PROTECTION OF POLYUNSATURATED FATTY ACIDS AGAINST RUMINAL BIOHYDROGENATION USING POLYPHENOL OXIDASE

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List of abbreviations

4-HR	4-hexylresorcinol
4-MC	4-methylcatechol
AVG	average
BDL	below detection limit
BH	biohydrogenation
CAS	casein
CP	continuous phase
CLA	conjugated linoleic acid
CR	crushed
D[v,0.5]	median volume-weighted distribution value
D[v,0.9]	90 % percentile of the volume-weighted distribution
D_{32}	surface-weighted mean diameter
D_{43}	volume-weighted mean diameter
df	degrees of freedom
DM	dry matter
DVE	intestinal digestible protein (Dutch: darm verteerbaar eiwit)
FA	fatty acid
FAME	fatty acid methyl ester
FFA	free fatty acid
FOR	formic acid
FT	frozen/thawed
GC	gas chromatography
LC	linear contrast
LUT	Lutrell Combi
MFD	milk fat depression

MOL	molasses
MS	mass spectroscopy
ND	undamaged
NE	Net energy of lactation
NL	neutral lipid
OEB	degraded protein balance
PBP	protein-bound phenols
PE	protection efficiency
PL	polar lipid
PPO	polyphenol oxidase
PROT	protected supplement
PUFA	polyunsaturated fatty acid
QC	quadratic contrast
RC+CAS	red clover extract plus extra casein
S/L	soybean/linseed
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SPB	sodium phosphate buffer
SPME	Solid Phase Micro-Extraction
SSA	specific surface area
t10c12	*trans*-10, *cis*-12 C18:2
TAG	triacylglyceride
TBARS	thiobarbituric acid reactive substances
TE	transfer efficiency
Tyr-eq	tyrosine-equivalents
UPROT	unprotected supplement (control)
VFA	volatile fatty acid

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GENERAL INTRODUCTION

General introduction

Consumers are increasingly aware about healthy food (Hoefkens *et al.*, 2011). One aspect of obtaining a healthier human diet is to increase the intake of polyunsaturated fatty acids (PUFA) (WHO, 2008). Increasing the PUFA content in milk and meat and decreasing the proportion of saturated fatty acids contributes to achieve these recommendations (Givens, 2008). In many countries, ruminant products represent a substantial part of the human diet. Unfortunately, lipids in milk (Chilliard *et al.*, 2007) and meat of ruminant origin (Wood *et al.*, 2008) is low in PUFA, despite the high PUFA content in forage lipids (Boufaïed *et al.*, 2003a). Improving the PUFA content of monogastric products can be achieved relatively easily by increasing the level of ingested PUFA-rich lipids. However, increasing PUFA in ruminant products is much more challenging (Kouba and Mourot, 2011). Indeed, due to microbial saturation of dietary PUFA in the rumen, a process called biohydrogenation (BH), very large proportions up to 900 g/kg of 'healthy' dietary PUFA are converted into more hydrogenated products, leading to the loss of their health features (Buccioni *et al.*, 2012). Besides the interest of PUFA for human health, *n-3* PUFA are also linked with better reproductive performances of animals (Gulliver *et al.*, 2012). Therefore, it is of interest to protect these PUFA against microbial BH in order to achieve an adequate bypass of PUFA to the distal parts of the gastro-intestinal tract, without conversion (Figure I.1).

A natural mechanism to achieve protection may be promising in this respect. Van Ranst *et al.* (2011) suggested that lipids could be effectively protected against pre-ruminal and ruminal lipid metabolism by encapsulation in protein-phenol complexes by the action of polyphenol oxidase (PPO), an enzyme abundantly present in nature, including red clover (Mayer, 2006). As red clover is not a popular fodder and has a low fat content, transfer of this natural mechanism to protect PUFA-rich sources (e.g. linseed or fish oil) against ruminal BH is also of interest.

