Results of the varimax rotated factor matrix*

Variable	Components	
	PC 1	PC 2
Temperature	0.259	-0.055
Protein	-0.178	-0.364
Fat	-0.030	0.368
K_4	0.372	-0.026
mt_2	0.389	0.108
$K_{f/g}$	-0.102	0.397

4


Extrinsic Factors: food to headspace volume ratio (Chapter 5)
Action
Controlled release of antimicrobials
Food
Packaging
Antimicrobial effects:
(P. fragi growth (Chapter 5)

5

Packaging design using mustard seeds as a natural antimicrobial: A study on inhibition of *Pseudomonas fragi* in liquid medium

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Abstract

Pseudomonas fragi is the dominant spoilage organism in various foods, especially in spoiled milk, fish, and meats. Its growth can be inhibited by releasing allylisothiocyanate (AITC) from ground mustard seeds in the food package. This paper aims to investigate the antimicrobial potential of ground mustard seeds against *P. fragi* growth and the effectiveness of released AITC concentration from mustard seeds on microbial inhibition of the spoilage bacteria growing in the liquid medium. The AITC concentration in the headspace and the liquid medium was measured, and the growth of *P. fragi* in the liquid medium was monitored. Depending on the concentration of AITC, not only growth was inhibited, but even a reduction of the total count of *P. fragi* was observed. The inactivation rate (k) of *P. fragi* was estimated using first-order inactivation kinetics, and the minimum gaseous-released AITC to inactivate *P. fragi* was determined. Higher AITC concentration in the headspace and the liquid medium was observed when using a higher amount of ground mustard seeds and a lower food to headspace ratio. Increasing the amount of ground mustard seeds (>100 mg per 10 ml liquid medium) led to full inactivation of *P. fragi* in 48 hours. By using an inhibition sigmoid E_{max} model, the minimum gaseous-released AITC for inactivation of *P. fragi* in 48 hours was observed around 15 µg/l headspaces. These results indicate that inhibition of the spoilage bacteria and extending the shelf life using ground mustard seeds is only possible by applying a careful design of the packaging system.

Keywords: Mustard Seeds; Allyl isothiocyanate; *Pseudomonas fragi*; Spoilage Bacteria; Microbial Inhibition; Shelf Life; Antimicrobial Packaging

5.1. Introduction

Considerable amounts of fresh food are lost during processing, storage, and distribution due to spoilage in the supply chain. Microbial spoilage of food and the resulting food waste continues to be a major sustainability concern. Spoilage bacteria can quickly grow at food surfaces (Hazards, 2016) and cause off-odours and off-tastes, so the deteriorated food is considered to be unacceptable to consumers from a sensory point of view and finally wasted. This wasted food could be minimized by modifying packaging that either extends the food shelf life or helps customers to decrease food waste (Garnett, 2013). Antimicrobial packaging concepts are currently being developed for the extension of food product shelf life. These antimicrobial packages are able to release antimicrobial agents, which might inactivate and/or prevent the growth of spoilage bacteria on the food products.

Pseudomonas fragi is the dominant spoilage bacteria and has the strongest spoilage potential in a variety of foods that are stored under aerobic conditions (Wang et al., 2017a; Wang et al., 2018). It can be found in fresh fish, fresh milk, milk products, and meat (Stanborough et al., 2018). Compared to other species of *Pseudomonas spp.*, this *Pseudomonas* species is the predominant bacteria that contribute significantly to food spoilage under aerobic refrigeration, followed by *Pseudomonas lundensis* and *Pseudomonas fluorescens. P. fragi* is a psychrotrophic and gram-negative bacterium that can grow at temperatures between 2 and 35^{o}C (Ercolini et al., 2010). Many studies have found *P. fragi* to be responsible for food spoilage in foods under aerobic, vacuum, and modified atmosphere conditions (Ercolini et al., 2010; Lebert et al., 1998; Wang et al., 2018), but there is no specific information about food preservation strategies with the help of antimicrobial packagings, such as the use of antimicrobial substances that can be added to the package and then released into packaging headspace. The release of antimicrobial compounds from a package has been proven to effectively inhibit the growth rate of microorganisms and extend the shelf life of foods (Yildirim et al., 2017).

Allyl-isothiocyanate (AITC) is a volatile compound showing strong antimicrobial activity and bacteriostatic effects on a wide variety of spoilage bacteria by attacking the cell membranes of the bacteria (Kim et al., 2015; Kramer et al., 2018; Saladino et al., 2017a). AITC penetrates the cells to disrupt the cytoplasmic membrane of bacteria. The AITC penetration leads to cell leakage of their intracellular compounds and disaggregation of cell walls which causes loss of the integrity of the cellular structure (Dufour et al., 2015; Lin et al., 2000; Luciano and Holley, 2009). This effective mechanism of bacterial inhibition has been investigated and reported to be able to inhibit the microbial growth of spoilage bacteria in packaged food by adding the AITC inside the packaging. Kanemaru and Miyamoto (1991), monitoring the growth of spoilage bacteria, e.g. *P. fragi*, for 24 hours in nutrient broth containing mustard extracts or/and pure AITC, found that 0.043% of mustard extract and 3.6 ppm of pure AITC were

sufficient to inhibit the growth of *P. fragi* in the broth for 24 hours. Pang et al. (2013) reported AITC combined with modified atmosphere packaging (49% CO_2/0.5% O_2/50.5% N_2) inhibited the growth of *P. aeruginosa* by extending the shelf life of the catfish fillets from about 12 to 27 hours (18 µg /l) and 41 hours (36 µg /l) at 20°C. Another form is the usage of mustard oil, showing its antifungal activity against foodborne moulds strains in the liquid with the minimum concentration values ranging from 0.8 to 50 ppm (Clemente et al., 2019). However, the antimicrobial effect on the spoilage bacteria was reported on the amount of added AITC/mustard extracts in the packaging system or into the bacterial medium, not on the actual gaseous concentration in the headspace exposed to the food. Furthermore, no literature related to the minimum gaseous concentration of AITC inhibiting *P. fragi* growth was found, whereas the *P. fragi* was identified to be the dominant bacteria, with an incidence between 56.7% and 79.0% on spoiled meat (Wang et al., 2018). The effectiveness of AITC on inhibition and reduction of *P. fragi,* therefore, needs to be further investigated to quantify the minimum headspace concentration of AITC required to inhibit the bacteria.

The release rate of AITC from natural sources, e.g. mustard seeds, can be increased by manipulating the properties of the sources (Dai and Lim, 2014). Mustard seeds contain sinigrin, a glucosinolate acting as a precursor for AITC formation (Okunade et al., 2015). Once the cells of mustard seeds are damaged and hydrated, myrosinase hydrolyzes the sinigrin, and then AITC is formed and released (Olaimat et al., 2018). Our previous study reported that a higher release of AITC was observed in mustard seeds ground into smaller sizes and with lower fat content. However, the capacity to control bacterial growth was not investigated (Bahmid et al., 2020b). In this study, we explored the antibacterial potential of the ground mustard seeds against *p. fragi,* simulating a food pack system and using Brain heart infusion (BHI) broth as an example of highly perishable food. To assess the antimicrobial activity of ground mustard seeds against *p. fragi*, the effects of volume ratio of the liquid medium to headspace and temperature on the released AITC concentration in the headspace and the liquid medium were monitored in relation to design a packaging system that effectively inhibits *P. fragi* in the food system. The effectiveness of headspace AITC was also investigated by determination of minimum concentration to inhibit and inactivate the *p. fragi* in liquid medium. These results provide valuable insight for the customers and the development of an antimicrobial packaging concept with mustard seeds that can be applied to a variety of food products.

5.2. Materials and Methods

5.2.1. Chemicals

Mustard seeds (*Brassica juncea*) were purchased from Natuurproduct.com, Jacob Hooy Brown Mustard seeds. Plate count agar (PCA) was from Merck KGaA. Brain heart infusion (BHI) broth powder was from Oxoid LTD. Disposable inoculation loops were from VWR

International. Glycerol was from Fisher Scientific. Diethyl ether was from sigma Aldrich. Sterile Cryovial was from Simport Scientific, and Peptone physiological salt solutions (PFZ) were from Tritium Microbiology.

5.2.2. Preparation of ground mustard seeds

Mustard seeds were freeze-dried (Martin Christ) for 5 days and then immediately stored in a desiccator for 3 hours. The freeze-dried seeds were ground by using a milling machine (Analysette 3 Pro ball mill, Fritsch group) with an amplitude of 2.2 mm. Afterwards, the ground seeds were sieved (Retsch) to obtain the size ranges: 200-315 µm and 600-800 µm. The ground seeds (25 g) were completely defatted for 6 hours by soxhlet extraction (Nielsen, 2010) using diethyl ether solvent that has a low boiling point (35^{o}C) to avoid the inactivation of myrosinase. The defatted ground seeds were collected and put in a desiccator for 1 day to remove any water or solvent left in the seeds to prevent the pre-hydrolysis of sinigrin due to moisture uptake. Finally, the ground seeds were stored in a freezer at -20^{o}C until usage.

5.2.3. Preparation of plates, media, and culture

PCA-plate and BHI broth preparation

To count $\log_{10}$ CFU/ml per ml of sample, non-selective PCA was used. PCA was prepared using a 2 litre Duran flask filled with 1 litre of MilliQ water and 22.5 grams of PCA powder. The flask was then heated while being stirred. After the powder was completely dissolved, these flasks were sterilized at 121°C for 15 minutes. Afterwards, the flasks were cooled down to approximately 45°C in a water bath (Salm en Kipp). The cooled down flask was brought to the biosafety cabinet and then disinfected with 70% ethanol. About 10 ml of liquid PCA-agar was poured into Petri dishes and left to dry. Finally, the Petri dishes were stored in a refrigerator at 5°C until usage for the microbial experiments. BHI broth was prepared to be used as a growth medium (liquid medium). BHI broth powder (37 grams) was dissolved with Milli-Q water (1 litre) at a Duran flask. The solution was then sterilized in an autoclave at 121°C for 15 minutes. Afterwards, the broth was cooled down to room temperature. Finally, the broth was stored in a refrigerator at 5°C until usage for the experiments.

Stock and bacterial strain of P. fragi culture preparation

A bacterial strain of *P. fragi* culture Gruber 1905 (DSM 3456) obtained from Food Microbiology group Wageningen University and Research was used. With a disposable inoculation loop (VWR International), a colony from the pure *P. fragi* culture was taken and suspended into 9 ml of sterilized BHI broth in a 12 ml Greiner tube. The tube was then incubated in an incubator (IKS) at 30°C for 48 hours. To prepare a stock culture of *P. fragi*, the incubated BHI broth (0.7 ml) containing inoculated bacteria was mixed 0.3 ml of glycerol in a 1.2 ml sterile Crovial. The glycerol was added to protect the bacteria from freeze damage, e.g. membrane leakage (De Paoli, 2005). Finally, the culture was stored in a freezer at -20°C till usage.

A suspension of *P. fragi* culture was prepared 12 hours in advance of the microbial experiments. A tube with 1 ml of stock culture was taken out of the freezer and suspended into a 12 ml Greiner tube containing 9 ml of sterile BHI broth. The tube was incubated in a stove at 30°C for 12 hours. Afterwards, the suspension was diluted to approximately 5 Log_{10} CFU/ml in pre-manufactured 9 ml peptone physiological salt solutions. The suspension was plated out on PCA plates in triplicate. The plates were incubated for 48 hours at room temperature. After incubation, three randomly chosen colonies from each plate were tested to confirm that the colonies were indeed *P. fragi* colonies. The presence of the enzymes oxidase and catalase were both tested, and a Gram stain test was performed.

5.2.4. Determination of allylisothiocyanate in both the headspace and the liquid medium, and total bacteria of the P. fragi

A closed system was used as a packaging simulation system to investigate the AITC release in the headspace and the antimicrobial effect of AITC on the growth of *P. fragi* (**Figure 5.1**). In this experiment, three different liquid medium to headspace ratios were investigated; (1) 10:90 (10 ml broth: 90 ml headspace), (2) 30:70 (30 ml broth: 70 ml headspace) and 50:50 (50 ml broth:50 ml headspace). Five different amounts of ground mustard seeds were used; 0 (control), 5, 20, 100, 300, 1000 mg. For 1000 mg ground seeds, this amount was initially used for preliminary experiments to investigate the effect of the sizes (200-315 and 600-800 µm) of ground seeds and temperatures (4 and 20 ^{o}C). The system was designed in an airtight system using a Duran flask (100 ml volume) and a cap with an airtight septum. Using these caps, samples of the broth (0.1 ml) could be taken out by a needle and syringe, which can then be inserted for headspace AITC measurement without the possibility of the gas to escape from the flask. In the disinfected biosafety flow cabinet, sterile Duran flasks were filled with 9, 27, and 45 ml of pure BHI broth (control) and BHI broth inoculated with the suspension of *P. fragi* (1, 3 and 5 ml) containing approximately 5 Log_{10} CFU/ml. The ground seeds (5, 20, 100, 300, and 1000 mg) covered by sterile tea bags were completely submerged for 5 seconds and then immediately placed in the Duran flasks closed with a septum lid. The broth/headspace samples were then stored in a refrigerator at 4°C and on a dark shelf at room temperature (20°C). To prevent contamination, the flasks, the tea bags, the Greiner tubes containing the liquid medium, pipette tips, and water were first sterilized at 121°C for 15 minutes before the start of the experiment.

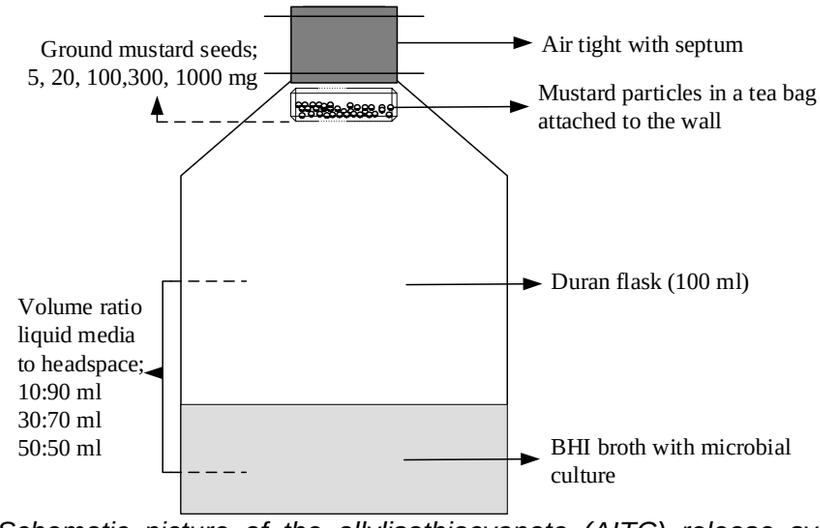

***Figure 5.1**. Schematic picture of the allylisothiocyanate (AITC) release system used for measuring the headspace AITC and the growth of P. fragi in the brain heart infusion (BHI) broth (liquid medium).*

AITC measurement in the headspace and the liquid medium

The concentration of volatile AITC in the headspace and the liquid medium of the closed system was measured at 6, 24, 48, 72, and 96 hours. For headspace measurement, the headspace of the samples was injected manually with Solid Phase Micro Extraction (SPME) (100µm polydimethylsiloxane, red fibre 23ga) fibre for 1 min, and the used SPME was then injected into the GC-FID.

For liquid AITC in the liquid medium, AITC was measured by a liquid injection method modified from Marton and Lavric (2013). The broth (0.1 ml) was taken out from the flask and then added into hexane (1.5 ml) in Eppendorf tubes. The mixtures were vortexed for 2 minutes and then centrifuged at 2627 g at 20^0C for 5 minutes. The solution was filtered by using a PTFE 45 um filter (Phenomenex) into the brown HPLC vials. Finally, the samples were measured by GC-FID in conjunction with a 10 µl syringe cemented needle (Hamilton Microliter) connected to an autosampler (Thermo-Scientific, TriPlus Autosampler).

During the AITC measurement in headspace and liquid, a Restek Rxi-5HT GC column (30-meter, 0.25 mm internal diameter, 0.25 µm stationary film thickness) was utilized. The inlet temperature was 250°C, a splitless mode for 1 minute (10ml/min flow) was maintained. The initial temperature of the oven was 40°C during the first minute of running. The temperature was increased until 280°C with a rate of 10°C per minute. The runtime was 26 minutes, and Helium gas was used as a carrier (1 ml/min). The detector had a temperature of 270°C, with a flow of 350 ml air and 35 ml H_2. AITC was analyzed with Xcalibur software, in which AITC is known to be detected as two separate peaks (Malabed and Noel, 2014). The calibration was quantified using pure AITC in concentrations 1 to 1000 ppm dissolved in hexane. This calibration was used to quantify the AITC concentration in the liquid medium. For the

headspace AITC concentration, the SPME values were converted by using an experimentally determined relation between the SPME and the direct injection of headspace samples and calibration with known amounts of AITC in hexane using liquid injection.

Microbial count of *P. fragi* growth

The number of cells, N (CFU/ml), surviving in the liquid medium, was monitored at 6, 24, 48, 72, 96, 120, 144 and 168 hours. In addition, the number of cell decrease during 48 hours were also observed in every 3 hours. The broth (1 ml) was taken from each Duran flask using a sterile syringe and needle passing through the septum without releasing AITC out of the flask. After serial dilution in 9 ml PFZ tubes, 100 µl of each dilution was plated out on PCA plates. Using this plate, the detection limit for *P. fragi* colony was 2 $\log_{10}$ CFU/ml. The plates were incubated at 37°C for 48 hours, and then colonies were counted. All plates containing at least 20 colonies to a maximum of 300 colonies were used in the calculations for the Log_{10} CFU/ml determination. The oxidase, catalase, and gram tests were performed on three random selected colonies per growth medium in order to confirm the presence of *P. fragi*.

5.2.5. Survival curves modelling

The concentration of AITC and the number of bacterial cells were measured in triplicate for each sample, and the data were presented as mean and standard deviation of three replicates using Microsoft office 2016. To describe the kinetics of the microbial survival from the obtained experimental data, first-order inactivation kinetics (Boekel, 2009) were used :

$$S(t) = 10^{\left(-\frac{t}{D}\right)} \quad \textbf{(5.1)}$$

$$Log\ S(t) = -\frac{t}{D} \quad \textbf{(5.2)}$$

where S(t) = N/N_0 is the survival fraction being N and N_0 the number of microorganisms at time t and time zero, respectively and the level of inactivation, $\log_{10}$ S(t). D is the decimal reduction time (the time needed to reduce the numbers/concentration by one $\log_{10}$):

$$D = \frac{ln10}{k} = \frac{2.303}{k} \quad \textbf{(5.3)}$$

in which k is a first-order rate constant (hours^{-1}).

Microsoft Excel was used to perform the estimation of the parameter, the rate constant (k) with the **Eq. (5.3)** substituted into **Eq. (5.2)**. The experimental data on the number of cells of *P. fragi* observed within 48 hours were fitted properly modelled using the solver in Microsoft Office Excel 365 ProPlus. **Eq. (5.2)** was best fitted to the data set by minimizing the sum of the squared differences between the empirical data and the fitted values provided by **Eq. (5.2)** using the Solver add-in (Hu et al., 2015).

5.3. Results

5.3.1. *The effect of particle size and temperature on AITC release in the headspace and the antimicrobial effect against P. fragi*

The preliminary results of the AITC released from mustard ground seeds (1000 mg) and its antimicrobial effect on the growth of *P. fragi* with the effect of particle size and temperature are shown in **Figure 5.2**. The temperature has a clear effect on AITC stability in the headspace, and the particle size shows an effect only in the shortest times. In **Figure 5.2a**, the higher AITC concentration at 20ºC was observed a few hours after rehydration. A higher temperature could initially increase the AITC volatility, which then causes an increase of the driving force of AITC from the ground seeds to headspace (Lim and Tung Marvin, 2006). However, the concentration was shifted after 24 hours, where the higher AITC was observed at 4ºC. These results demonstrate that the temperature is crucial for the stability of AITC needed to prolong bacterial growth inhibition.

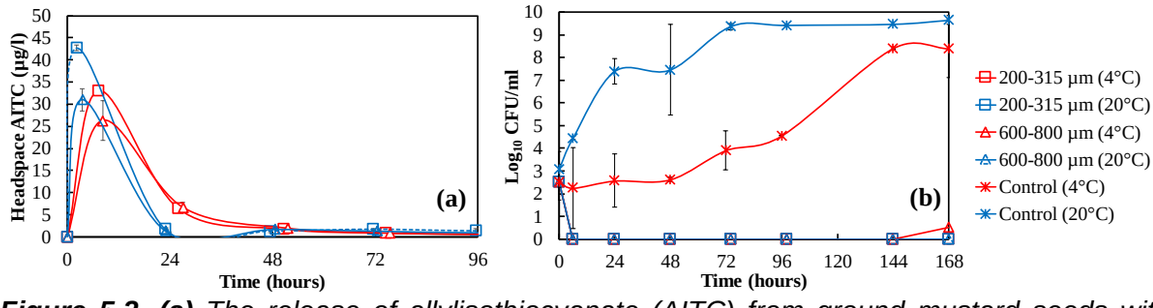

Figure 5.2. (a) *The release of allylisothiocyanate (AITC) from ground mustard seeds with different particle sizes (200-315 and 600-800 µm) stored at different temperatures (4 and ~20ºC) for 7 days;* ***(b)*** *the antimicrobial effect of AITC against P. fragi from the released AITC*

In **Figure 5.2b**, the antimicrobial effect of AITC against *P. fragi* was observed. The initial bacterial count of *P. fragi* was around 3 $\log_{10}$ CFU/ml. In control (sample without ground seeds), *P. fragi* grew quickly, and the bacterial population reached the stationary phase after 48 hours at 20^{0}C, compared to the control sample at 4^{0}C, taking 144 hours to reach the maximum growth. The presence of AITC released from the ground seeds at 4 and 20^{0}C completely inactivated the bacterial cells in the liquid medium within 6 hours, so the bacteria were no longer detectable. In an extended period (two weeks), the *P. fragi* growth was not observed in the BHI broth. These results indicate that the concentration of AITC released from 1000 mg ground seeds into the headspace kills all the *P. fragi*. Therefore, for the next experiment, lower amounts of ground seeds were studied to evaluate the antimicrobial effect for the particle size (200-315 µm) and temperature (20^{0}C).

5.3.1. *The effect of ground mustard seeds and the liquid medium to headspace ratio on AITC concentration*

Figure 5.3 shows the concentration of AITC released from different amounts of added ground mustard seeds in the closed containers containing different volumes of the liquid

medium. A reduction in the AITC concentration occurred in the presence of less ground seeds. In the headspace, AITC concentration was apparently decreased in the higher liquid medium to headspace ratio (**Figure 5.3**). About 27 µg/l AITC was released from 300 mg of ground mustard seeds into 90 ml of headspace at 6 hours, while only 10 and 20 µg/l was released into 50 and 70 ml of packaging headspace. These results indicate that besides the fat content and particle sizes of ground seeds (Bahmid et al., 2020b), AITC release into headspace is also influenced by the food to headspace volume ratio in the packaging system.

The liquid medium was a better phase to keep AITC remaining in higher concentration rather than in the headspace. In **Figure 5.3**, the AITC in both headspace and liquid medium reached peaks at 6 hours. Afterwards, the headspace AITC dropped down to an almost undetectable level after 24 hours, while at the same time, the AITC in the liquid medium reduced to about 3 µg/l that was above the headspace concentration. This concentration was declined only by 1 µg/l to 7 days. It can be concluded that AITC stability was also influenced by the phase where AITC partitions.

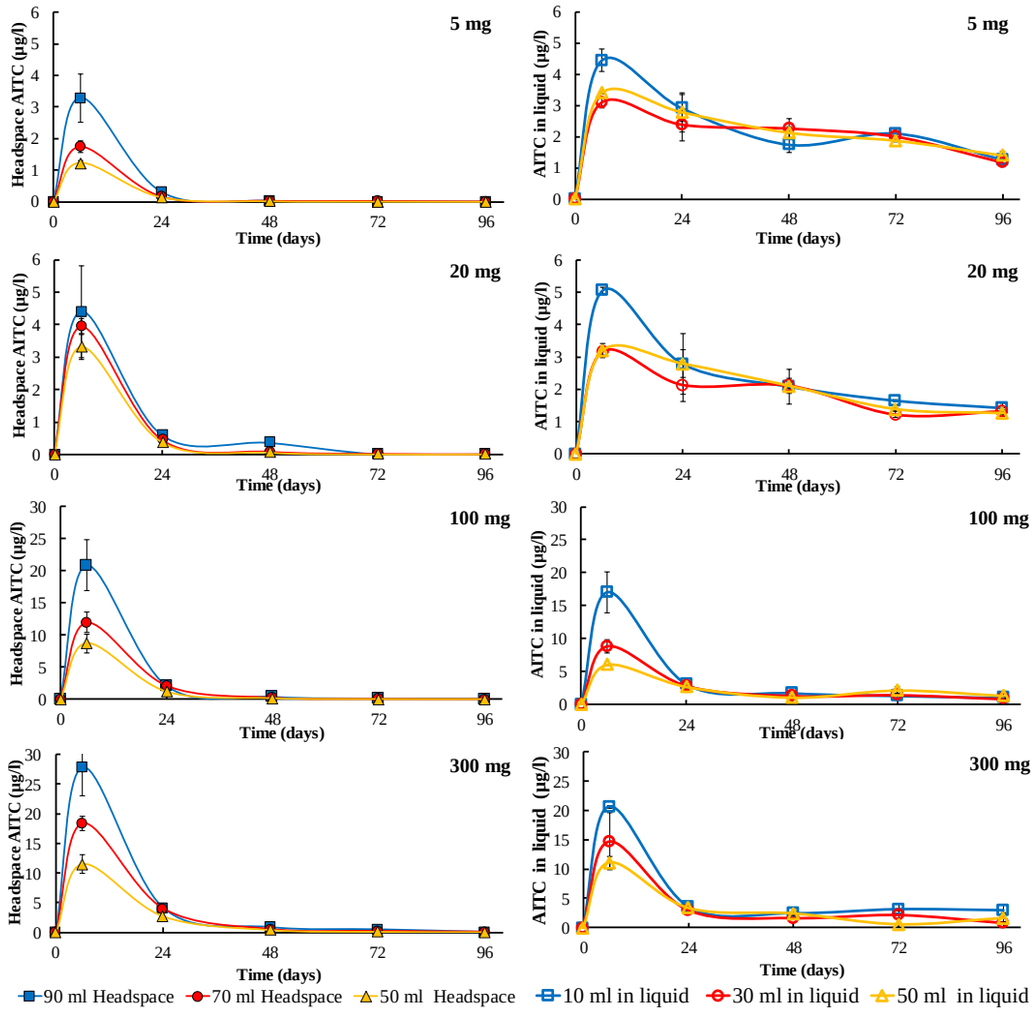

Figure 5.3. *The concentration of allylisothiocyanate (AITC) in the headspace (90, 70 and 50 ml)* ***(left side)*** *and the liquid medium (10, 30 and 50 ml)* ***(right side)*** *with an increase of amount of ground mustard seeds (5, 20, 100, 300 mg) added into the closed system.*

5.3.2. *The effect of ground mustard seeds on the growth of P. fragi inoculated in the different volumes of the liquid medium*

Figure 5.4 shows the total bacteria of *P. fragi* affected by the amount of ground seeds and the ratio of the liquid medium to headspace volume in the packaging system. The total bacteria of *P. fragi* was dependent on the amount of ground mustard seeds in the closed system; more ground seeds in the system speeded up the inhibition process of the bacteria. **Figure 5.4** shows a complete microbial inactivation in 48-72 hours with the highest amounts of ground seeds (100 and 300 mg) in the package setup. The use of the smaller liquid medium to headspace ratio speeded up the inactivation rate of *P. fragi,* as clearly observed with 100 mg ground seeds. In 10 ml of BHI liquid medium, 100 mg of ground seeds were able to completely inactivate the bacteria in 48 hours, but in 30 and 50 ml liquid medium, the bacteria population was reduced to be the undetectable level after around 72 hours.

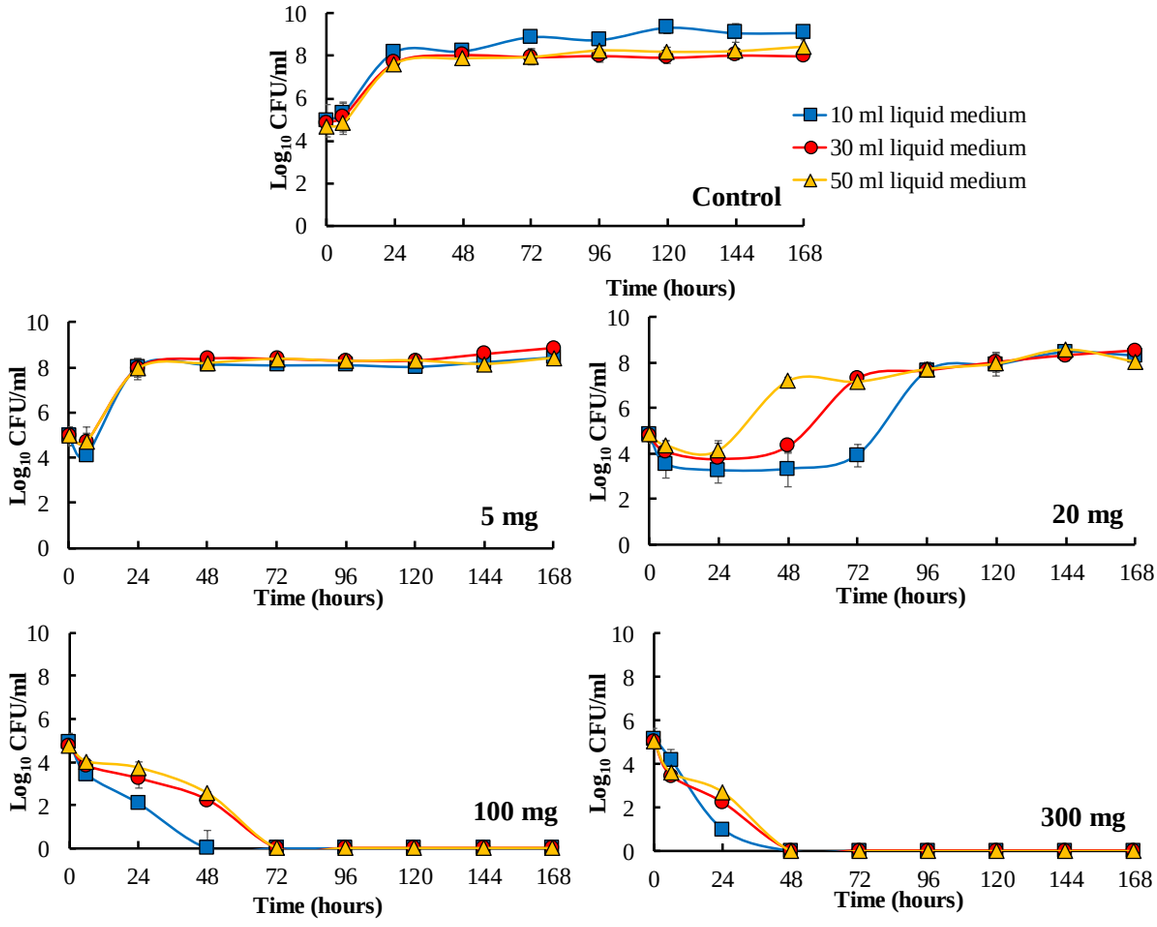

Figure 5.4. *The total bacterial count of P. fragi for 7 days influenced by the amount of ground mustard seeds added into the package and the liquid medium volume present in the package*

On the other hand, 5 mg of ground mustard seeds only reduced the bacterial population by around 1 $\log_{10}$ CFU/ml before *P. fragi* grew as fast as the control samples, reaching the maximum total bacteria after 24 hours. Ground seeds (20 mg) first reduced the population of *P. fragi* by around 1-3 $\log_{10}$ CFU/ml and subsequently also prolonged the lag phase before the

bacteria started to regrow. The growth of *P. fragi* was postponed longer for the smaller liquid medium to headspace ratio. *P. fragi* inoculated in the 10 ml, 30 ml and 50 ml of the liquid medium started to grow again after 1,2 and 3 days of incubation time, respectively.

5.3.3. The inactivation rate constant of inoculated P. fragi within 48 hours of contact time with AITC

Figure 5.5 shows the inactivation rate constant, a parameter estimated by the first-order inactivation kinetics model (**Eq. 5.2**) to mathematically describe the kinetic microbial inactivation for 48 hours (Appendix 5.1). The results clearly show that the larger amount of ground mustard seeds present in the packaging system increased the rate constant k. Consequently, the time needed to reduce the bacterial count by one decimal $\log_{10}$ becomes shorter (lower D-value). In **Figure 5.5**, with 20 mg ground seeds, the inactivation rate constant of *P. fragi* at 10 ml of liquid medium solution was around 0.13 $hours^{-1}$. With 300 mg ground seeds, the rate constant increased by 2.5 fold (0.33 $hours^{-1}$), leading to a reduction of the total bacterial count to an undetectable level after 48 hours (**Figure 5.4**). These estimated rate constants decreased with an increase of liquid medium to headspace ratio. For 20 mg ground seeds and 50 ml liquid medium, the rate constant cannot be estimated because the first order inactivation model did not fit with the observed data due to bacterial regrowth after 24 hours (Appendix 5.1). These results clearly show that the ratio of food to headspace volume for inoculating the bacteria and the amount of added ground seeds in the packaging system take an important role in the reduction and inactivation of the bacteria.

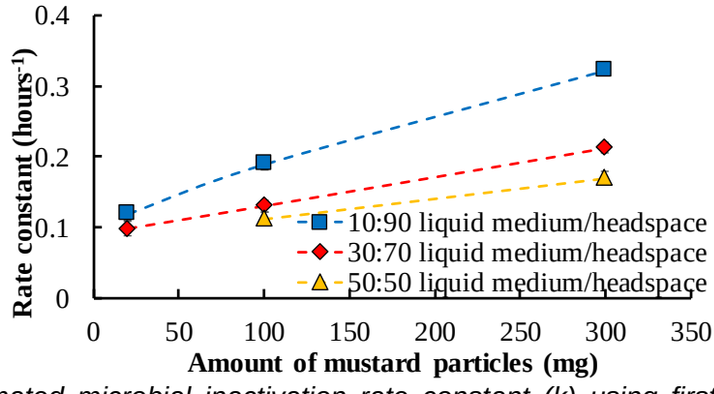

Figure 5.5. *Estimated microbial inactivation rate constant (k) using first-order inactivation kinetics for different amounts of ground mustard particles and liquid medium to headspace ratio.*

5.3.4. The fits of the inhibitory sigmoid E_{max} model to the highest concentration-inhibition curve

In this study, the highest (peak) concentration of AITC at 6 hours, shown in **Figure 5.3,** can be linked to the total bacteria of *P. fragi* (initially inoculated ±5 $\log_{10}$ CFU/ml) in order to determine the minimum headspace concentration to effectively inactivate the bacteria in 48 hours. The highest concentration-inhibition curve can be described using a modified inhibitory sigmoid E_{max} model (**Eq. 5.4**) (Dayneka et al., 1993; Parng et al., 2018) as shown in **Figure 5.6a**,

$$E = E_0 - \frac{{C_{6h}}^N E_{max}}{{C_{6h}}^N + {EC_{50}}^N} \quad \textbf{(5.4)}$$

where E is the total bacteria (log_{10} CFU/ml) at 48 hours showing the effect of the highest concentration of gaseous AITC; E_0 is the baseline total bacteria (log_{10} CFU/ml) without AITC (0 µg/l); E_{max} is the maximum effect (log_{10} CFU/ml) of the highest concentration of gaseous AITC, which assumed to be equal to E_0 due to full inactivation; EC_{50} is the highest concentration of gaseous AITC causing half of E_{max}; N is slope factor (hill factor) that determines the steepness of the concentration-inhibition curve; C_{6h} is the highest (peak) concentration of AITC at 6 hours.

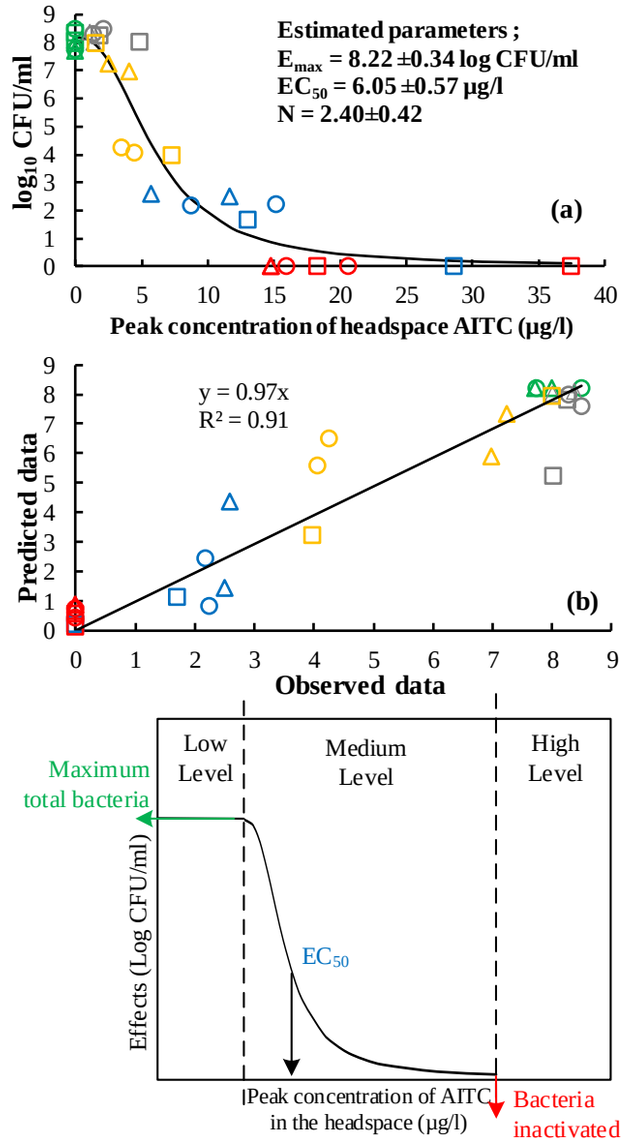

Figure 5.6. (a) *the total bacteria of P. fragi at 48 hours as a function of the peak concentration of allyl isothiocyanate (AITC) in the headspace, followed by inhibitory sigmoid E_{max} and the estimated parameters,* ***(b)*** *The goodness of fit of the E_{max} model, and* ***(c)*** *schematic overview of inhibitory E_{max} model. The different shape shows different volumes; square (10 ml), circle (30 ml), triangle (50 ml) and different colours depict the different amount of mustard seeds; red (300 mg), blue (100 mg), yellow (20 mg), grey (5 mg), and green (control).*

The inhibitory sigmoid E_{max} model fitted well with the highest concentration-inhibition curve. As shown in **Figure 5.6b**, the estimated parameters provide low standard deviations with no correlation amongst the parameters. Furthermore, high goodness of fit (R^2=0.93) was obtained from this model. It indicates the inhibitory sigmoid E_{max} model accurately described the relation of AITC with the total bacteria and can be employed to determine the minimum gaseous released AITC to inactivate the bacteria. The minimum headspace concentration estimated by using the model is discussed below.

5.4. Discussion

In this work, different AITC concentrations in the headspace and liquid medium were evaluated, with varying amounts of ground seeds and the ratio of the liquid medium to headspace volume. As expected, the concentration of released AITC increased with the amount of ground seeds present in the packaging system. A higher AITC concentration was also observed in the decreased liquid medium volume. The smaller volume of liquid medium absorbed less AITC from the headspace causing higher concentrations for the lower ratio of liquid medium-to-headspace volume. Upon partitioning of AITC to the smaller volume of the liquid medium, the concentration of AITC became higher, but the amount of AITC will be less. Therefore, it can be concluded that in a food pack system, if less food is present, the concentration in both food and headspace will be higher.

The bacterial growth and inhibition depend on the concentration of antimicrobial compounds in the headspace and the liquid medium in the package. The relation between the antimicrobials and growth inhibition was described by Clemente et al. (2019) that the increasing mustard oil (to 7 ppm) inhibited almost 50% of the growth of *R. stolonifer,* and the complete inhibition concentration was at almost 15 ppm. Comparing the effectiveness of AITC between both gaseous and aqueous phases, Several studies show that AITC is less effective to inhibit the bacteria in the (model) food phase compared to the gaseous phase (Kim et al., 2015; Lin et al., 2000). Lin et al. (2000) claimed the better bactericidal activity of AITC vapour due to (a) better penetration of gaseous AITC into the food, (b) low water solubility of AITC and high volatility of AITC and (3) AITC degradation in the aqueous phase. The claim was based on the comparison of antibacterial activity of AITC in gaseous (indirect contact with food) versus liquid (direct contact with food) form against the bacterial growth on food, in which AITC was more effective at a lower concentration in the gaseous form, compared to the liquid form. On the other hand, no study reports a comparison of AITC concentration in the packaged food and in the headspace to determine whether microbial inhibition occurred because of the gaseous AITC or AITC in the food. In our study, the actual AITC concentration in the headspace and the liquid medium was daily measured for a few days. The result shows that the AITC was released from ground mustard seeds into the headspace, then partitioned into

the food. In Appendix 5.2, AITC concentration in the headspace and the liquid medium at 6 hours have a linear correlation, but the AITC concentration was slightly higher in the headspace than AITC in the liquid medium. The higher AITC in the headspace does not mean that headspace AITC gave a better effect on microbial inhibition against *P. fragi* because the liquid medium also contains AITC. Instead, the headspace AITC might have a direct effect on the bacterial growth on the surface, while in the food, the AITC should first partition from the headspace into the liquid medium to then inhibit the bacteria in the liquid medium (Sekiyama, 1996) by attacking the growing cells of *P. fragi* over time. In addition, the observed better effect of gaseous AITC against the spoilage bacteria is observed for aerobic bacteria (Gill, 2003). The aerobic bacteria normally grow on the surface part of the packed food because of the dependence on oxygen availability. For these reasons, the gaseous AITC is more effective to inhibit the spoilage bacteria on the surface of the packed food.