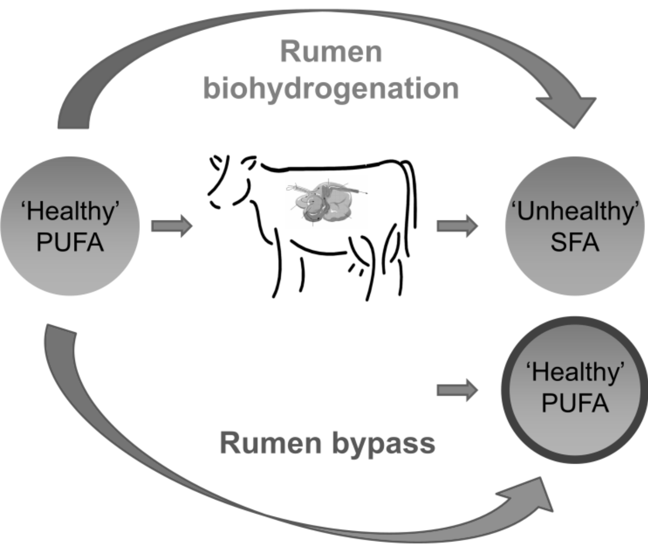


Figure I.1 Principle of protecting 'healthy' polyunsaturated fatty acids (PUFA) against ruminal biohydrogenation in order to prevent the formation of 'unhealthy' saturated fatty acids (SFA)

Objectives and outline of this study

This work focusses on the protection of PUFA against ruminal BH using the natural and omnipresent enzyme PPO. The objectives of this thesis can be divided in two major parts:

In **PART I**, the objective is to further explore the protection of PUFA in roughages. ***Chapter 1*** first introduces the role of PPO in reducing ruminal BH. The aim of ***Chapter 2*** was to assess whether BH of red clover FA is linked with polar lipid levels of the (conserved) material, to clarify the possible role of *in silo* microbial activity on polar lipid disappearance and to improve the current understanding of the role of PPO in reducing BH.

The objective of **PART II** is to assess the protection of emulsified fatty acids as a new means to protect non-roughage PUFA against BH through the action of PPO. First, an overview is given of the current state of the art in rumen lipid bypass technologies, focusing on both patent-described protection mechanisms, possible advantages or drawbacks of the technologies and protection results being described in recent scientific literature (***Chapter 3***). In this chapter, the potential of a novel rumen lipid protection technology, based on interfacial

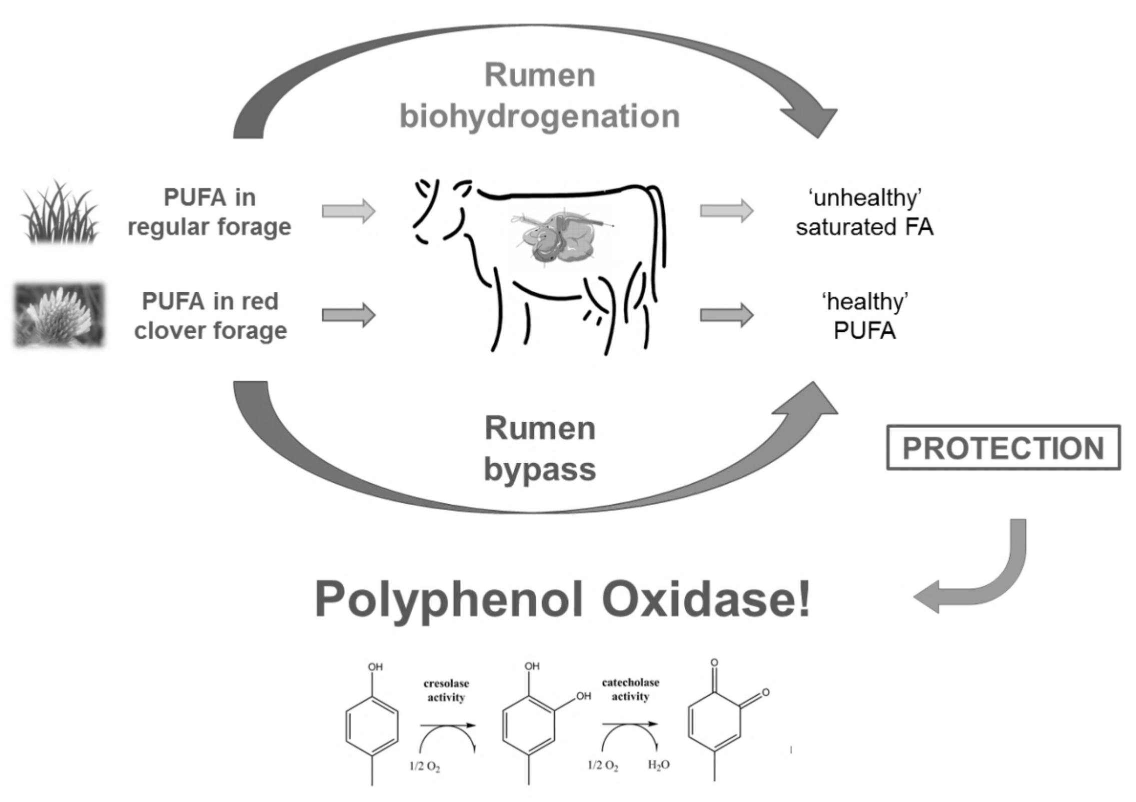
cross-linking of emulsions, is introduced. The experimental work related to PPO-induced cross-linked emulsions is described in three subparts of PART II. In **PART II A** the protection of PUFA using the new PPO-based rumen bypass technology is explored. The first evaluation of the concept of protecting PUFA against rumen BH with PPO from a red clover extract was made in ***Chapter 4***. Broadening the PPO technology to protein extracts of other plant sources is described in ***Chapter 5***, where enzymatic and interfacial factors affecting the rumen protection efficiency were also addressed in order to deepen the understanding of this technology. The goal of ***Chapter 6*** was to evaluate if the protective properties of the technology could be expanded to pre-ruminal processes and to other lipophilic compounds. As such protection of PUFA and vitamin E against aerobic oxidation has been assessed. Further, the post-ruminal availability of rumen protected PPO-based emulsions was examined in **PART II B**. Indeed, rumen protected PUFA have to be liberated again during post-ruminal passage in the gastro-intestinal tract. Otherwise the added value of protection is meaningless. Post-ruminal availability was first assessed by means of a mice trial (***Chapter 7***) and an *in vitro* method (***Chapter 8***), but the ultimate *in vivo* proof of concept for the transfer of dietary PPO-protected PUFA to milk was given by a trial with dairy cows (***Chapter 9***). Finally the objective of **PART II C** was to put the first steps towards upscaling of the protection technology (***Chapter 10***).