The first-order inactivation kinetic model (**Eq. 5.2**) describes well the inactivation of *P. fragi* for 48 hours. From the model, the rate constant (k) parameters ware estimated, as shown in **Figure 5.5**. The minimum inactivation rate (k) to fully inactivate the total bacteria (inoculated at ±5 $\log_{10}$ CFU/ml) for 48 hours is over 0.15 $hours^{-1}$, which was determined from 100 and 300 mg of ground mustard seeds that totally reduced the total bacteria (**Figure 5.3**). In agreement with our results, although no results on the inactivation rate were given, microbial inactivation in the presence of AITC at over 20^{o}C was reported by Guo et al. (2017). This study reported a fully reduced population from 5 logs *L. innocua* in Tryptic soy broth (TSB) at 22^{o}C with an edible coating containing 2 to 4% of AITC and 1% of AITC for 2 and 3 days of inactivation, respectively. These indicate the AITC has a strong antimicrobial activity to inactivate the total population of spoilage bacteria in the (model) food and the inactivation rate is faster at higher AITC concentration in the packaging system.

The total bacteria is highly related to the concentration of antimicrobial compounds added to the headspace of food packaging (Dufour et al., 2015). The initial concentration of pure compound added into packaging at 0 hours can be assumed to be considered equal with the concentration peak (after few hours) of antimicrobial compounds released from the naturally antimicrobial carrier added into the packaging system. To determine the headspace AITC concentration that is sufficient to inhibit the *P. fragi*, the peak headspace concentration-inhibition curve was fitted with the inhibitory sigmoid E_{max} model (**Eq. 5.4**) as shown in **Figure 5.6**. The model gives an understanding of the headspace concentration of AITC released from mustard seeds required to reduce and inactivate the bacteria in packed food products. **Figures 5.6a** and **5.6c** define the level of peak AITC concentration that can affect the total bacteria. There are three levels of concentration; low, medium and high. In the low level, the concentration resulted in no effect in total bacteria. The medium level can be defined as a sensitive concentration because the AITC reduces the total bacteria, but the bacteria might

still re-grow after reduction. At the high level, the concentration induced a complete reduction or inactivation of the bacteria. From this model, the minimum-gaseous released AITC causing inhibition and inactivation of *P. fragi* in 48 hours can be defined, which are around ±6.05 µg/l (1.49 ppm) for microbial inhibition and at least 15 µg/l (3.70 ppm) for inactivation. The inhibitory concentrations were lower than the minimum concentration of pure AITC (3.6 ppm) initially added into the nutrient broth to inhibit the growth of *P. fragi* for 24 hours (Kanemaru and Miyamoto, 1990). Comparable minimum vapour concentrations of AITC for inactivation against other microorganisms inoculated at 4-5 $\log_{10}$ CFU in agar plates for 2 days was also observed; *Pseudomonas fluorescens* (36-50 µg/l), mould (16-22 µg/l), yeast (16-31 µg/l) (Dai and Lim, 2014; Isshiki et al., 1992). The different results of minimum concentration might be because of the difference in experimental design, like using agar as culture media or added directly AITC into food products, while in our study the the liquid medium was used to grow the bacteria. In summary, AITC concentration,15 µg/l (3.70 ppm), released from mustard seeds (>100 mg) into the 90 ml headspace and 10 ml liquid medium, is sufficient to totally reduce *P. fragi* in the liquid medium to an undetectable level for 48 hours.

For the application of mustard seeds in a food package, the required ground mustard to inhibit the bacteria in a food, e.g. fresh catfish fillet (Pang et al., 2013), can be estimated using the first-order inactivation model to calculate the inactivation rate (k). The catfish fillet initially contained around 3.5 $\log_{10}$ CFU/g of the microbial population of *P. aeruginosa* (Pang et al., 2013). To inhibit the total bacteria *P. aeruginosa* in 50 mg of catfish fillet for 48 hours, the calculated k was observed, around 0.168 $hours^{-1}$. In relation to **Figure 5.5**, the obtained k indicates that 300 mg of ground mustard seeds are assumed to effectively inhibit the bacteria in the catfish fillet for 48 hours. Nevertheless, this AITC concentration may vary due to several other factors to be considered, e.g. particle sizes, temperatures, fat content, and food components influencing the AITC release and stability in the headspace.

The biggest challenge of mustard seeds in the application in packaged foods is the sensory effects on food products. The sensory impact highly depends on the AITC concentration in the packaged food. This sensory effect of AITC concentration has been reported in some studies. AITC concentration (0.1 to 2.5%) in hummus (Olaimat et al., 2018) and in kimchi (J.A. Ko, 2011) was organoleptically accepted by the panellists. Lopes et al. (2018) also evaluated sensory the AITC concentration (0.5 to 2.5 µl/l) on Brazil peanuts, in which the highest doses (2.5%) of AITC did not change the sensory properties of Brazil peanuts. In this study, we used 20 to 300 mg of ground mustard seeds in 100 ml packaging volume (0.02 to 0.3% w/v), releasing around 1-30 µg/l in the headspace and 3-20 µg/l in the liquid medium, which is a lower concentration than those in the aforementioned literature, assuming less effects of food sensory. However, regardless of high dependence on the type of food, sensory evaluation needs to be performed for further confirmation of gaseous AITC concentration acceptable by consumers.

5.5. Conclusions

The aim of this study was to explore the antimicrobial potential of ground mustard seeds and the effectiveness of released AITC concentration from mustard seeds on microbial inhibition of *P. fragi* growing in the liquid medium. The concentration of AITC in the headspace and the liquid medium is dependent on the amount of ground mustard seeds added into the packaging system; more ground seeds released more AITC in the headspace and liquid medium. Consequently, the higher the concentration of AITC, the stronger the inhibition of *P. fragi* growth. The AITC released from 100 mg of ground seeds in the package with 10 ml liquid medium fully inactivated the cells of *P. fragi* in 48 hours. The amounts of AITC released in a package with 20 mg of ground mustard seeds did not completely inactivate the bacteria but was able to effectively reduce the bacterial count by 1-3 $\log_{10}$ CFU/ml before the bacteria re-grew after 1-3 days of incubation, depending on the liquid medium to headspace ratio. For a smaller liquid medium to headspace ratio, an increased AITC concentration in the headspace is required to extend the microbial inhibition of *P. fragi*. By using the sigmoid E_{max} inhibition model, the minimum-released AITC concentrations required to inhibit *P. fragi* are determined, around 6 µg/l (1.49 ppm) for reduction and 15 µg/l (3.70 ppm) for full inactivation. These results show that ground mustard seeds are effective to control the spoilage by *P. fragi* on food products at a concentration not influencing the food sensory. This study shows the effectiveness of AITC released from ground mustard seeds to control the spoilage bacteria, thereby giving insight for the customers in the design of food packaging system to extend the shelf life of a variety of packaged food products

Acknowledgements

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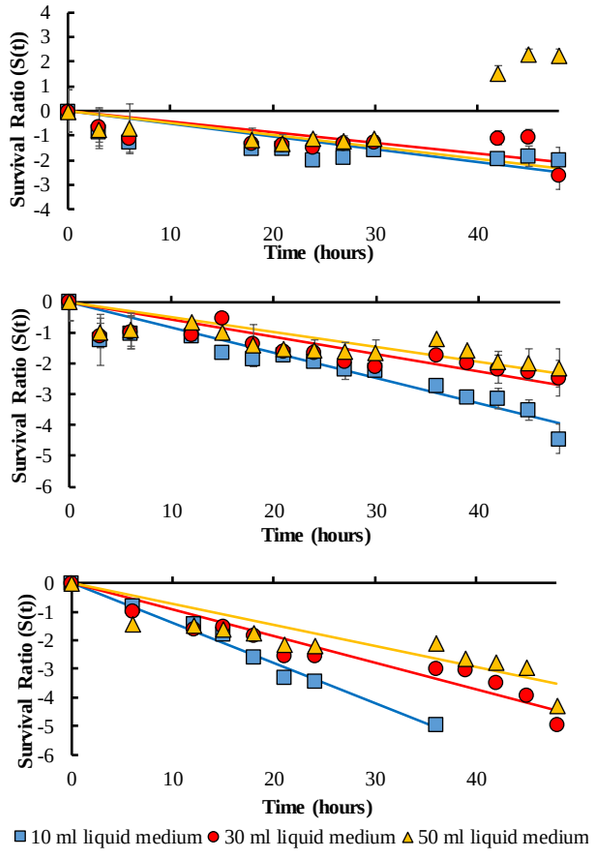
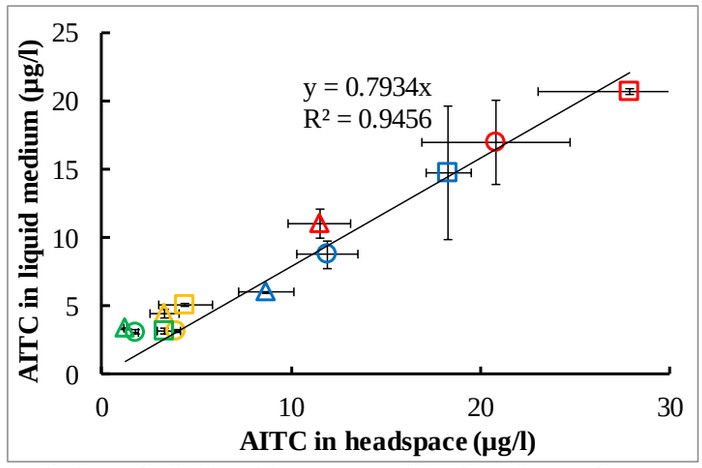
Appendix 5. Supplementary Materials

The following are the supplementary data to this article

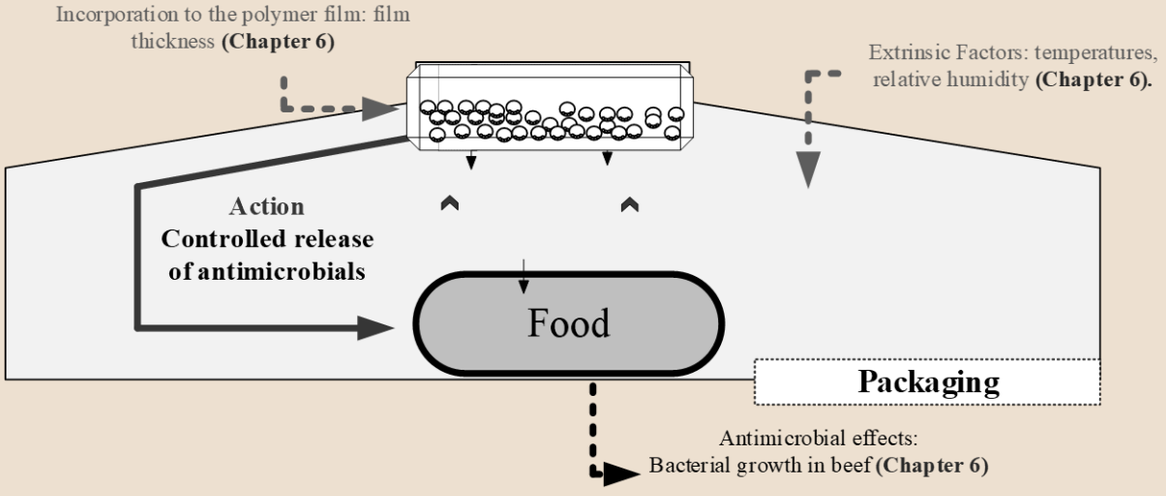


Appendix 5.1. *The kinetic microbial inactivation with* ***(a)*** *20,* ***(b)*** *100 and* ***(c)*** *300 mg of ground mustard seeds on the Pseudomonas fragi inoculated in different liquid medium volume.*



Appendix 5.2. *The relation of allyl isothiocyanate (AITC) release from ground mustard seeds in the headspace and in the liquid medium at 6 hours. The different shape shows different volumes; square (10 ml), circle (30 ml), triangle (50 ml) and different colours depict the different amount of mustard seeds; red (300 mg), blue (100 mg), yellow (20 mg) and green (5 mg).*

5


Incorporation to the polymer film: film thickness (Chapter 6)
Extrinsic Factors: temperatures, relative humidity (Chapter 6).
Action
Controlled release of antimicrobials
Food
Packaging
Antimicrobial effects:
Bacterial growth in beef (Chapter 6)

6

Development of a moisture-activated antimicrobial film containing ground mustard seeds and its application on meat

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Abstract

An antimicrobial cellulose acetate film containing finely ground mustard seeds was developed. The effect of the film properties on the allyl isothiocyanates (AITC) formation and release into the headspace was investigated. Less porous structure and larger thickness caused slower moisture absorption and consequently a slower AITC formation and release rate. For its application in food, the film was tested on whether the concentration of AITC was sufficient to inhibit bacterial growth in ground beef with different fat contents. The AITC concentration for the low-fat ground beef was lower in the product and higher in the headspace compared to medium fat ground beef. The shelf life was extended by 3.7 and 0.6 days for low fat and medium fat ground beef, respectively. Besides a novel way to control the compounds' release to the packaging system, this study also shows the importance of the food composition for tailoring the effective active packaging.

Keywords: Allyl isothiocyanate; Controlled Release; Sinigrin; Mustard Seeds; Antimicrobial Packaging; Shelf Life; Ground Beef.

6.1. Introduction

Spoilage bacteria and microbial contamination are major issues for food industries in maintaining the quality and safety of food products (Roodhuyzen et al., 2017). Antimicrobial packaging is being developed by incorporating specific compounds into the packaging material to protect the food from spoilage bacteria during storage. The inhibition of spoilage can be done either by direct contact between packaging material and foods or by indirect contact, in which the antimicrobial packaging releases a volatile antimicrobial agent into the headspace of the packaging (Quintavalla and Vicini, 2002; Yildirim et al., 2017). The volatile antimicrobials, e.g. essential oils (Rao et al., 2019) and allyl isothiocyanates (AITC) (Luciano and Holley, 2009), have a greater antimicrobial activity at a lower concentration on the inhibition of microbial growth than the non-volatiles that are normally applied by direct addition into food products (MejÍA-Garibay et al., 2015; Suhr and Nielsen, 2003). The direct addition of compounds causes a quick interaction between the compounds and food components, which can negatively affect the sensory food characteristics (Kapetanakou and Skandamis, 2016). The types of antimicrobial compounds, the release of compounds into the headspace, and their interaction with food products should be considered to effectively inhibit the bacteria.

AITC is a lipophilic volatile with strong antimicrobial properties that effectively inhibits the growth of a wide range of microorganisms (Kurek et al., 2017; Quiles et al., 2015). For application in an antimicrobial packaging system, an active carrier is required to release the AITC into the packaging headspace, where the AITC can effectively suppress the bacteria growth in the food surface. Mustard seeds can be an active carrier that can release AITC in a packaging system. Mustard seeds are a good natural source of sinigrin (Sharma et al., 2018), a precursor for the AITC formation, and contain an endogenous enzyme called myrosinase (Nakano et al., 2014). Myrosinase and sinigrin are located in different cells; Myrosinase in myrosin cells and sinigrin in s-cells (vacuoles) (Kissen et al., 2009). The AITC formation occurs once the myrosinase enzymatically interacts with the sinigrin after cell disruption, e.g. through fat extraction or milling process, in the presence of water (Oliviero et al., 2014). The moisture is essential to trigger the AITC formation (Chen et al., 2019). In a packaging system, the packaged (model) foods can vaporize the moisture that can be absorbed by ground mustard seeds to cause a release of AITC into the headspace. Such a packaging system is defined as a triggered release system created to prevent the premature release of volatiles until the release is triggered by an internal stimulus in a closed packaging system (Chen et al., 2019).

The incorporation of the antimicrobial agent into the packaging polymer prevents the premature excessive release of the AITC (Appendini and Hotchkiss, 2002; Fang et al., 2017). Methods with the incorporations of pure AITC into film polymers, like an encapsulation of AITC /β-cyclodextrin complex encapsulated in electrospun nanofibers (Aytac et al., 2014) and

encapsulation of AITC in poly(lactic-co-glycolic acid) nanoparticles (Encinas-Basurto et al., 2017), have been studied to control the AITC volatility. These methods resulted in a sustained release of AITC due to a slower diffusion rate of AITC through the packaging material. These researches mainly investigated the release of volatiles using pure (chemical) AITC in the package. In the current study, ground mustard seeds were used as a natural source of AITC to be incorporated into the packaging polymer. This method can potentially enable a triggered release system by moisture by controlling both the AITC formation and release, which is not possible in the incorporation of pure AITC in the packaging materials.

For the application of an antimicrobial package on food products, in-depth knowledge of the food, e.g. food composition and structure, is important because interactions between the applied antimicrobial compounds, the package, and the food components are possible. The interactions affect the antimicrobial activity of the compound against the bacteria growing on and in a food product (Keppler et al., 2017). For example, AITC that was added into a food model system containing whey protein isolate caused covalent binding between AITC and the protein, and no antimicrobial effect of AITC on the bacteria was observed (Keppler et al., 2017). Other components, like fat, could also affect the distribution of AITC between the fat phase and other phases in food due to its solubility in the fat phase (Giroux et al., 2007; Tsao et al., 2000). Besides the factors influencing the AITC release, the understanding of the effects of fat influencing the absorption of AITC by food products could help to improve the growth inhibition of spoilage bacteria by antimicrobial packaging.

In this study, the ground mustard seeds were applied in a hygroscopic polymer film made of cellulose acetate (Białopiotrowicz and Jańczuk, 2002) to be used as a triggered release system in a packaging system. The current study aims to develop moisture activated antimicrobial film containing ground mustard seeds and understand the release mechanism of AITC from the ground mustard seeds incorporated in the film by investigating the effect of thickness of the film, temperature, and relative humidity, on AITC release in the packaging system. This study also investigated the antimicrobial effect on ground beef. The effect of the fat content on the AITC release from the film and absorption by the food and the resulted antimicrobial effects against the spoilage bacteria in the ground beef was studied. The shelf life extension of ground beef containing different fat content was also predicted by modelling microbial growth. This study shows that active packaging design can be optimized by tailoring the properties of active sources and food products to reach the desired and extended shelf life of food products.

6.2. Materials and Methods

6.2.1. Materials

Mustard seeds were purchased from Natuurproduct.com, Jacob Hooy Brown. Lean ground beef (around 9% fat) and normal ground beef (around 19% fat) with the same production dates were supplied from a local supermarket, Albert Heijn. Cellulose acetate (39.8 wt% acetyls) used as a film polymer, and glycerol (≥99.5%) were purchased from Sigma Aldrich. Sterile Cryovial was from Simport Scientific, and Peptone physiological salt solutions (PFZ) were from Tritium Microbiology. Disposable inoculation loops were from VWR International. Other chemicals used in this study were of analytical grade and purchased from Sigma Aldrich or Merck.

6.2.2. Film preparation by solution casting

Films incorporating mustard seeds were produced by using a solution casting method modified from Gonçalves et al. (2019). To obtain a varying thickness of the film, different weights of cellulose acetate (300, 700, 1100, and 1500 mg) were used with 500 mg of ground mustard seeds with particle sizes <100 µm (Bahmid et al., 2020b). For the control, 700 mg of cellulose acetate was used. The cellulose acetate was solubilized in 25 mL acetone as a solvent in 50-mL Duran flasks and then stirred for 10 minutes using magnetic stirrers at room temperature until well dissolved. Glycerol (3 mL) acted as a plasticizer was also added to the solution and stirred for 10 minutes until well dissolved. Finally, the ground mustard seeds (500 mg) were slowly added into the solution while stirring continuously, and then the Duran flasks were closed tightly with caps to avoid evaporation of the solvent during stirring. The stirring was stopped after 3 hours when the mustard particles, glycerol, and cellulose acetate turned into a homogenous mixture. The mixtures were subsequently poured into glass Petri dishes (diameter 100 mm) and stored in a fume hood for 3-5 hours to evaporate the solvent at room temperature. Afterwards, the formed films were removed from the Petri dishes and then cut into pieces with a size of 2 by 2 cm. Each film was weighed, and the thicknesses were measured using a calliper, with 0.001 mm precision, at four points for each side of the films. To prevent premature release of AITC during storage before its usage, the films were stored in an impermeable glass Duran flask at -20^{o}C until usage.

6.2.3. Confocal microscopy and Scanning Electron microscopy

The distribution of ground seeds in the film was measured by confocal microscopy. Confocal microscopy was used to characterize the phase composition of the substrate in the film polymer (Lattante et al., 2014). Fluorescent dye calcofluor white (0.002 wt%) were prepared to stain the films containing the ground seeds and also the control film (without incorporation of ground seeds). The dye mixture (30 µl) was added to the films (2x2 cm) that were placed on a glass microscope slide. The films were visualized by a confocal laser

scanning microscope (LSM) type 510 (Zeiss, Oberkochen, Germany) using a 405 nm blue diode laser for calcofluor white. All pictures were analyzed with the ZEN blue edition (Carl Zeiss Microscopy).

The surface of the films was investigated by scanning electron microscopy (SEM). The films were snap-frozen in liquid nitrogen and subsequently freeze-dried for SEM analysis. A film was attached on SEM sample holders using carbon adhesive tabs (EMS, Washington, USA), sputter-coated with a 15 nm thick tungsten layer (EM SCD 500, Leica, Vienna, Austria) and subsequently analyzed with a field emission scanning electron microscope (Magellan 400, FEI, Eindhoven, Netherlands) with SE detection at 2 kV and 6.3 pA.

6.2.4. Experimental packaging design with a mustard seeds-incorporating film

Glass vials (10 mL) were used to simulate an impermeable package, which was adapted from the previous study (Bahmid et al., 2020a). A 2-by-2 cm film (with varying thickness) was put inside the glass vials containing water or a saturated salt solution (1 mL) by attaching the film under the closed lid. The vials containing a cellulose acetate film without ground mustard seeds were used as a control. The vials were held underwater for a minute to ensure there was no leakage in the package. Different liquids were added into the flasks to study the effects of relative humidity (RH) on the AITC release; Milli-Q water for RH 100% and a saturated salt solution (Natrium Chloride, NaCl) for RH 75.5% (Cerisuelo et al., 2012). Besides, the vials were stored at different temperatures (5, 10, and 20^{o}C). Each sample was prepared in a new batch for each daily measurement. To understand how moisture can be an internal stimulus for AITC release in the packaging system, the moisture absorption of the films, sinigrin content in ground mustard seeds, and AITC concentration in the headspace were measured for 4 days.

Determination of moisture absorption into the film

Moisture plays a role in the hydrolysis reaction of sinigrin and therefore provokes AITC formation. The films were weighted using a balance (Mettler A-150, Columbus USA) to measure the moisture absorption of the film. The percentage of moisture absorption was calculated using the following **Eq. (6.1)**;

$$Wa = \frac{W_t - W_o}{W_o} x100\% \qquad \textbf{(6.1)}$$

where W_a is the percentage of absorbed moisture (%), W_t is the weight of the film in different time points (g), and W_o is the initial weight of the film (g).

Determination of sinigrin content in the film

The films are firstly cut in pieces of about 0.2-0.3 cm to enlarge the surface of the films, so the solvent can penetrate the films to extract the sinigrin content of the film. The sinigrin was extracted from the small pieces of film using the method described in the previous study (Bahmid et al., 2020a). The extracted solutions were collected in an HPLC vial and analyzed

using HPLC (Thermo Scientific UHPLC focused, Dionex UltiMate 3000) with a C18 reversed-phase HPLC column (Merck; Lichrocart 100 LP18). The measurement method and software settings of the HPLC was, according to Bahmid et al. (2020b). To quantify the sinigrin concentration in the films, the ratio of sinigrin to glucotropeolin (as an internal standard) areas were multiplied with the mol of the glucotropeolin per weight of the films and the relative response factor (RRF) of sinigrin (1.053).

Determination of Allyl isothiocyanate in the headspace

The AITC concentrations in the headspace were measured with Gas Chromatography - Flame Ionization Detection (GC-FID) (Thermo-Scientific Focus GC), using direct injection. A sample of 500 µl gas was taken out of the headspace of the 10 mL-vial containing the film and then manually injected into the GC apparatus. The method and software performed in the GC apparatus were the same as described in the previous study (Bahmid et al., 2020b). To calculate the concentration of AITC in the headspace, the obtained area was converted to concentration (µg/L) by using the calibration quantified using pure AITC (Allyl isothiocyanate, 97%) dissolved in Hexane, with the range of used concentrations 1 – 1000 ppm.

6.2.5. Experimental design of antimicrobial packaging containing the mustard seeds-incorporating film and ground beef

The antimicrobial effect of the film incorporated with ground mustard seeds was tested on real food, ground beef. Antimicrobial packages with ground beef were prepared as described in **Section 6.2.4**. The film thickness used in this experiment was the thickest film produced with 1500 mg of cellulose acetate. As shown in Appendix 6.2, 1 g of extra-lean ground beef (around 9% fat content) or medium fat ground beef (around 19% fat content) was taken out of the package under aseptic conditions, weighed, given a flat-round shape and placed in the bottom part of the sterilized glass vials. The films were attached under the lid of the vials. The vials without films were used as control. These preparations were conducted under an aseptic condition in a disinfected safety flow cabinet to ensure no cross-contamination during the preparation of samples and packaging. Glasses and pipettes used in the experiments were sterilized at 121^{o}C for 15 minutes, and the flow cabinet was disinfected under UV light and using ethanol (70%) before sample preparations. The vials were then kept at refrigerated temperature (around 5^{o}C). The measurement of AITC concentration in the headspace and ground beef and microbial growth of the ground beef was conducted for 10 days.

Determination of AITC in the headspace and ground beef

For headspace AITC, the measurement was conducted as described in the previous part (determination of Allyl isothiocyanate in the headspace). The AITC concentrations in the ground beef were measured by using the adapted method from Marton and Lavric (2013). The

ground beef was taken out from the flask and then added into 10 mL hexane in 15-mL tubes. The mixtures were vortexed for 5 minutes and then centrifuged at 2627 g at 20 °C for 5 min. The supernatant (1 mL) was filtered using a PTFE 45 um filter (Phenomenex) into brown HPLC vials. The samples were measured using GC-FID in conjunction with a 10 µL-syringe cemented needle (Hamilton Microliter), connected to an autosampler (Thermo-Scientific, Waltham, MA, USA, TriPlus Autosampler). The method of measurement using the GC apparatus was the same as described in section 'determination of Allyl isothiocyanate in the headspace'.

Microbiological analysis of ground beef

Non-selective plate count agar (PCA) was used as a medium to count the bacteria of the ground beef. The pipettes and other materials used in the experiments were sterilized at 121ºC for 15 minutes, and the flow cabinet was disinfected under UV-light and using ethanol (70%) before the analysis was started. The ground beef was taken out of the packaging (glass vial) and then put in a sterile *Stomacher* bag. Sterile peptone water (9 mL) was added into the bag to achieve 10^{-1} dilution. Afterwards, the content in the Stomacher bag was homogenized for 1.5 min at 260 rpm by using Stomacher® *400 Circulator Lab Blender*. After the ground beef was completely homogenized, the homogenate was transferred into a sterile *Greiner* tube. A 1-mL sample was diluted in 9 mL of peptone physiological salt solutions (PFZ) up to $10^{-6,}$ and 100 µl of each dilution was plated out on PCA plates. The plates were incubated at 30°C for 48 hours, and then the colonies were counted. Only the plates containing 20 to 300 colonies were counted to determine Log_{10} CFU/mL.

Kinetic modelling of microbial growth and determination of ground beef shelf-life

The kinetics of microbial growth of the spoilage bacteria in ground beef was described by using using the Gompertz model **Eq. (6.2)** modified by Zwietering et al. (1990):

$$ln\frac{N}{N_o} = A_s.exp\left[-exp\left(\frac{\mu_{max}.e}{A_s}.(\lambda - t) + 1\right)\right] \quad \textbf{(6.2)}$$

where N (CFU/g) is the number of bacteria at time t, N_o (CFU/g) is the total population of bacteria at time 0, A_s is asymptotic value, μ_{max} is maximum specific growth rate (h^{-1}), ƛ is lag time (hours) and t is time (hours).

The shelf life of the ground beef was determined by using the **Eq. (6.3)** derived from the **Eq. (6.2)** as the following equation

$$t_s = \lambda - \frac{A_s.\left\{ln\left[-ln\left(\frac{\ln(N_S)-ln(N_O)}{A_S}\right)\right]-1\right\}}{\mu_{max}.e} \quad \textbf{(6.3)}$$

where t_s is the shelf life of a product, N_s is the maximum number of spoilage bacteria at the end of the shelf life of the food. The level of spoilage bacteria at the end of the shelf life of ground beef (N_s) was defined to be 10^7 CFU/g (Koutsoumanis et al., 2006).

6.2.6. Statistical analysis

All data of headspace AITC concentration, sinigrin content, and the total population of bacteria was performed in triplicate. The data of the survival ratio (N/N_0) of total bacteria was fitted with the non-linear regression model in **Eq. (6.2)** by minimizing the sum of squared differences (SSR) with the Solver add-in in Microsoft Excel to estimate the growth parameters consisting of μ_{max}, A_s, and ƛ. A_s is assumed to be constant for all samples. The standard deviation of the parameters and correlation amongst parameters were calculated using the macro SolverAid (Hu et al., 2015). Significant differences (at the level of $p < 0.05$) of the experimental data and parameters were evaluated.

6.3. Results and Discussion

6.3.1. Thickness, morphology of the film, and distribution of ground seeds in the film

The thickness of the film with ground mustard seeds incorporated is shown in Appendix 6.1. The average thicknesses of the films made using 300 mg (thinnest film) to 1500 mg (thickest film) of cellulose acetate were around 134 ± 13 to 293±25 µm, while the thickness for the control film was around 121± 10 µm. The results revealed a linear increase of the thickness with the increasing content of cellulose acetate in the film.

The morphology of the thickest and the thinnest films, as well as the control film without ground mustard seeds, was observed using SEM (**Figure 6.1a-f**). **Figure 6.1a** reveals a sponge-like porous structure (white arrows) on the surface of the film control (without the addition of ground mustard seeds). The sponge-like porous structure can also be seen in the cross-section image, as shown in **Figure 6.1d**. With the incorporation of ground mustard seeds into the polymer solution, a strong reduction of the porosity on the surface was observed, and the morphology of the film surface became more compact, homogenous, and smoother, as shown in **Figures 6.1b** and **6.1c**. The porous structure indicated by white arrows was possibly due to the evaporation of acetone during the drying process. The sponge structure is also known as “breath-figure” structures on the surface obtained from the evaporation of the solvent droplets on cellulose acetate-glycerol (as plasticizer) solution of the film (Furtado et al., 2020; Kalaycıoğlu et al., 2020). The smoother surface and low porosity in the film containing ground mustard seeds are in line with the results obtained by Rajeswari et al. (2020), who investigated the effect of the addition of polysaccharide (fine powder of sodium alginate and carrageenan) on the film formation. The ground mustard seeds were well embedded in the cellulose acetate, so the film was relatively smooth, flat, and had an unbroken matrix in the absence of pores.

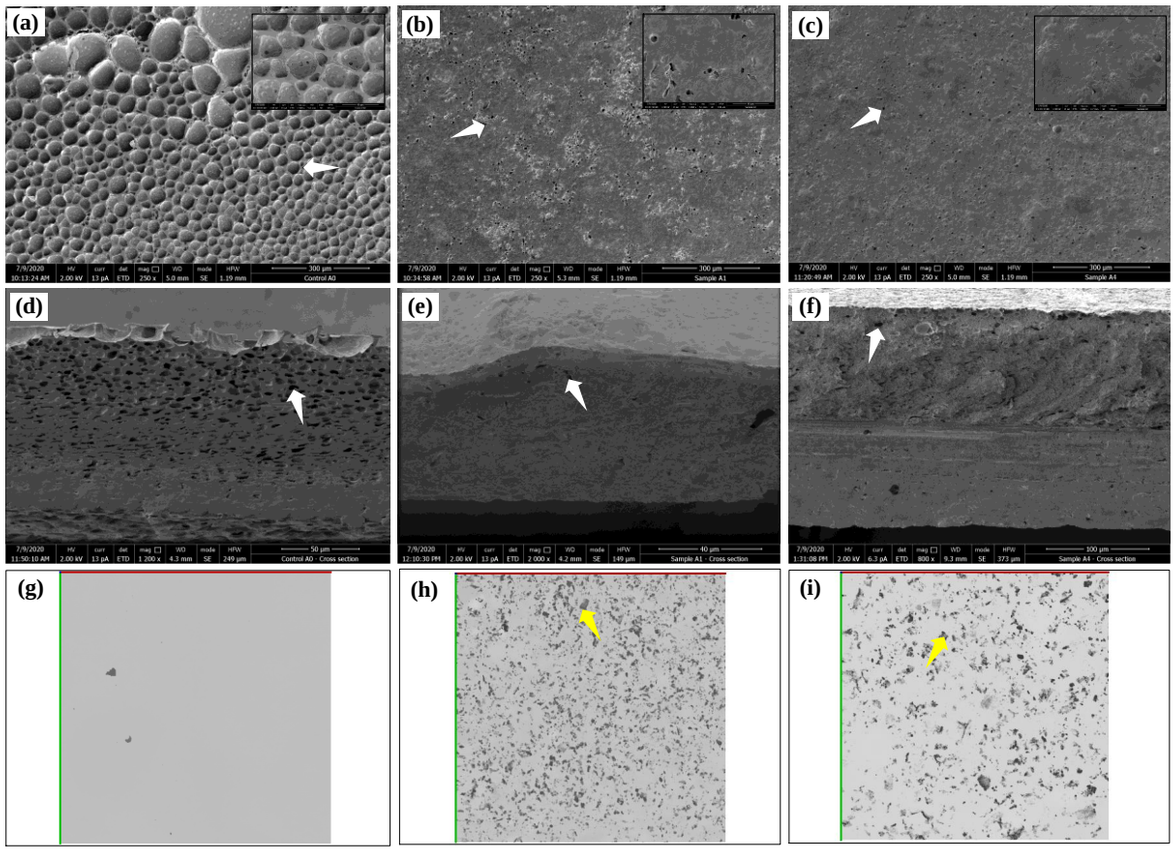

Figure 6.1. *Morphology of film surfaces* ***(a-c)*** *and film cross-section* ***(d-f)*** *measured using scanning electron microscopy (SEM) and confocal laser scanning micrographs of ground mustard seeds dispersions* ***(g-i)*** *in the films measured using confocal microscopy.* ***Left, middle and right*** *pictures depict the control film made with 700 mg of cellulose acetate and without ground mustard seeds, the film with 500 mg mustard seeds produced with 300 mg of cellulose acetate, and the film with 500 mg mustard seeds produced with 1500 mg cellulose acetate, respectively. White and yellow arrows point to the porous structure and black flakes, indicating ground mustard seeds distribution in the film, respectively.*

The film with 1500 mg cellulose acetate (threefold more than the ground seeds) into the film led to a nonhomogeneous structure of the film. In the cross-section image of the films containing the ground seeds, **Figure 6.1f** depicts two distinct layers; a layer of cellulose acetate polymer for the bottom part and a layer of a mixture of ground mustard seeds and the cellulose acetate for the upper part. These bilayers did not appear in the film with a lower amount of cellulose acetate (**Figure 6.1e**). The bilayers were possibly due to the excessive amount of cellulose acetate polymer added into the mixture, resulting in a separate homogenous layer of cellulose acetate. In general, the results show that the ground mustard seeds could be used as a filler of the pores in the cellulose acetate.

Figure 6.1g-i shows the images of confocal microscopy of the films. The black flakes indicated with yellow arrows represent the ground mustard seeds phase dispersed in the films. Without the addition of ground mustard seeds, no black flakes were expectedly found in the images (**Figure 6.1g**). In the samples with ground mustard seeds, the flakes were visible and

homogenous in almost the whole area of the film (**Figure 6.1h-i**). More black flakes can be seen in the film in **Figure 6.1h**, which was expected since the film contains less cellulose acetate and a relatively higher content of ground mustard seeds.

6.3.2. Moisture absorption, sinigrin content remaining in the film, and AITC release in the headspace

Figure 6.2a depicts the percentage of moisture absorbed by the film with and without ground mustard seeds monitored for 5 days at 100% RH and 5^{o}C. The thickest film (made using 1500 mg cellulose acetate) absorbed moisture up to 15% on day 1, which was half of the percentage of moisture absorbed by the thinnest film (made using 300 mg cellulose acetate). The relative decreasing content of cellulose acetate in the film with ground seeds increased the moisture absorption, but the control film had the highest percentage of moisture absorption. On day 1, the percentage of water absorption increased to 40%. The absorption of the moisture in the film might be related to thickness (Appendix 6.1) and surface roughness (**Figure 6.1**). Valente et al. (2005) found that the percentage of moisture absorption did not depend on the cellulose acetate content, but it was a function of thickness. Furthermore, they also observed that the percentage of water sorption was slightly higher for the membrane containing a more porous surface structure, which was consistent with our results. The porous structure of the control film shown in **Figure 6.1d** might cause the moisture to easily penetrate through the porous structure to the intrinsic structure of the film, while the smoother surface, more compact structure and low porosity (**Figures 6.1b** and **6.1c**) in the film containing ground seeds stimulate slower moisture absorption. This result confirms the capability of the film in absorbing the vaporized water from the (model) food that could trigger the enzymatic hydrolysis of sinigrin in the film to form and then release AITC into the headspace.

Figure 6.2b shows the sinigrin content remaining in the ground mustard seeds. The sinigrin content of ground seeds in the thinner film reduced more quickly than that in, the thicker film. From day 0 to 2, the sinigrin content of ground seeds in the thinnest film dropped from 25 µg/g to less than 2 µg/g, compared to that in the thickest film, reducing from 12 to only 7 µg/g. The faster reduction of sinigrin was related to the higher percentage of moisture diffusing into the thinner film. A higher amount of water absorbed in the film stimulates the enzymatic hydrolysis of sinigrin resulting in the formation of a higher amount of AITC.

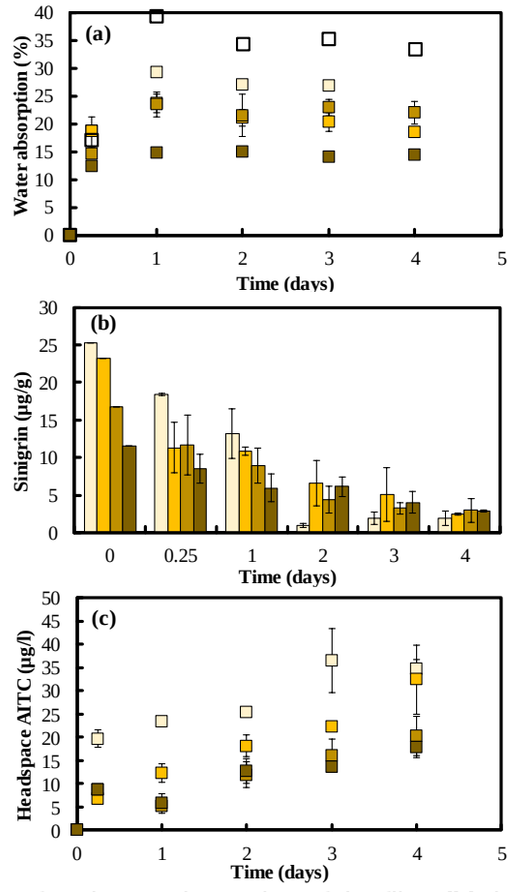

Figure 6.2. (a) *percentage of moisture absorption of the film,* ***(b)*** *the sinigrin content remaining in the ground mustard seeds, and* ***(c)*** *the concentration of Allyl isothiocyanate in the headspace with storage at 5°C. The control film (700 mg of cellulose acetates) without ground mustard seeds (□); the film with mustard seeds produced with 300 mg (□); using 700 mg (□); 1100 mg (□); and 1500 mg (□) of cellulose acetates.*

The concentration of AITCs in the film and the headspace are a consequence of the sinigrin degradation in the film and the release of the formed AITC. In Appendix 6.3, AITC concentration in the thinnest film peaked at 0.85 µg/g after 3 hours and quickly decreased to reach almost the same level of AITC content of the thickest film after 4 days, while the AITC remaining in the thickest film kept increasing to 0.3 µg/g for 2 days and then followed a gradual decrease. As a result, the thicker film resulted in a lower AITC concentration in the headspace (**Figure 6.2c**). After 4 days, the AITC concentration in the headspace of the package with the thickest film just reached around 20 µg/L, which was equal to the concentration for the packaging with the thinnest film after 6 hours. A lower AITC concentration in the headspace of the package containing the thicker film was related to the slower degradation of sinigrin because of limited moisture absorption and/or a slower AITC release rate because of the higher polymer thickness, as was also found by Seo et al. (2012); the release rate of allyl isothiocyanates from a sachet of low-density polyethylene (LDPE) increased significantly with reduced LDPE thickness. In general, the AITC release in the headspace was influenced by the morphology and thickness of the film.

The results show that moisture absorption triggered AITC formation in the film and its release in the headspace. The steps of diffusion and reaction mechanisms in the triggered release packaging system are proposed to be; (1) the absorption and diffusion of water vapour through the film, (2) enzymatic sinigrin hydrolysis and AITC formation, (3) the diffusion of AITC through the film, (4) the release of AITC into the headspace, and (5) the possible absorption of AITC by the (model) food as discussed in the next sections.