PART I

PROTECTION OF FATTY ACIDS IN ROUGHAGES

Chapter 1

Introduction part I - The role of polyphenol oxidase in reducing ruminal biohydrogenation



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Chapter 1

Introduction part I - The role of polyphenol oxidase in reducing ruminal biohydrogenation

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1 What is ruminal biohydrogenation?

Ruminants have the unique ability to utilize structural carbohydrates and non-protein nitrogen due to the presence of a wide range of microorganisms in the rumen. However, a consequence of this symbiosis is the biohydrogenation (BH) of unsaturated fatty acids by ruminal bacteria. This is believed to be caused by the action of micro-organisms to protect themselves from the toxic effects of polyunsaturated fatty acids (PUFA) (Jenkins, 1993). As a consequence, the majority of PUFA passing through the rumen is transformed into more saturated FA due to BH. This limits the transfer of dietary PUFA to the milk or peripheral tissues. The key steps of ruminal PUFA BH are illustrated for C18:2*n-6* in Figure 1.1 (Buccioni *et al.*, 2012). First, lipolysis of unsaturated FA from glycerol is a prerequisite before BH by ruminal micro-organisms can take place, as microbial isomerases can only attack FA with a free carboxyl group. The number of bacteria capable of excreting extra-cellular microbial lipases to hydrolyze PUFA from its glycerol backbone is limited to some bacterial strains of *Butyrivibrio fibrisolvens* and *Anaerovibrio lipolytica* (Buccioni *et al.*, 2012). These enzymes act mainly on the *sn*-1 or *sn*-3 position of the triacylglyceride, but hardly no accumulation of mono- or diacylglycerides is generally observed (Jenkins, 1993). Full hydrolysis to free FA can be the result of microbial lipases acting also on the FA in the sn-2 position or by a positional redistribution of FA (Karupaiah and Sundram, 2007). Free PUFA may also arise from the action of several bacterial galactosidases and phospholipases (Buccioni *et al.*, 2012), as well as through plant hydrolases prior to ingestion (Van Ranst *et*

al., 2009c; Van Ranst *et al.*, 2010). Then, the initial step of the actual BH is the conversion of the *cis*-12 double bond to a *trans*-11 isomer. Microbial reductases are further responsible for the elimination of double bonds. The hydrogenating activity of the rumen micro-organisms is mainly associated with bacteria, with a negligible role for ciliate protozoa (Lourenço *et al.*, 2010). The attachment of free PUFA to feed particles seems essential (Buccioni *et al.*, 2012). It has been suggested that enzymes responsible for biohydrogenation are found in membranes of bacteria such as *Butyrivibrio fibrisolvens*, which are attached to feed particles (De Beni Arrigoni *et al.*, 2016).