6.3.3. The effect of temperature and relative humidity on AITC release

The AITC concentrations in the headspace at different temperatures and film thickness are given in **Figures 6.3a** and **6.3b**. The AITC release and degradation from the thinnest films at 20ºC were faster than the AITC release and degradation from the thicker film and at lower temperatures. For example, at 20ºC, AITC concentration in the packaging containing the thinnest film peaked at around 150 µg/L in 6 hours and followed a quick decrease to almost 0 µg/L at day 4 (**Figure 6.3a**), and at 5ºC the AITC kept increasing to 40 µg/L until day 4. This is consistent with the result obtained by Seo et al. (2012) with pure AITC, who found a more rapid release of AITC from an LDPE sachet at 25ºC than that at 4ºC.

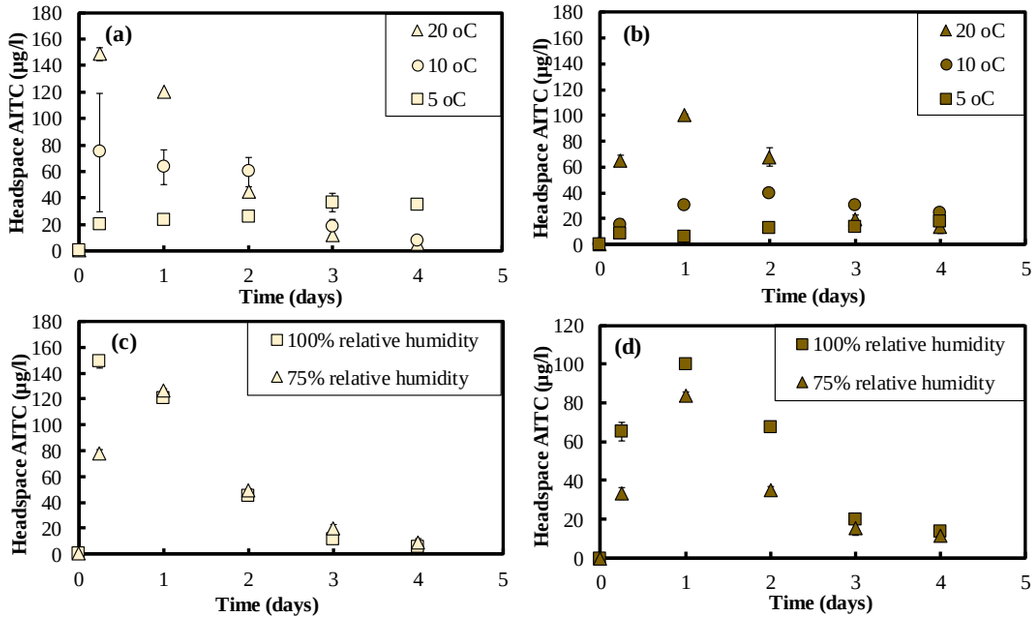

Figure 6.3. *The effect of* ***(a,b)*** *temperature and* ***(c,d)*** *relative humidity on the concentration of allyl isothiocyanate (AITC) released from the thinnest films made using 300 mg of cellulose acetate* ***(left)*** *and the thickest films made using 1500 mg cellulose acetate* ***(right)****.*

Figures 6.3c and **6.3d** depict the effects of relative humidity to understand the importance of moisture to AITC release in the packaging system. A slower rate of AITC release and less AITC concentration in the headspace at 75.5% RH was observed compared to 100% RH. Lower water-vapour presence in the packaging system causes less water being absorbed by the film and then less sinigrin degradation to form less AITC, resulting in a slower release of AITC to the headspace. Understanding the effects of the relative humidity gives an insight

into which food products this packaging might be effective. For example, this packaging system might not be effective to pack dried food because the low amount of water vapour might not be enough to humidify the packaging. Consequently, a limited effect of the addition of the antimicrobial film in the packaging system is expected. The obtained results of the effect of RH emphasize the role of moisture to trigger the AITC formation and release in a food package.

6.3.4. The antimicrobial effectiveness of the developed film in a ground beef package

Partitioning of AITC between the packaging headspaces and the ground beef

Figure 6.4 depicts the concentration of AITC from the thickest film in the headspace and the ground beef at 5°C. Generally, the AITC concentration increased continuously in the headspace for 4 days and in the ground beef for 3 days and then decreased gradually. A higher AITC concentration in the headspace was observed in the packaging containing extra-lean ground beef (9% fat) compared to the packaging with medium-ground beef (19% fat) (**Figure 6.4a**). This result was opposite to the results of the AITC concentration in ground beef (**Figure 6.4b**), showing a lower absorbed AITC concentration in the presence of less fat in ground beef (9% fat). This opposite trend can be explained by the solubility of AITC in a higher fat content of ground beef. In a similar packaging system, Wang et al. (2020c) found that higher fat content in ground beef enables more carvacrol to penetrate ground beef due to the higher solubility of carvacrol in the fat phase.

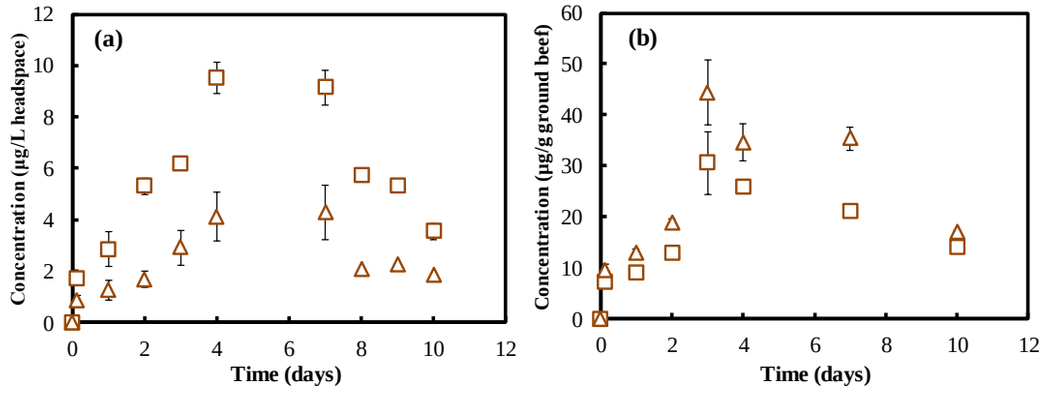

Figure 6.4. *The allyl isothiocyanate (AITC) concentration in* ***(a)*** *the headspace and* ***(b)*** *ground beef containing (□) 9% fat and (△)19% fat*

The effect of AITC on microbial growth in the ground beef

The total bacterial count in ground beef is shown in **Figure 6.5**. For control samples, the total bacteria in extra-lean ground beef reached the maximum (almost 9 Log_{10} CFU) faster than that in medium ground beef. The AITC release from the film reduced bacteria around 3 log_{10} after 6 days and 4 log_{10} after 8 days for the lean ground beef, while for medium ground beef reduced the bacteria only a log_{10} CFU after 8 days.

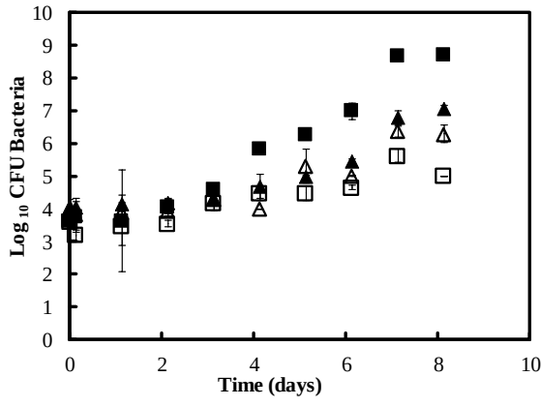

Figure 6.5. *The curve of bacterial growth in (■) extra-lean ground beef and (▲) medium ground beef for the control group, and (□) extra-lean ground beef and (△) medium ground beef for the antimicrobial packaging*

Kinetic modelling of bacterial growth in the ground beef was used to understand the growth rate and lag phase of the bacterial growth. The used kinetic model was the Gompertz model (**Eq. 6.2**), which showed a good fit for the observed data (Appendix 6.4). The value of the asymptotic parameters of the model was set as a constant for the different samples since the maximum total bacterial count was assumed to be similar in the different ground beef samples. For the control samples, **Table 6.1** depicts that medium ground beef has a significantly more prolonged lag phase of bacterial growth and slower growth rate than those of the extra-lean ground beef. The higher fat content in ground beef caused a 2 days longer shelf life for the medium ground beef. The fat in food products is well-known as a growth-restricting factor (Wilson et al., 2002), therefore, the larger amounts of fat reduced the microbial growth, like *L. monocytogenes,* at 4 ºC, leading to a longer lag phase and lower maximum specific growth (Verheyen et al., 2018).

Table 6.1. *The estimated parameters of the Gomperts model as calculated by fitting the model to observed PCA count data of the ground beef and the predicted shelf life (at 7 log_{10} CFU)*

Samples	**As** (log_{10} CFU)	**Lag phase** (h)	**μmax** (h^{-1})	**Shelf life** (days)
Extra-lean ground beef (control)	15.53±2.71	52.45±7.23^{a}	0.09±0.01^{c}	3.84±0.56^{a}
Extra-lean ground beef		83.40±19.32^{ab}	0.04±0.01^{a}	7.52±1.38^{b}
Medium-ground beef (control)		93.10±11.11^{b}	0.07±0.01^{bc}	5.28±0.65^{ab}
Medium-ground beef		102.14±13.12^{b}	0.06±0.01^{ab}	5.84±0.73^{b}

Values represent means ±standard deviation. Small letters indicate significant differences between the samples

Table 6.1 shows that the film caused extended lag phases for both extra lean and medium ground beef and reductions of the growth rate of microorganisms in extra lean ground beef by about 0.05 h^{-1} and by around 0.01 h^{-1} for the medium ground beef. Therefore, the shelf life of extra-lean ground beef was significantly extended by around 3.5 days, and it could be concluded that the application of this antimicrobial package almost doubled the shelf life of the lean ground beef. The medium ground beef had an extended shelf life of 0.56 days. These

results confirm the effect of food composition on AITC concentrations and its antimicrobial efficacy in the packaging system, where the higher AITC concentration was observed in the headspace for the ground beef with lower fat (**Figure 6.4**). Initially, the film releasing 1-2 µg AITC per litre headspace of packaging containing both types of ground beef (**Figure 6.4**) was able to reduce the total bacterial count by 0.5-1 $\log_{10}$ CFU for 6 hours (**Figure 6.5a**). However, the obtained AITC concentration (around 5 µg/L) during 4 days in the packaging might not be sufficient to effectively suppress the bacterial inhibition in medium ground beef because the MIC of AITC against *P. fragi* is around 6.05 µg/L (Bahmid et al., 2020a). Whilst the AITC concentration was increasing to 10 µg/L (exceeded the minimum inhibitory concentration) during 4 days in the headspace of the packaging with extra-lean ground beef. The AITC was able to prolong the lag phase and reduce the growth rate in the extra lean ground beef. These results show that the concentration of the compounds should be above the MIC of the bacteria. Otherwise, the compounds might have a limited antimicrobial effect on packaged food.

On the other hand, despite the higher AITC concentration in high-fat ground beef, as shown in **Figure 6.4**, the AITC could be solubilized mostly in the fat phase of the ground beef, where the AITC is not effective to inhibit the bacteria in the ground beef. The solubility of AITC in the fat phase limits the interaction between AITC and the bacteria growing in the aqueous phase of the food, as also shown for other lipid-soluble antimicrobial compounds (Wang et al., 2020c). From the results of this study, it can be concluded that the food composition does not only influence the antimicrobial film properties but also influences the AITC concentration in different phases of the package, and this can affect the bacterial growth in the food products. Other factors might also affect the effectiveness of AITC against the bacteria in ground beef, as the ground beef contains, for example, proteins that might covalently interact with the AITC (Keppler et al., 2017) which was not considered in this study.

6.4. Conclusion

This study shows the development of a moisture-activated antimicrobial film containing ground mustard seeds. The properties of the active carrier (ground mustard seeds incorporating film), storage conditions (RH and temperature), and food composition (fat content) influenced the AITC concentration in different phases of the package, which then affected the bacterial growth and shelf life of ground beef. The higher cellulose acetate content and the thicker films reduced the moisture absorption and, in turn, decreased the rate of AITC formation and release into the packaging system. The formation of AITC in the ground mustard loaded into the film polymer was triggered by the vaporized moisture absorbed by the film and was therefore influenced by the rate and amount of absorbed moisture. The formation and release of AITC were also dependent on the storage conditions where higher temperatures and relative humidity enhance the rate of AITC release into the packaging system. Controlling those factors

in the packaging system is important to result in a sufficient and sustained AITC concentration in the packaging system. The packaging system with the thickest film (made using 1500 mg cellulose acetate) was tested to study the effects of the fat content of ground beef influence the AITC concentration and absorption and the antimicrobial effect of AITC against the bacterial growth in the ground beef. The packaging with ground beef with lower fat content gave a higher AITC concentration in the headspace but a lower AITC concentration in the ground beef. However, the shelf life of the low-fat ground beef can be very effectively extended by 3.68 days, compared to a limited extension of only 0.56 days of the shelf life of the medium ground beef. In this case, differences in fat composition cause the efficiency of food packaging from very effective to barely noticeable. Besides showing the novel way to control compound release, these results give an insight that tailoring the effective active packaging system should consider not only the properties of the active carrier but also the storage conditions and food characteristics.

Acknowledgement

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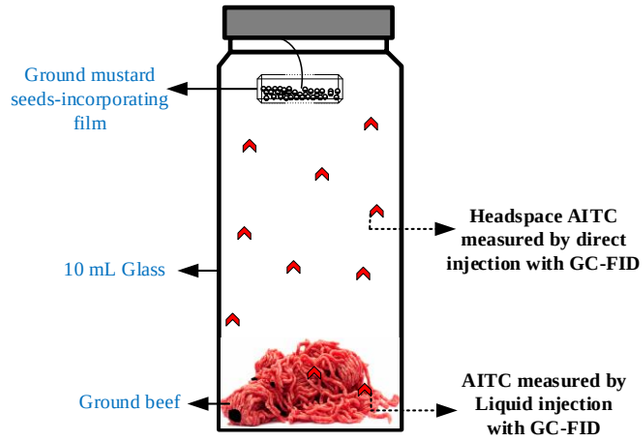
Appendix 6. Supplementary Materials

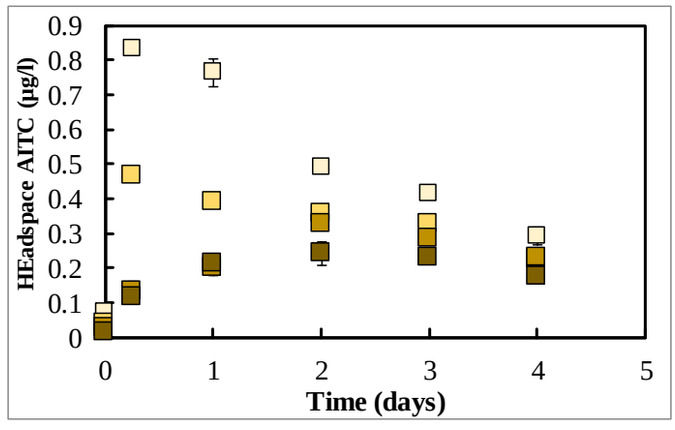
Appendix 6.1. *Thickness and weight of the 2-by-2 cm film with different cellulose acetate weight*

Samples	**Control***	**Weight of cellulose acetate** (with 500 mg ground mustard seeds)			
		300 mg	700 mg	1100 mg	1500 mg
Weight (µg)	59.37± 6.54	62.05 ± 8.67	64.6 ± 2.06	118.35±9.05	151.30±7.43
Thickness (µm)	121± 10	134 ±13	237±24	273±27	293±25

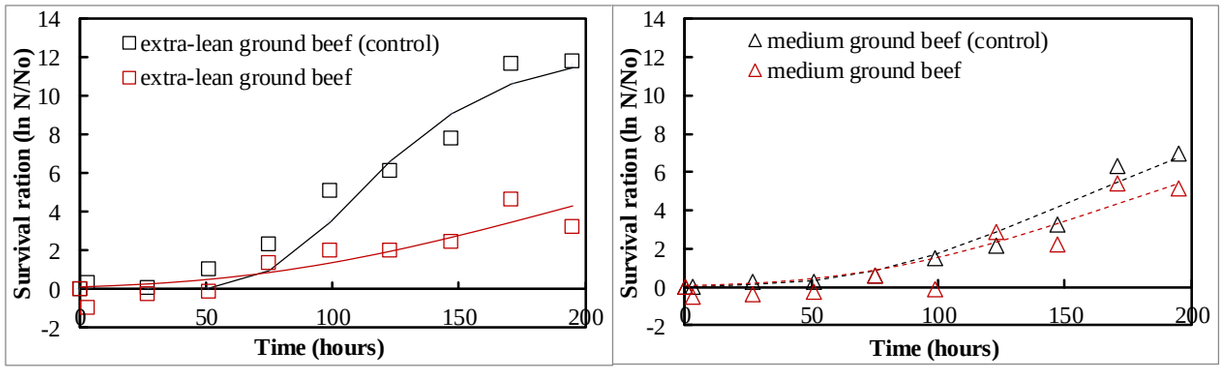
* Control sample is a packaging system containing film produced with 700 mg cellulose acetate and without ground mustard seeds



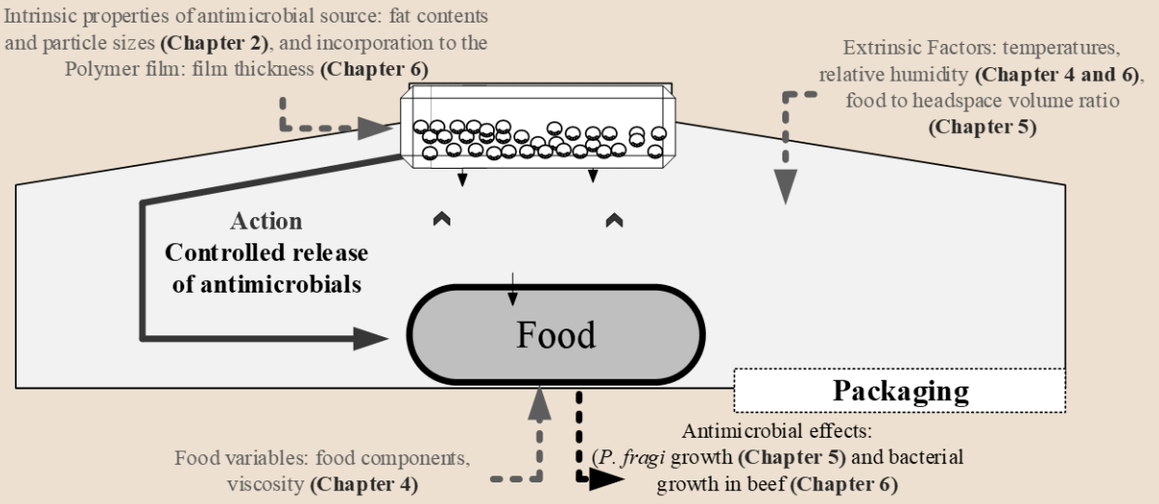
Appendix 6.2. *The set-up of an antimicrobial packaging system with a 10 mL vial containing film incorporating ground seeds and ground beef.*



Appendix 6.3. *The concentration of Allyl isothiocyanate in the film with storage at 5 °C. * the film control without ground mustard seeds (□); the film made using 300 mg (□), using 700 mg (□), using 1100 mg (■) and using 1500 mg (■) of cellulose acetates.*



Appendix 6.4. *The curve of survival ratio (N/N_0) of total bacteria in (□) extra-lean ground beef and (△) medium ground beef for control group, and (□) extra-lean ground beef and (△) medium ground beef for the antimicrobial packaging.*


Intrinsic properties of antimicrobial source: fat contents and particle sizes (Chapter 2), and incorporation to the Polymer film: film thickness (Chapter 6)
Extrinsic Factors: temperatures, relative humidity (Chapter 4 and 6), food to headspace volume ratio (Chapter 5)
Action
Controlled release of antimicrobials
Food
Packaging
Food variables: food components, viscosity (Chapter 4)
Antimicrobial effects: (P. fragi growth (Chapter 5) and bacterial growth in beef (Chapter 6)

7

General Discussion

The antimicrobial packaging reported in this thesis was designed by using ground-seeds as a natural antimicrobial source, releasing allyl isothiocyanate (AITC) to the packaging system. The mustard seeds enable control of the compound release to effectively suppress the growth of microorganisms for an extended shelf life of the packaged food.

This thesis aimed to explore the potential of AITC release from the mustard seeds in antimicrobial food packaging. The factors influencing the AITC concentration in a packaging system and its efficacy on the microbial growth of a packed food were investigated. The investigated factors consisted of the intrinsic properties of mustard seeds (particle size and fat content), the extrinsic factors (temperature and relative humidity), the food composition (fat and protein content), and the incorporation of ground mustard seeds in a polymer film. The AITC concentrations were measured in each phase of the packaging system (antimicrobial source, headspace, and (model) food) and then described by a kinetic model containing a set of combined equations to understand and describe the mechanism of mass transfer and reactions. The antimicrobial effects of the AITC on the growth of spoilage bacteria in a food model (liquid medium) and real food were studied to understand the effectiveness of an antimicrobial package in microbial inhibition and the extension of the shelf life of the food. The thesis overview is depicted in **Figure 7.1**. The main findings of the study are interpreted and discussed in **Section 7.1**. Methodological consideration (**Section 7.2**), multiresponse kinetic model consideration (**Section 7.3**), and future researches and recommendations (**Section 7.4**) are also provided.

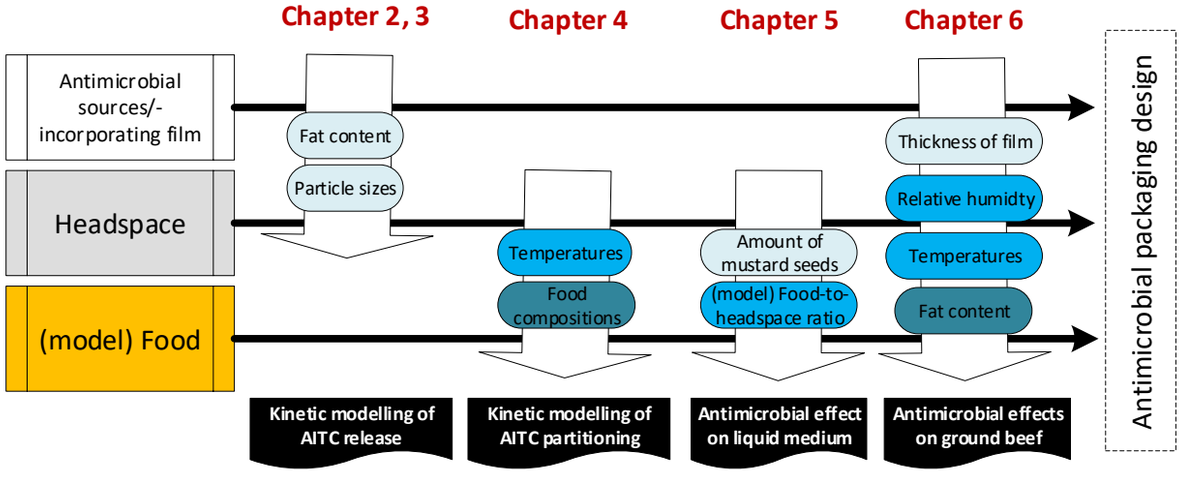


Figure 7.1. *The overview of the thesis: the factors that were influencing allyl isothiocyanate concentration in an antimicrobial packaging system were studied in each chapter and the outcome for each chapter. The* ***down arrows*** *contain the factors; the properties of antimicrobial sources (mustard seeds or - film incorporated with ground mustard seeds) (soft blue), extrinsic factors (light blue), food matrix (dark blue)****,*** *and* ***the black shapes*** *at the bottom are the outcome for each chapter.* ***Three rectangles*** *coloured in white, grey, and yellow on the left side are the phases of the packaging system included in the study.*

7.1. Interpretation and discussion of the main findings

7.1.1. Characteristics of mustard seeds have a key role in the controlled release of AITC

Mustard seeds can be used to control the AITC release in the headspace by modifying the mustard seeds properties, e.g. fat content and particle size (Dai and Lim, 2014; Sharma et al., 2012). In **Chapter 2**, a decreased fat content and particle size of the ground mustard seeds resulted in more sinigrin conversion to AITC and a higher AITC concentration in the headspace. The AITC release rate was, thus, controlled by the rate of sinigrin hydrolysis reaction and by the fat content of mustard seeds (**Chapter 3**). This is an additional mechanism for the controlled release of AITC in comparison with previously published research. For the conventional antimicrobial packaging with the usage of pure AITC added into the packaging material, the AITC release rate was only controlled by the packaging material thickness (Quintavalla and Vicini, 2002). Seo et al. (2012) found that thicker low-density polyethylene (LDPE) reduced the rate of AITC release from the packaging material. The reason for the AITC controlled release was the rapid migration, which causes a loss of its efficiency of the packaging on microbial inhibition during the storage of food products (Nazareth et al., 2020; Rehman et al., 2020).

The main challenges to apply AITC for antimicrobial packaging are the high volatility and low stability of AITC in the headspace during storage (Lashkari et al., 2017; Nazareth et al., 2020; Rao et al., 2019). **Chapter 2** reported the low stability of AITC in the headspace at 20 ^{o}C where the AITC concentration quickly reduced to below 2 µg/L after 48 hours due to its reaction with the hydroxyl group of the water molecule from water/moisture from the food or present in the packaging headspace. In **Chapter 3**, a multiresponse kinetic model containing a set of differential equations was developed to understand the AITC formation, release, and degradation in the packaging system. Based on the result of this chapter, a simulation was performed using this established kinetic model to monitor the AITC concentration with varying values for the rate constants of AITC formation (k_1) and its degradation in the headspace (k_3), and the mass transfer coefficient of AITC from the mustard seed particles to the headspace (m_t). As shown in **Figure 7.2a**, the black curve representing a reference curve, that is simulated using parameters of mustard seeds particles with 50-100 µm and 0% fat in **Chapter 3**, shows a higher AITC concentration than the green curve representing the AITC concentration of a simulated sample with a 10-times lower m_t than the reference m_t. This indicates that the mass transfer to the headspace is not a limiting factor for building up the AITC concentration in the headspace. With a 10-times decreased k_3 value, the AITC concentration (red line) increased quickly to twice the concentration of the reference but then decreased quickly. The rapid decrease in AITC concentration could be caused by two mechanisms; the AITC degradation and the partition of AITC concentration between headspace and ground mustard seeds phases. The partition coefficient controlled the AITC concentration between both

phases at equilibrium, so the AITC could partly partition back into the seed again and degraded there with the still faster rate. Interestingly, decreasing k_1 to 10-times lower than the reference k_1 caused a lower AITC concentration (yellow) in the headspace, but this concentration can be maintained for a more extended period. From the simulation could be concluded that the AITC concentration in the headspace is controlled by the rate constant of AITC formation and sinigrin hydrolysis.

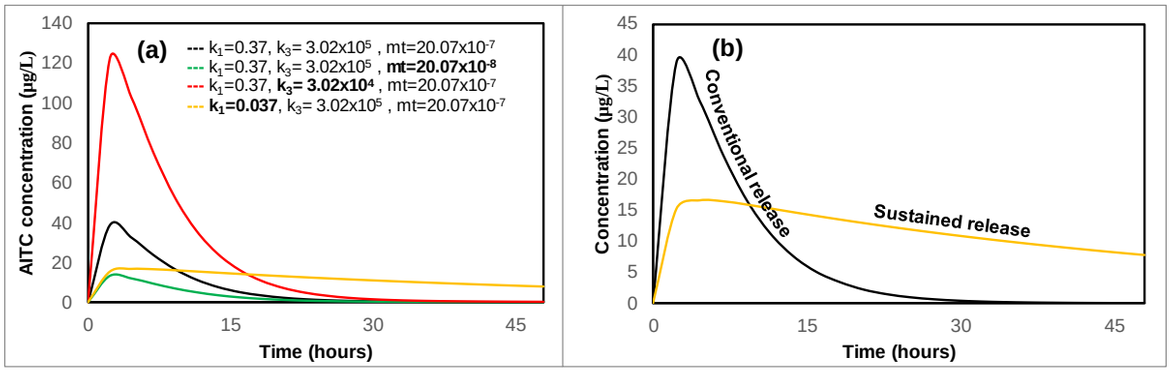


Figure 7.2. (a) *Simulation of allyl isothiocyanate (AITC) concentration using the multiresponse kinetic with varying rate constants of AITC formation (k_1 in h^{-1}), AITC degradation (k_3 in m^3/mol/h) and AITC release (m_t in m/h),* ***(b)*** *the curves of the simulated AITC concentration, that can be defined as a conventional and sustained release.*

The fat content of ground mustard seeds influenced the AITC formation rate and the amount of sinigrin that is accessible for hydrolysis in ground mustard seeds (**Chapter 3**). The fat in the ground mustard seeds protected cells in the internal part of the seeds (where the myrosinase and sinigrin containing cells were located) during the milling process that mainly disrupted cells in the external part of the seeds (where the myrosinase containing cells only were located). The more disruption in the cells in the external part caused more damage in myrosinase containing cells. This cell disruption in the external part could result in high myrosinase release but with a low sinigrin content available in fatty ground mustard seeds. Less sinigrin available to be hydrolyzed by more myrosinase caused a rapid formation of AITC in the ground seeds, but, due to the limited sinigrin availability, at a lower level. Furthermore, the presence of the fat in ground seeds caused AITC to be solubilized in the lipid phase resulting in a lower amount of AITC to be released into the headspace (Giroux et al., 2007; Tsao et al., 2000). Therefore, defatted ground mustard seeds were used for the next study to optimize the release of AITC from the defatted ground seeds into the headspace.

Another way to control the AITC release to the headspace is by using an external stimulus to trigger the release, e.g. moisture (Chen et al., 2019). This trigger release method could be interesting to control the AITC formation with moisture inside in a food application since moisture is essential for the AITC formation in the ground mustard seeds. In **Chapter 6**, the ground mustard seeds were incorporated into the film packaging material, and then this film was attached under the lid of an impermeable package. The result showed that a thicker

film had slower moisture absorption and sinigrin hydrolysis; consequently, AITC kept being released, as shown in **Figure 6.2.** This trend of the prolonged AITC release from the film containing ground seeds can be described as a sustained-release (yellow line) shown in **Figure 7.2b**. The sustained release trend and the linear correlation between water absorption and sinigrin hydrolysis in the film confirm that moisture can be used as an external stimulus to control the AITC formation and sinigrin hydrolysis. A sustained release was also described by using another mechanism by Encinas-Basurto et al. (2017), who encapsulated pure AITC in nanoparticles, e.g. poly(lactic-co-glycolic acid) (PLGA). The water uptake triggered the degradation of PLGA to produce acidic oligomers via hydrolysis of the ester bonds in the polymer backbone. The AITC molecules were therefore released slowly under acidic conditions. The efficacy of the moisture trigger release method to obtain a sustained release gives a new insight into a novel way to effectively control the antimicrobial release (volatility) and concentration. Therefore, by using this concept, the antimicrobial packaging can effectively maintain microbial inhibition and this can increase the shelf life of a packed food.

7.1.2. Minimum and maximum concentrations of Allyl isothiocyanates in the packaging system

The AITC concentration profile determines how long the microbial growth is inhibited and, therefore, determines the extension of the food shelf life (J.A. Ko, 2011; Nazareth et al., 2020; Nazareth et al., 2019b; Torrijos et al., 2019). It is necessary to maintain the concentration above the minimal concentration limit to inhibit microbial growth, as shown in **Figure 7.3**. This limit is the minimum AITC concentration needed against the growth of *Pseudomonas fragi*. In **Chapter 5** was described that a minimum inhibitory concentration (MIC) of AITC, >6 µg/L, was able to effectively inhibit the growth of *P. fragi* in a liquid medium and at a headspace AITC concentration of >15 µg/L, AITC killed (totally reduced) *P. fragi* in 48 hours at 20^{o}C. The minimum inhibitory concentration (MIC) value of AITC cannot be generally applied for other microorganisms and antimicrobial compounds (Dufour et al., 2015), as each microorganism has different resistance and variation in its chemical constituents and components against antimicrobials (Gonelimali et al., 2018) (**Tabel 1.1** in **Chapter 1**). The MIC value obtained could be different for real food products due to the interaction of AITC with the food components in the food products (Gavara et al., 2015). Therefore, the MIC should also be determined for individual food products. In **Chapter 6,** the AITC concentrations from the film incorporated with ground mustard seeds and the bacteria growth in extra-lean and medium ground beef were measured. The AITC concentration in the headspace increased to 10 µg/L. It was capable to reduce the bacterial growth by prolonging the lag phase and reducing the growth rate, thereby extending the shelf life of the extra-lean ground beef by almost 4 days at 5^{o}C. While an AITC concentration in the headspace of ~4 µg/L only prolonged the shelf life of medium ground beef by around 16 hours. Pang et al. (2013) also obtained a 2 days-extended shelf life of catfish

fillet by inhibiting the *P. aeruginosa* growth from the addition of 18 µg/L AITC into the headspace. These results show that the release kinetics of antimicrobials has to be tailored to the food and spoilage microorganisms that are likely to be present (Quintavalla and Vicini, 2002) to maintain the concentration above the MIC value.

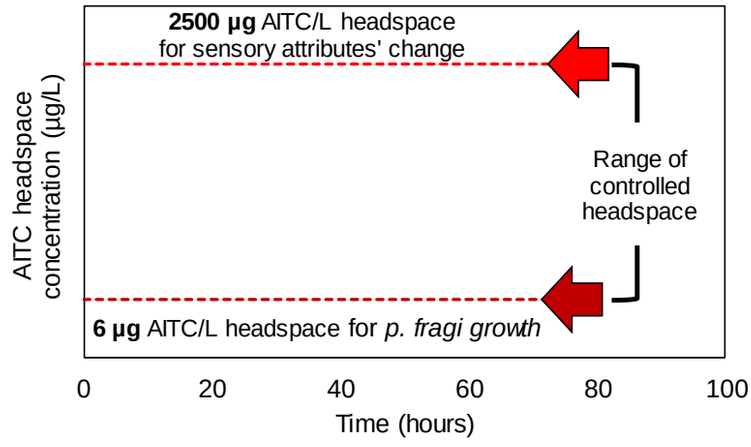


Figure 7.3. *The concentration of allyl isothiocyanate (AITC) should be controlled within these limits to avoid a change of sensory attributes and bacterial growth*

However, the previous discussion does not mean the AITC concentration in the package should be released and maintained as high as possible to inhibit or even kill the bacteria in food products. Too high AITC concentration can affect the sensory attributes and quality of food products before their expiration date is reached (Dai and Lim, 2014; Kim et al., 2015). The change of the sensory attributes of packaged food also needs to be taken into account when determining the desired AITC concentration in the packaging headspace, as shown in **Figure 7.3**. If the AITC concentration exceeds the limit, the smell of the compounds can give a bad experience to the consumers (Fang et al., 2017). In this thesis, the maximum limit was not investigated, but according to a sensory test in brazil nuts done by Lopes et al. (2018), the highest acceptable dose is around 2.5 µL AITC per litre headspace (2500 µg/L). Kim and others (2015) recommended the concentration in the range 0.02 to 2500 µg/L for the application of AITC in the headspace of packaging to avoid negative impact on the sensory properties of- food. According to the result obtained by Marcinkowska and Jeleń (2020), the minimum AITC concentration that was detected by minimally one panellist, called experimental threshold concentration, was around 100 µg/L. This result might be different in their application to other foods depending on the aroma profile of the foods.

For the practical application of antimicrobial packaging to food products, the selection of an antimicrobial compound and effective delivery method is the key point of consideration regarding their effects on the microbial growth and sensory attributes of the packaged products.

7.1.3. Food characteristic and storage conditions determine the partitioning of antimicrobial compounds

In this thesis, food characteristics, consisting of the composition of food, viscosity, the surface area of the food, and the packaging volume, influence the AITC concentration in the packaging system. **Chapter 4** described that the lower fat content and higher protein content of the food matrix decreased AITC absorption into the food. Besides the food composition, higher viscosity reduced the AITC penetration into the matrix. The surface area of the food products was not investigated in this experimental study but was included in the set of equations of the developed kinetic model in **Chapter 4**. The broader area of the food product opens the area for antimicrobial compounds to diffuse more quickly into food products (Guo et al., 2017). **Chapter 5** shows the effect of the ratio between headspace and BHI broth used as a (model) food. The bigger headspace volume resulted in a higher AITC concentration in the headspace and the food matrix. All these factors influence the AITC partition between headspace and food products in the packaging system. Understanding all the mechanisms of the different food characteristics is essential to consider how much ground mustard seeds required to incorporate into the packaging film or to add in the food packaging. In **Chapter 4**, a kinetic model has been developed as a case study to understand the effect of fat and protein content on the partitioning of AITC between the headspace and food products. This kinetic modelling approach can be broadened by including other factors in the set of equations, like temperature.

The food composition influences the AITC partition between the headspace and food product in the package; a different composition results in a different ratio of concentrations between both headspace and food product, known as the “partition coefficient”. **Figure 7.4**. shows the different partition coefficients of AITC between headspace and different food matrices. The partition coefficient between the food matrix containing oil (emulsion) and headspace ($K_{fmo/g}$) was the largest, which meant that AITC is preferentially retained in the oil phase and the higher oil content in the food matrix caused an increase in the partition coefficient (**Chapter 3**). In **Chapter 4**, higher viscosity and a protein content >5% in the matrix resulted in the volatile only binding the free water molecules (Nahon et al., 2000), so the thermodynamic equilibrium conditions for the package containing the food matrix with the higher protein content ($K_{fmhp/g}$) was determined between the volatile in the aqueous phase and the headspace (Ammari and Schroen, 2018). Therefore, $K_{fmhp/g}$ was the smallest, then followed by the partition coefficients between water and headspace ($K_{w/g}$) and between the food matrix with the lower protein content and headspace ($K_{fmlp/g}$). The partition coefficients of compounds in the package containing different food composition show that the effectiveness of the antimicrobial is different for different food products because the characteristic of foods determines the partition coefficients and therefore the concentration of the antimicrobial compounds in the headspace.

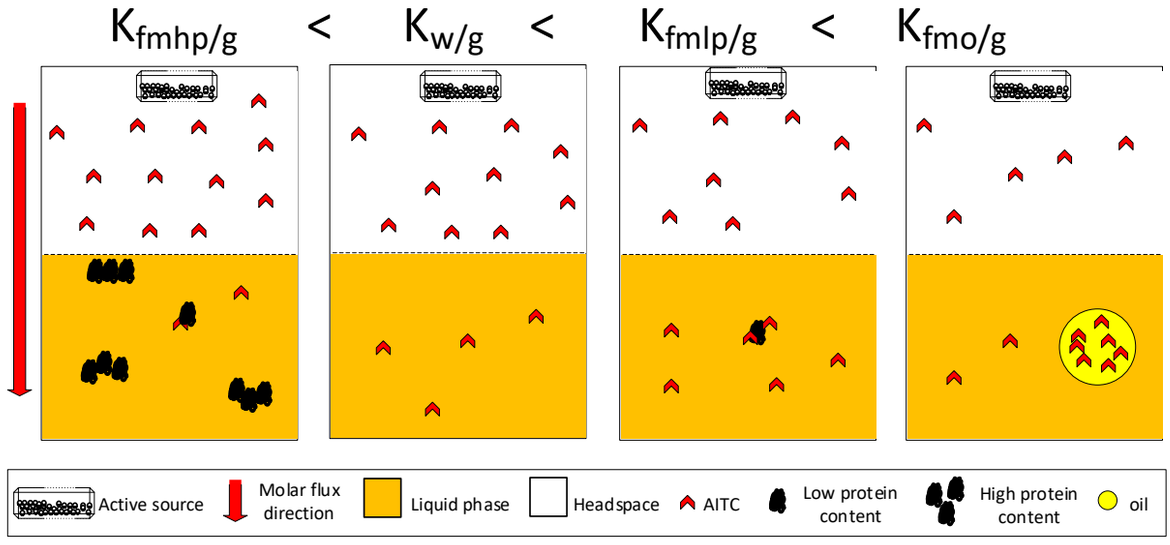


Figure 7.4. *Schematic representation of the effect of different components in the food matrix on the partition coefficient of allyl isothiocyanate (AITC). K is partition coefficient, fmhp and fmip is food matrix containing a high protein content and low protein content, respectively, fmo is food matrix containing oil, w is water, and g is headspace.*

Figure 7.4 also shows the interaction of the AITC with the components inside the food matrix. The interactions of the AITC compounds with the food components can be a disadvantage for the antimicrobial activity on microbial growth in food products. The interaction can lead to a reduction in concentration in the headspace and/or in the aqueous phase of food products where the microorganisms grow, which consequently reduces its antimicrobial activity against the growth of bacteria. The interaction between the antimicrobial compounds and the food components can be chemical or physical. For the chemical interactions, antimicrobial reacts or complexes to other compounds, reducing the concentration and stability. For example, AITC can be degraded in the aqueous phase because the hydroxyl groups of water react with sulfur groups in the allyl isothiocyanates (Pang et al., 2013). The chemical interactions were also found between AITC and proteins; AITC covalently binds the protein, e.g. with whey protein isolates (Keppler et al., 2018). The most important interaction with the food matrix is the physical interaction between AITC and oil in the food matrix, where the AITC was solubilized in the oil. The dependence of the interaction on the food composition also gives an insight that besides the antimicrobial source characteristic, the effectiveness of active packaging depends on the food characteristic.

Another factor on the AITC concentration that should be understood in antimicrobial packaging design is the storage condition, such as the relative humidity and temperature (Dai and Lim, 2015), and the chemical and biological changes in the food product during storage. The effect of temperature was reported in **Chapter 4-6**; an increase in temperature increased the rate constant of AITC formation, release, degradation and absorption. The higher temperature increased the volatility of AITC, so more of the AITC is released to the headspace phase, which

indicates a lower partition coefficient at the higher temperature. These results are in line with Quintavalla and Vicini (2002) and Liu et al. (2020). The relative humidity, as reported in **Chapter 6,** functioned to provide moisture to trigger the AITC formation in the film (with ground mustard seeds incorporated), after which the AITC was released into the headspace. A higher relative humidity increased the AITC formation and released more AITC into the headspace, so a higher AITC concentration was observed in the headspace (Lashkari et al., 2017; Vega-Lugo and Lim, 2009; Wang et al., 2017b).

7.1.4. Application of the designed antimicrobial packaging system

The antimicrobial packaging containing the film with ground mustard seeds can be interesting for application on perishable food products, especially foods containing a high water content (**Chapter 6**). **Figure 7.5** shows the package containing the film placed under the lid of the impermeable food packaging. In the packaging system, the perishable food product releases moisture. The film then absorbed the moisture to start enzymatic hydrolysis of sinigrin to form AITC. In this case, a high enough relative humidity inside the packaging created by the food products is important for the release of the compounds (Vega-Lugo and Lim, 2009). In this thesis, this packaging system was tested on a perishable food product, extra-lean ground beef, and the result showed an effective inhibition on bacterial growth and an extension of the shelf life of about four days. In **Chapter 6**, it was recommended to apply the film with ground mustard seeds to the package with food products containing a lower fat content due to the high solubility of AITC in the fat.

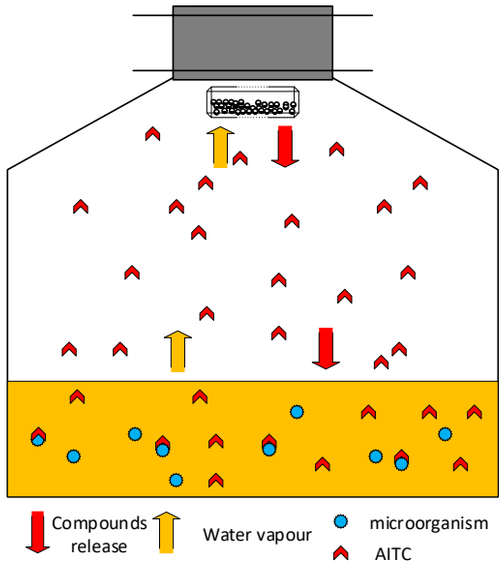


Figure 7.5. *The schematic antimicrobial packaging system where the vaporized water acts as an external stimulus for the allyl isothiocyanate (AITC) formation*

This packaging system can be commercialized to fulfil the consumers' needs for a longer shelf life of products and food waste reduction. On the industrial level, the film loaded with natural antimicrobial sources can be widely produced and marketed to protect the food from

spoilage bacteria during the storage or distribution of the food products. On the consumer level, the natural antimicrobial source, e.g. mustard seeds, can also be used by the consumers by adding the ground mustard seeds to their home food storage that used to store the food after the package is open. After opening the package of the food product within the period of consumption, the secondary shelf life will start. During the period, the expired date of the food will be not the same as or earlier than the date seen in the package. The addition of mustard seeds can therefore help the consumer to prolong the secondary shelf life of the food. This way of shelf-life extension is also more sustainable and low-cost. Besides mustard seeds, other natural antimicrobial sources that are available in their home can also be used by the consumers. So, the consumers are free to select an acceptable natural antimicrobial source to be used in their home food storage. With more knowledge of the advantage of using natural antimicrobial compounds, the consumer can be involved and contribute more to food waste reduction.

In terms of the regulation, AITC is considered a GRAS compound (generally recognized as safe) by the Food and Drug Administration (FDA) in the United States and is considered safe as a natural food additive by EFSA European Food Safety Authority (EFSA) in Europe. For the application of AITC vapour (not intended to be added to food product) released from the antimicrobial source or packaging material, EFSA regulates the AITC level for a certain type of foods with the range of concentration around 3.5-10 mg/L (EFSA, 2010). This concentration is very high, compared to the minimum concentration of AITC to inhibit (6 μg/L) and inactivate (15 μg/L) the bacteria observed in **Chapter 5**. Besides the acceptability of the compounds regarding the safety issue, the use of released active substance, like AITC, should be labelled on the packaged as an ingredient, regarding Commission Regulation (EC) No 450/2009 (EFSA, 2009). Regarding the acceptable concentration, applying this packaging system with ground mustard seeds or film with ground mustard seeds is possible. Other potential natural antimicrobial sources containing antimicrobial agents can be explored to be applied to antimicrobial food packaging, for example, cloves with eugenol (Aguilar-González et al., 2015; Reyes-Jurado et al., 2019), wasabi with isothiocyanates (Lu et al., 2016), and carvacrol from oregano (Garcia-Diez et al., 2017).

7.2. Methodological considerations

In **Chapter 2**, AITC content in ground mustard seeds was quantified using the HPLC method modified from Oliviero et al. (2014) with minor adjustment. The extraction method for the HPLC measurement consisted of many steps, and it was time-consuming before the supernatants were finally collected in HPLC vials to be measured in the HPLC. The long process of the extraction potentially reduces the AITC concentrations due to its high volatility. Marton and Lavric (2013) developed a method to determine the AITC concentration using GC

without a time-consuming extraction method, leading to time efficiency and simplicity. Performing the GC method for AITC measurement in the headspace and other phases in the food matrix (**Chapter 4**), in liquid medium (**Chapter 5**), ground beef, and a film containing ground mustard seeds (**Chapter 6**) was found to be more accurate to determine the partition coefficient. The GC method was also efficient since hexane was used to extract the AITC from the samples (ground mustard seeds, film, or (model) food), in which hexane was also used as a solvent in making the calibration standard to quantify the AITC concentration in the headspace.

When considering the determination of AITC in the packaging headspace, Solid-Phase microextraction (SPME) and direct injection with needles are typically used to detect volatile compounds in the headspace. The SPME method was used initially (**Chapter 2)**; however, it was decided to use direct injection to determine the real AITC concentration in the gaseous phase (**Chapters 4, 5, and 6**). The quantification with SPME depended on the equilibrium of AITC between the fibre and headspace or gas phase (Cheong et al., 2010; Zhang et al., 2014). However, AITC can also be released from the (model) food and (active) packaging materials during the absorption time. Moreover, it is an easily degraded compound in the headspace, which makes the equilibrium between the SPME fibre and headspace difficult to be determined; therefore, the SPME method is not suitable to quantify the real concentration in the headspace. Therefore, the direct injection was used to determine the real AITC concentration in the packaging system in **Chapters 4, 5, and 6**. As an alternative for the performed AITC measurement with the SPME method, the AITC concentration was quantified by using an equation obtained from the relation between the SPME and direct injection results. Therefore, the SPME method was recommended to be used to detect the types of compounds in the packaging, and the direct injection method could be more relevant to be applied to quantify the amount or concentration of compounds in the packaging system.

The homogeneity of myrosinase and sinigrin in the mustard seeds needs to be considered by selecting the right particles of ground mustard seeds. In **Chapter 2**, the release rate of AITC in the samples with sizes 400-500 µm was very low, but the AITC concentration was very high in the headspace. A possible reason for this special behaviour could be due to more sinigrin cells damage and less myrosinase cells damage in that particular fraction. The milling process and fat extraction damaged the cells in the internal part and external parts of the mustard seeds. However, during cell disruption, it was still not known whether those processes damaged the myrosin cells or S-cell. According to Kissen et al. (2009), the cell structure in the external part of the seeds, including testa (seed coat/skin), contains more myrosin cells, and the inner structure contains well-distributed myrosin cells and S-cells. The selected particles with testa might contain a higher myrosinase and lower sinigrin content, which then caused a faster AITC formation rate and caused a lower sinigrin content remaining in the particles. To have a more

homogenous distribution of myrosinase and sinigrin in the particles, the particles separate from the testa were recommended to be used. **Figure 7.6** shows the appearance of the testa appointed by layer number 2 and the intrinsic part of particles. Further studies are needed to investigate the best method in separating the testa from mustard seeds without premature release and the effects of testa separation from ground mustard seeds on the AITC release and formation.

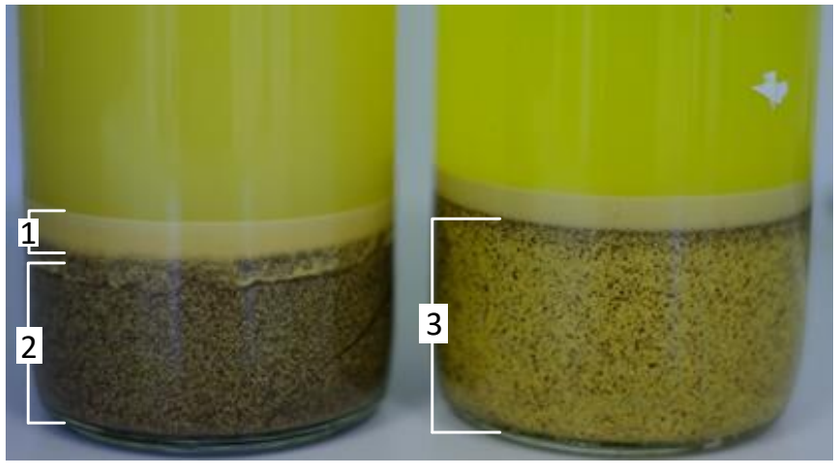


Figure 7.6. *The ground mustard seeds in hexane solvent.* ***Layer number 1*** *is the smaller ground mustard seeds,* ***layer number 2*** *is the ground mustard seeds containing the majority of the brown testa (seed coat/skin), and* ***layer number 3*** *is the bigger ground mustard seeds containing less brown testa (seed coat/skin).*

7.3. Multiresponse kinetic model considerations

Antimicrobial packaging with a closed system contains 3 phases; antimicrobial source, headspace, and (model) food phase. The food product is viewed as a target, the headspace is an intermediate phase, and the antimicrobial source is the place where the antimicrobial compounds are produced before the release into the headspace. The AITC can react or interact with other components in the packaging system; at the same time, the AITC is transferred from a phase to another phase. To understand the changes of AITC in each phase in the packaging system, like AITC formation and release, it could not be described for each mechanism separately since there was an interlinked system between AITC formation, release, absorption and degradation. In this thesis, the rates of each reaction and mass transfer of the AITC in the package were estimated using a multiresponse kinetic model with a good fit between the observed and predicted data (**Chapters 3** and **4**). The fits indicate that the models are appropriate (Verkempinck et al., 2019). The advantage of the multiresponse kinetic model is that the model can be used to describe the whole system (reaction and mass transfer) and results in a better understanding of the reaction and mass transfer mechanisms in the packaging system. Describing those mechanisms with the multiresponse kinetic model, the amount of antimicrobial compounds that need to be added to the packaging system containing a specific food can be determined. For example, the kinetic model describing the AITC

concentration shown in **Figure 7.7a** can be used to predict the growth of bacteria based on the amount of ground mustard seeds required to add into the packaging (**Figure 7.7b**). Finally, the model can be used to optimize the concentration of antimicrobial compounds in each phase of the packaging to achieve the extended shelf life of food products.

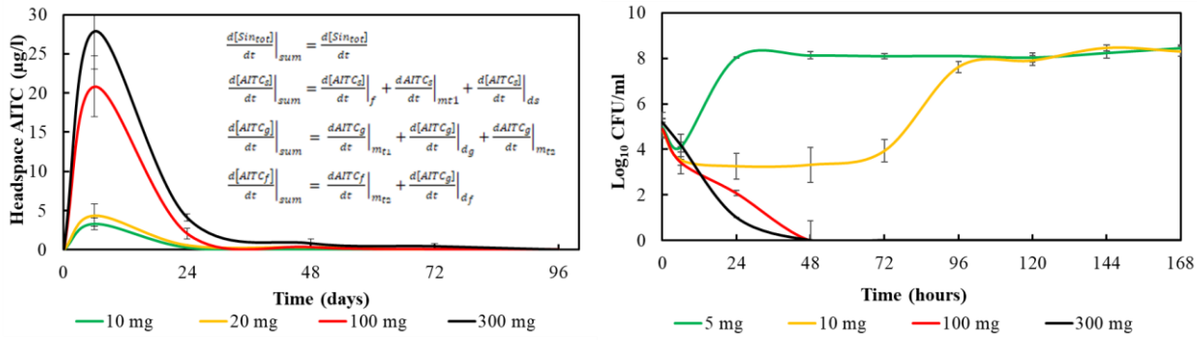


Figure 7.7. *The allyl isothiocyanate (AITC) compound release* ***(left)*** *into the headspace as a function of the quantity of ground mustard seeds added in the packaging and correlated to the effect on the growth of pseudomonas fragi* ***(right)****.*

However, some considerations are needed for the interpretations of the predictions when using the kinetic model. Some unexpected results containing a number of outliers in a group of data needs to be considered to be included in the model. In our study **(Chapter 2)**, we found an unexpected result of the AITC headspace concentrations. The particles with a size range (400-500 µm) resulted in a much higher concentration of AITC in the headspace compared to other samples. The possible reason for the phenomena was explained in **Chapter 2**. In **Chapter 3**, the model was developed, and the unexpected response was included in the developed multiresponse model, which then causes the predicted lines to be forced to follow and fit the unexpected response. Consequently, the model produced high standard deviations for some parameters and high correlations amongst those parameters.

In the design of antimicrobial packaging, it is better to measure all phases in the packaging system in which the antimicrobial compounds partitions. The more experimental data are included in the multiresponse kinetic modelling, the more accurate parameter estimates will be obtained. A lack of data for one of the phases causes a high correlation and high standard deviation in some parameter estimations. For instance, the concentration of AITC in ground mustard seeds containing fat and aqueous phases (**Chapter 2**) and in the food matrix containing the fat, protein, and aqueous phases (**Chapter 4**) were measured. However, the AITC was not measured in the specific phases within the seeds and food, e.g. fat and aqueous phases. No measurement method in a specific phase was still accepted. However, when the AITC concentration is very low, the equilibrium between headspace and ground mustard seeds or food matrix competes with the equilibrium between the phases inside ground mustard seeds, consequently resulting in the high standard deviations in the parameter

estimations. Therefore, it is recommended to further study the antimicrobial partition in all phases to obtain a more accurate fitting and estimation of the kinetic parameters.

7.4. Future research and recommendations

This thesis gained insights into the factors influencing the concentration of antimicrobial compounds and their antimicrobial effects on packaged food and understanding the mechanism of mass transfer and reactions in the packaging system. However, there is still much more to explore in this field, and additional research is required to enable the actual application of allyl isothiocyanate released from mustard seeds in an antimicrobial packaging system. The following paragraphs are further elaborating on various possible recommendations for further research.

With the use of a (model) food system, the organoleptic and sensory effect of applying AITC in food packaging systems could be studied (Pang et al., 2013). This acceptability of the consumers' needs to be taken into account in the packaging design. Studies on Catfish (Pang et al. 2013), cheese (Winther and Nielsen, 2006), poultry (Alanazi et al., 2018; Shin et al., 2010), and bread (Jideani and Vogt, 2016) indicate product-specific AITC thresholds regarding organoleptic issues. Additionally, product sensory attributes affect the purchase decision of consumers (Fang et al., 2017). The AITC in the packaging system has interactions with food components leading to a sensory change (**Chapter 4**). Future studies are needed to understand better the effects of AITC interactions leading to various changes, such as texture changes, undesired flavour formation, and colour formation.

In **Chapter 4**, the partitioning of AITC into the headspace and food products has been investigated. However, in this study, partitioning of AITC inside the (model) food phases, e.g. fat and water, was not investigated. This partitioning is also important to understand the mass transfer of AITC in the packaging system. The experimental data for each phase in the packaging system makes the parameter estimations of the developed kinetic model more accurate and reliable. In addition, the structure of the food products, e.g. viscosity, the porosity of the food products, droplet size, can influence the partitioning of AITC into food products (Wang et al., 2020a; Wang et al., 2020b). Those factors are also needed to be studied to gain further insight into the application of AITC in food products with different structures.

Chapter 2 reported that the smaller particles could result in a higher concentration of AITC in the headspace. In this study, the smallest particle is ranged from 50-100 µm. It could be favourable to do a study with smaller particles on the nanoscale, which can optimize the cell damage in the particles and might help to optimize the AITC formation. Nanoparticles of ground mustard seeds (<100 nm) improve or optimize the sinigrin available to be hydrolyzed by myrosinase. In the incorporation of the nanoparticles to polymer film or packaging material, the nanoparticles are expected to be easily mixed with a packaging polymer to form a better

surface with less porosity (Hammed, 2018). However, the milling process method to reduce the size could be a challenge for further study.

The active packaging system could be applied in combination with intelligent packaging that can give information to consumers about the quality of the packaged food. A combination with intelligent packaging can be an advanced application for the pre-detection of the expired date. For applying this system, the concentration of compounds in the packaging system can be used as an indicator to indicate the remaining capacity of the compounds to inhibit the bacterial growth of the foods. For example, a colour sensor can be used to indicate the compound concentration in the packaging system; once the concentration is not sufficient anymore to inhibit the bacteria, the colour changes. The colour change indicates a change in the product's quality decline rate, which gives a warning to consumers to consume packaged food as soon as possible. This concept needs to be investigated further and could be promising to apply since the packaging system can provide safety and quality information about the packaged food.

With the in-depth knowledge about the antimicrobial sources, food products, mass transfer, and reaction of the antimicrobial compounds, the effectiveness of active packaging can optimally protect the food products from spoilage bacteria. In tailoring the packaging, not only the characteristic of antimicrobial sources or packaging material needs to be considered, but also the type and characteristic of food products. The kinetic modelling containing mathematical equations can be established to describe potential mass transfer and reactions of compounds occurring inside the packaging and its effect of the compounds on the food. At the industrial level, the established kinetic modelling can be used to select the right packaging material or antimicrobial compound and its concentration for a particular type of food packaged.

7.5. Main Conclusion

This thesis describes a case study of an antimicrobial packaging design by exploring the potential of AITC release from the mustard seeds. The factors that influence the release and stability of antimicrobial compounds and the antimicrobial effect on (model) food products were investigated to optimize the effectiveness of the antimicrobial packaging on food products. A multiresponse kinetic model was developed to understand the mechanism of the reaction, e.g. formation and degradation of the compounds, and mass transfer of the compounds, e.g. release, partitioning, and absorption, in the packaging system.

Mustard seeds are promising to be used as an antimicrobial carrier in active packaging because the mustard seeds can form antimicrobial compounds AITC inside the package. Optimizing the headspace concentration of AITC released from the ground mustard seeds is important to reach an extended food shelf life. Therefore evaluating the factors affecting the concentration change and the effects on bacterial growth is essential. The concentration of the

compounds in the headspace determines the microbial growth in food products. Defatted ground mustard seeds and a thinner thickness of film incorporated with mustard seeds results in a higher AITC release into the packaging headspace. At lower temperature and relative humidity, AITC was slower released from the antimicrobial source and degraded less in each phase in the packaging system. The food composition influences the AITC partition between the headspace and the food product and also the absorption of AITC in the food product. Influencing these factors gives possibilities to obtain the desired AITC concentration in the packaging system, which result in more prolonged inhibition of spoilage bacteria in bacterial growth media and real foods. This new antimicrobial packaging approach can, therefore, extend the shelf life of the packaged food products without influencing the sensory attributes of the food.

The case study shown in the thesis gives an insight into an antimicrobial packaging design by using a sustainable, natural, low-cost antimicrobial source that can effectively inhibit spoilage bacteria in food products. The packaging system can be applied by food industries to fulfil the consumers' demands for healthy and fresh food products and to reduce global food waste.

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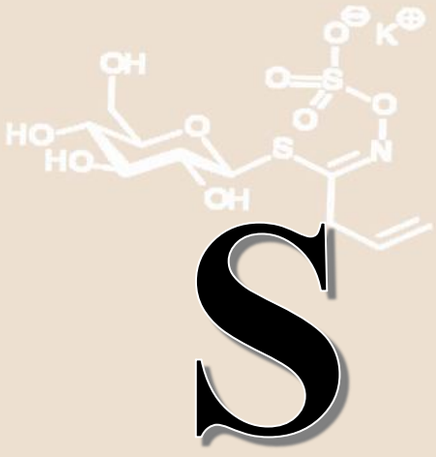

Summary

Summary

The Food and Agriculture Organization (FAO) reported 1.3 billion tons of food produced for human consumption are globally wasted each year. Perishable products mishandling by retailers, consumer behaviour, inappropriate quality control, overstocking, and high safety expectancy by consumers are known as the main factors contributing to food spoilage or contamination. Antimicrobial food packaging can inhibit the growth of spoilage and pathogenic bacteria by releasing antimicrobial compounds into food products. Allyl isothiocyanate (AITC) is acknowledged as a volatile and strong antimicrobial compound that can inhibit the growth of a variety of microorganisms in food products. Mustard seeds containing a high content of sinigrin, which is the precursor of AITC, can be used as an antimicrobial carrier in antimicrobial packaging.

This thesis aimed to explore the potential of allyl isothiocyanate release from mustard seeds to design an antimicrobial packaging. The factors influencing the released AITC concentration and its efficacy on microbial growth in a packaging system were investigated. The AITC concentrations were measured in each phase of the packaging system (antimicrobial source, headspace and (model) food). Mass transfer and reactions occurring inside the package were described by a kinetic model containing a set of combined mathematical equations to understand the mechanism of formation, release, absorption, and degradation of AITC. The antimicrobial effects of AITC on the growth of spoilage bacteria in a food medium and/or real food were analyzed to understand the effectiveness of the antimicrobial package in microbial inhibition and the extension of the shelf life of foods.

Chapter 1 reviews the allyl isothiocyanates as an antimicrobial agent, consisting of mustard seeds as a source of AITC, its formation and mode of action. Mass transfer of antimicrobial agents in the packaging system was explained. Lastly, the factors controlling the concentration of antimicrobial compounds and their effects on food quality for the application in the packaging system were reviewed.

Chapter 2 reports the effects of particle size and fat content of mustard seeds on the AITC formation and release into the headspace. Particle size reduction and fat extraction caused more damages to mustard seeds' cells leading to a higher amount of hydrolyzed sinigrin by myrosinase. Consequently, a higher amount of AITC was formed in the mustard seeds, and a higher AITC concentration was observed in the headspace. Furthermore, the higher fat ground mustard retained more AITC in the seeds and was releasing less to the headspace.

Chapter 3 reports how the mechanisms of formation, degradation, and release of AITC could be described by using a multiresponse kinetic model. This established model produced a good prediction for those mechanisms. The defatted mustard particles had a significant

influence on some parameters, e.g. increasing the accessible sinigrin and lowering the formation rate constant and increasing the mass transfer coefficient. The particle size significantly affected accessible sinigrin in the particles but had no significant effect on the release of AITC into the headspace.

Chapter 4 reports the effects of food composition and temperature on AITC partition between the packaging headspace and a (model) food. A food matrix with a higher fat content absorbed a higher amount of AITC and caused a lower concentration of AITC in the headspace. Opposite to these results, a higher AITC concentration in the headspace and lower concentration in the food matrix were observed in the package containing a food matrix with higher protein content. These effects were explained by the increased viscosity of the food matrix. Multiresponse kinetic modelling was used to describe the mechanism of AITC partitioning in the food packaging system. The AITC concentration was more stable in the higher fat-based food matrix, and a higher mass transfer coefficient from headspace to food matrix was observed. An increase in the protein content of the food caused a slower AITC penetration into the food matrix, but the complex between proteins and AITC stabilized AITC in the food matrix. Furthermore, a higher temperature resulted in faster AITC absorption by the food matrix and faster degradation in the packaging system.

Chapter 5 describes the antimicrobial effects of the AITC released from the ground mustard seeds into the headspace. The antimicrobial effect against *Pseudomonas fragi* was tested in a liquid medium. An increasing amount of ground mustard seeds added into the packaging system resulted in a higher AITC concentration in the headspace and liquid medium. The higher AITC release from ground seeds (>20 mg) inhibited the growth of *P. fragi* and prolonged the lag phase of the bacterial growth curve. When a larger quantity of ground mustard seeds (100 mg) was added, the bacteria population was fully eliminated in the liquid medium for 48 hours. The relation between the total bacterial count ($\log_{10}$ CFU/mL) at 48 hours and the initial AITC concentration in the headspace at 6 hours was used to determine the minimum headspace concentration to inhibit and inactivate the bacteria. The curve was fitted using the Emax model. Finally, the minimum inhibitory concentration was determined to be around ~6 µg/L and the minimum concentration to inactivate the bacterial population was around 15 µg/L.

Chapter 6 reports the development of a moisture-activated-antimicrobial film containing ground mustard seeds and reported the AITC release and its antimicrobial effects in ground beef. The morphology of the thicker film was smoother and had fewer pores, causing slower water absorption, sinigrin degradation, and AITC release. Furthermore, a higher temperature and relative humidity stimulated a faster AITC release in the packaging system. The thicker film (about 0.3 mm) was used to determine the antimicrobial effects of AITC on the microbial growth in ground beef containing 9%- or 19%-fat. The lower fat content of ground beef caused

a higher AITC concentration in the headspace compared to the higher fat- ground beef. Consequently, it resulted in a longer period of inhibition of bacterial growth. The longer inhibition resulted in an extension of the shelf life of 4 days for the lower fat ground beef (9% fat), while the higher fat ground beef (19% fat) had an extension of the shelf life of only 16 hours. These results show that besides a controlled release of AITC, the composition of food products should be considered in tailoring effective antimicrobial packages.

Chapter 7 discusses the main findings of this study, the application of the antimicrobial packaging system in food products, some considerations of the methodology and kinetic modelling, and gives recommendations for further researches.

In conclusion, the design of antimicrobial packaging using in-situ formation to control the release of antimicrobial compounds into the headspace gives new opportunities to effectively inhibit microbial growth and prolongs food shelf life. The study gives an insight into a natural antimicrobial source as sustainable and low-cost concepts to be applied in the food packaging system.

Ringkasan

Organisasi pangan dan pertanian dunia atau dikenal sebagai *Food and Agriculture Organization (FAO)* melaporkan 1.3 milliar ton pangan yang diproduksi untuk konsumsi manusia seluruh dunia telah menjadi limbah. Penanganan yang kurang tepat yang dilakukan oleh pengecer terhadap produk pangan yang mudah mengalami kerusakan (*perishable food*), perilaku konsumen, kontrol kualitas yang tidak tepat, kelebihan stok dan tingginya ekspektasi oleh konsumer terhadap keamanan produk diketahui sebagai faktor utama yang berkontribusi terhadap pembusukan dan kontaminasi pada produk pangan. Kemasan pangan antimikroba dapat menghambat pertumbuhan bakteri patogen dan pembusuk melalui pelepasan senyawa antimikroba kedalam produk pangan yang dikemas. Allyl isothiocyanate (AITC) dikenal sebagai senyawa antimikroba yang volatile (mudah menguap) dan kuat yang dapat menghambat laju pertumbuhan berbagai mikroorganisme dalam produk pangan. Biji *mustard* (sesawi) mengandung sinigrin yang tinggi dimana sinigrin tersebut merupakan sumber senyawa (*prekursor*) pembentukan AITC. Biji *mustard* ini dapat digunakan sebagai pembawa antimikroba dalam kemasan antimikroba.

Disertasi ini bertujuan untuk menggali potensi pelepasan AITC dari biji *mustard* dalam mendesain sebuah kemasan antimikroba dan faktor-faktor yang mempengaruhi konsentrasi dan efektifitas AITC terhadap pertumbuhan mikroba dalam sistem kemasan pangan. Konsentrasi AITC tersebut diukur di setiap fase dalam sistem kemasan (sumber antimikroba, *headspace* dan (matriks) pangan. Perpindahan massa dan reaksi yang terjadi didalam kemasan tersebut digambarkan dengan menggunakan model kinetik yang terdiri dari kombinasi persamaan matematika untuk memahami mekanisme pembentukan, pelepasan, absorpsi dan degradasi AITC. Efek antimikroba AITC terhadap pertumbuhan bakteri pembusuk pada pangan atau matriks pangan dianalisis untuk memahami efektifitas kemasan antimikroba dalam menghambat pertumbuhan mikroba dan memperpanjang umur simpan pangan.

Bab 1 membahas AITC sebagai agen antimikroba, yang terdiri dari beberapa bagian yaitu biji *mustard* sebagai sumber AITC, pembentukan AITC dan cara kerja AITC dalam menghambat pertumbuhan mikroba dan transfer massa senyawa antimikroba dalam sistem kemasan pangan. Yang terakhir yaitu faktor-faktor yang mengontrol konsentrasi senyawa antimikroba dan pengaruhnya terhadap kualitas pangan ketika diaplikasikan dalam sistem kemasan pangan.

Bab 2 melaporkan tentang pengaruh ukuran partikel *mustard* dan kandungan lemak biji pada *mustard* terhadap pembentukan AITC dan pelepasannya dari biji *mustard* ke *headspace*. Pengecilan ukuran partikel dan ekstraksi lemak pada biji *mustard* menyebabkan terjadinya lebih banyak kerusakan pada sel-sel biji *mustard* sehingga menyebabkan jumlah sinigrin yang

terhidrolisis oleh myrosinase lebih banyak. Sebagai dampaknya, jumlah AITC yang terbentuk dalam biji *mustard* lebih banyak dan konsentrasi AITC di *headspace* kemasan juga lebih tinggi. Selain itu, kandungan lemak yang tinggi pada biji *mustard* mempertahankan lebih banyak AITC dalam biji sehingga hanya sedikit AITC yang dilepaskan ke *headspace* kemasan pangan.

Bab 3 melaporkan bagaimana mekanisme pembentukan, degradasi dan pelepasan AITC dapat digambarkan dengan menggunakan "*multiresponse* kinetik model". Model yang telah dikembangkan ini menghasilkan prediksi yang baik terhadap mekanisme tersebut. Partikel *mustard* tanpa kandungan lemak memiliki pengaruh signifikan pada beberapa parameter, seperti peningkatan jumlah sinigrin yang dapat diakses oleh myrosinase, penurunan konstanta laju pembentukan AITC, dan peningkatan koefisien perpindahan massa. Ukuran partikel secara signifikan mempengaruhi jumlah sinigrin yang dapat diakses oleh myrosinase, tetapi tidak memiliki dampak yang signifikan terhadap laju pelepasan AITC ke *headspace* kemasan.

Bab 4 melaporkan pengaruh komposisi pangan dan suhu pada partisi senyawa AITC antara *headspace* kemasan dan (matriks) pangan. Matriks pangan dengan kandungan lemak yang lebih tinggi mampu menyerap jumlah AITC lebih banyak dan menyebabkan konsentrasi AITC di *headspace* lebih rendah. Sebaliknya, konsentrasi AITC yang lebih tinggi di *headspace* dan konsentrasinya yang lebih rendah dalam matriks pangan dihasilkan oleh kemasan pangan dengan matriks pangan yang mengandung protein yang lebih tinggi. Pengaruh kandungan protein yang tinggi pada matriks pangan disebabkan oleh viskositas matriks yang juga meningkat. Permodelan *multiresponse* kinetik digunakan untuk menggambarkan mekanisme partisi AITC dalam sistem pengemasan pangan. Konsentrasi AITC lebih stabil dalam matriks pangan yang mengandung lemak lebih tinggi dan koefisien perpindahan massa yang lebih tinggi (dari *headspace* ke matriks pangan). Peningkatan kandungan protein pada matriks pangan dapat menyebabkan laju penetrasi AITC lebih lambat ke dalam matriks pangan, tetapi ikatan antara protein dan AITC menyebabkan AITC lebih stabil dalam matriks pangan. Selain itu, suhu yang lebih tinggi menyebabkan laju penyerapan AITC lebih cepat oleh matriks pangan dan laju degradasi AITC lebih cepat dalam sistem kemasan pangan tersebut.

Bab 5 menjelaskan tentang pengaruh antimikroba dari AITC yang dilepaskan dari biji *mustard* ke *headspace* kemasan. Pengaruh senyawa antimikroba terhadap *Pseudomonas fragi* telah diuji dalam media cair. Peningkatan jumlah biji *mustard* yang ditambahkan ke dalam sistem kemasan menghasilkan konsentrasi AITC yang lebih tinggi di *headspace* kemasan. Pelepasan AITC yang lebih tinggi dari biji *mustard* (> 20 mg) menghambat pertumbuhan *p. fragi* dan memperpanjang fase lag pada kurva pertumbuhan bakteri tersebut. Ketika jumlah biji *mustard* yang lebih banyak (100 mg) ditambahkan ke dalam kemasan pangan, AITC dapat menonaktifkan bakteri dalam 48 jam. Kurva hubungan antara total bakteri ($\log_{10}$ CFU/mL) pada 48 jam dan konsentrasi AITC saat 6 jam di *headspace* digunakan untuk menentukan

konsentrasi AITC minimum di *headspace* yang menghambat dan menonanktifkan bakteri. Kurva tersebut dapat digambarkan dengan menggunakan model Emax. Dengan demikian, konsentrasi minimum untuk menghambat pertumbuhan mikroba dapat ditentukan, yaitu sekitar ~6 µg/L dan konsentrasi minimum untuk menonaktifkan bakteri dalam media cair sekitar 15 µg/L.

Bab 6 melaporkan hasil pengembangan film yang mengandung partikel *mustard*, dimana sistem pelepasan antimikroba diaktifkan oleh kelembaban dalam kemasan. Mekanisme pelepasan AITC dan pengaruh AITC pada daging giling dilaporkan juga pada bab ini. Film dengan morfologi lebih halus dan memiliki pori-pori yang lebih sedikit menyebabkan laju penyerapan air yang lebih lambat, yang berdampak pada penurunan laju degradasi sinigrin dan pelepasan AITC. Selain itu, suhu yang lebih tinggi dan kelembaban relatif memicu pelepasan AITC yang lebih cepat dalam sistem kemasan. Film dengan ketebalan 0.3 mm digunakan untuk menginvestigasi efek antimikroba dari AITC pada pertumbuhan mikroba dalam daging giling dengan kandungan lemak sekitar 9 dan 19%. Kandungan lemak yang lebih rendah pada daging giling menyebabkan konsentrasi AITC yang lebih tinggi di *headspace* dibandingkan dengan daging giling yang berlemak lebih tinggi. Akibatnya, waktu yang dibutuhkan untuk menghambat pertumbuhan bakteri lebih lama. Penghambatan pada pertumbuhan antimikroba yang lebih lama mengakibatkan perpanjangan umur simpan selama 4 hari untuk daging giling dengan kandungan lemak 9%, sedangkan untuk daging giling yang mengandung lemak sekitar 19%, umur simpan hanya mampu memperpanjang selama 16 jam. Hasil ini menunjukkan bahwa selain pelepasan AITC yang perlu untuk dikontrol, komposisi produk pangan harus dipertimbangkan dalam menyesuaikan kemasan antimikroba yang efektif.

Bab 7 membahas tentang penemuan utama (novelty) dalam penelitian ini, penerapan sistem kemasan antimikroba pada produk pangan, beberapa pertimbangan dari aspek metodologi dan permodelan kinetik, serta rekomendasi untuk penelitian selanjutnya. Hasil penelitian ini dapat disimpulkan bahwa desain kemasan antimikroba yang menggunakan mekanisme pembentukan secara in-situ untuk mengontrol pelepasan senyawa antimikroba ke *headspace* memberikan peluang untuk menghambat pertumbuhan mikroba secara efektif dan memperpanjang umur simpan pangan. Studi ini memberikan wawasan tentang sumber antimikroba alami sebagai konsep yang berkelanjutan dan berbiaya rendah yang dapat diterapkan untuk kemasan pangan di masa mendatang.

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Nur Alim Bahmid

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About the Author

Author Biography

Nur Alim Bahmid was born on December 17th, 1989, in Pare-pare, Southern Sulawesi Province, Indonesia. He completed his BSC in Food Science and Technology Department at Hasanuddin University, Makassar, in 2011 and his MSc in Agroindustrial Technology Department, IPB University, Bogor, in 2014. His master thesis focused on the development of bioplastic from nanofiber of oil palm empty bunches for packaging application. After graduation, he began his work as a lecturer at Universitas Sulawesi Barat, Majene.

After receiving a Letter of Acceptance from Wageningen University and Research (WUR) and LPDP scholarship in 2015, he started his PhD in November 2016 in Food Quality and Design WUR. During his PhD, he worked on food packaging by designing an antimicrobial food packaging using allyl isothiocyanate from mustard seeds. The results of the study are presented in this thesis.

Besides working on his PhD, he also contributed to some non-profit organizations. He worked as Vice President of the Indonesian Student Association in The Netherlands (PPI Belanda) in 2017-2018, appointed as Vice-Chair of Association of Hasanuddin University Alumni in The Netherlands (IKA UNHAS Belanda) in 2019-2021, and as Chair of Agricultural and Environmental Institutions PCINU in The Netherlands (LPLH-PCI NU Belanda) in 2019-2021. He also led the organizing committee of a bilateral symposium, Wageningen Indonesian Scientific Exposure (WISE) 2019, held for the fourth consecutive year by the Indonesia PhD Association in Wageningen in collaboration with WUR. WISE is a platform to exchange information on the current research projects of Indonesian PhD and postdoc candidates in WUR.

He enjoys watching every football match, playing football and badminton.

Alim can be contacted by email: alimbahmid@yahoo.com.

List of Publications

Bahmid, N.A., Pepping, L., Dekker, M., Fogliano, V., Heising, J., (2020). Using particle size and fat content to control the release of Allyl isothiocyanate from ground mustard seeds for its application in antimicrobial packaging. Food Chemistry 308.

Bahmid, N.A., Heising, J., Fogliano, V., Dekker, M. (2020). Packaging Design Using Mustard Seeds as a Natural Antimicrobial: A Study on Inhibition of Pseudomonas fragi in Liquid Medium. Foods 9(6).

Bahmid, N.A., Heising, J., Dekker, M. (2021). Multiresponse kinetic modelling of the formation, release, and degradation of allyl isothiocyanate from ground mustard seeds to improve active packaging. Journal of Food Engineering 292, 110370.

Bahmid, N.A., Dekker, M., Fogliano, V., Heising, J., Modelling the effect of food composition on antimicrobial compound absorption and degradation in an active packaging. Journal of Food Engineering 300, 110539.

Bahmid, N.A., Dekker, M., Fogliano, V., Heising, J., Development of a moisture-activated antimicrobial film containing ground mustard seeds and its application on meat. (submitted to a Journal)

Overview of completed training activities

Discipline specific activities

Courses

Advanced Food Analysis[3], VLAG, Wageningen, The Netherlands, 2017

Management of Microbiological Hazards in Foods, VLAG and FSM, Wageningen, The Netherlands, 2017

Healthy Food Design, VLAG, Wageningen, The Netherlands, 2018

Reaction Kinetics in Food Science, VLAG, Wageningen, The Netherlands, 2019

Conferences

Wageningen Indonesian Scientific Exposure (WISE) 2017[3], Indonesian PhD student community and WUR, Wageningen, The Netherlands, 2017

32nd EFFoST International Conference[3], EFFoST, Nantes, France, 2019

Wageningen Indonesian Scientific Exposure (WISE) 2019[3], Indonesian PhD student community and WUR, Wageningen, The Netherlands, 2019

2nd International Conference of Innovations in Food Science and Technology[2], AIIC, Amsterdam, The Netherlands, 2019

2nd Food Chemistry Conference[3], Elsevier, Seville, Spain, 2019

33rd EFFoST International Conference[2,3], EFFoST, Rotterdam, 2019

2nd International Conference ICROEST[1], PMC Hasanuddin University, Online, 2020

34rd EFFoST International Conference[2], EFFoST, Israel (Online), 2020

General courses

Philosophy and Ethics of Food Science and Technology, VLAG, Wageningen, The Netherlands, 2017

PhD Week, VLAG, Wageningen, The Netherlands, 2017

Data Management Planning, WGS, Wageningen, The Netherlands, 2017

The Essential of Scientific Writing and Presenting, WGS, Wageningen, The Netherlands, 2018

Scientific Writing, WGS, Wageningen, The Netherlands, 2018

Introduction to R, VLAG, Wageningen, The Netherlands, 2018

Applied Statistics, VLAG, Wageningen, The Netherlands, 2018

Scientific Publishing, WGS, Wageningen, The Netherlands, 2019

Career Assessment, WGS, Online, 2020

Optional courses and activities

Scientific meetings, seminars, colloquia, FQD, Wageningen, The Netherlands, 2016-2021

VLAG research proposal, VLAG, Wageningen, The Netherlands, 2016-2017

Indonesian Scientific Exposure and Round Table (INSERT) Committee, Indonesian Student Association in the Netherlands (PPI Belanda), Amsterdam, The Netherlands, 2018

WISE Committee, Indonesian PhD student community and WUR, Wageningen, The Netherlands, 2018-2019

Teaching Obligation

Supervision of BSc- and MSc students, FQD, Wageningen, The Netherlands, 2017-2021

Food Packaging Design FQD-21306, FQD, Wageningen, The Netherlands, 2017-2020

Quality System Operations FQD-20804, FQD, Wageningen, The Netherlands, 2020

Predicting Food Quality FQD31306_2020, FQD, Wageningen, The Netherlands, 2021

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[1]Invited speaker.

[2]Oral presentation.

[3]Poster presentation.

Abbreviations: VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Science; WGS: Wageningen Graduate School; FQD: Food Quality and Design; FSM: Food Safety and Microbiology; EFFoST: European Federation of Food Science and Technology; AIIC: The Acad Indus International Conventions; PMC: Publication Management Center.

About the cover

The cover describes a graphical representation of the packaging system.

The mustard seeds are located at the top. AITC is released from mustard seeds through the headspace (the brown colour) to the beef (the bottom parts).

The front book cover shows the packaging containing AITC and fresh beef, while the back cover shows the packaging containing no AITC and spoiled beef.

In the front book cover, there is also a hidden AITC molecule describing the invisibility of AITC, and the line under the mustard seeds shows a fitting line of a model to the AITC concentration.

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