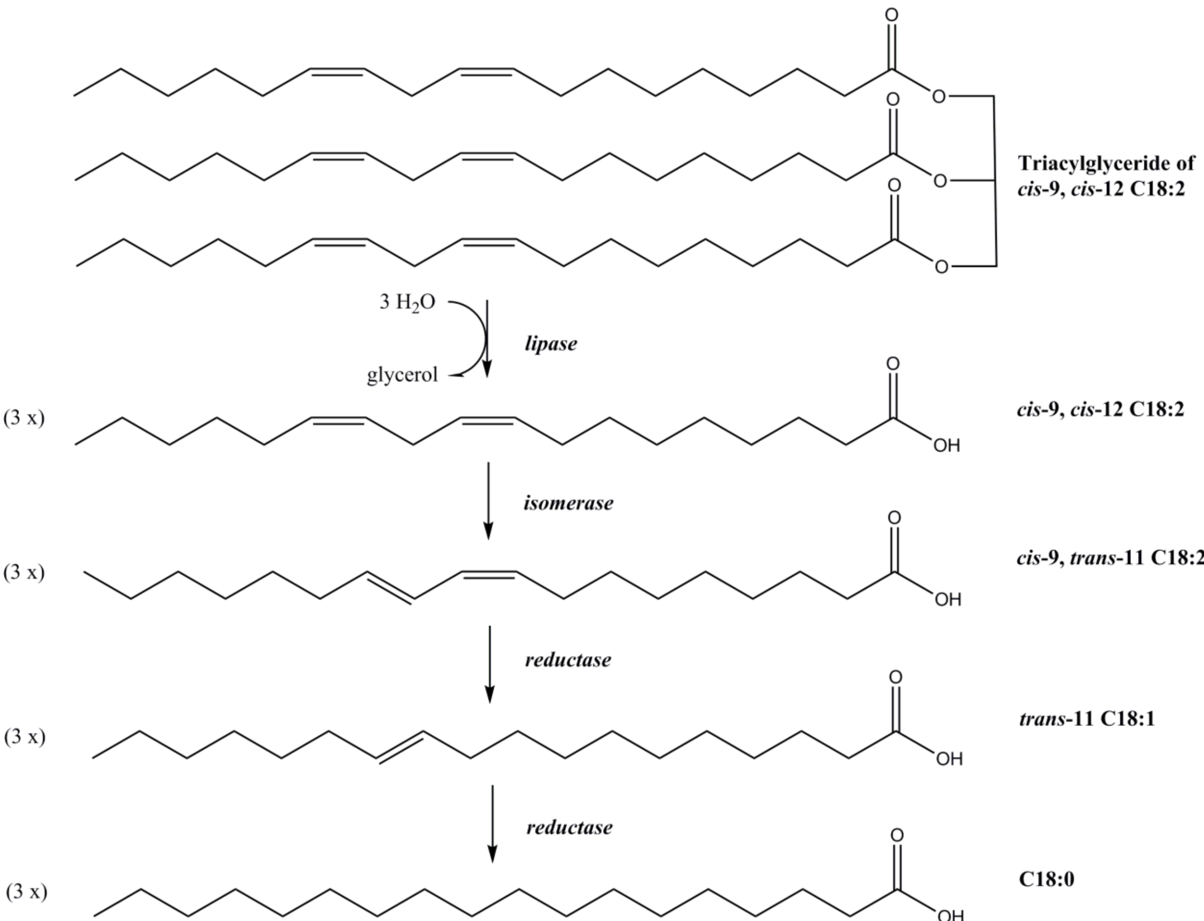


Figure 1.1 Key steps in the conversion of esterified linoleic acid by ruminal lipolysis and biohydrogenation (Buccioni *et al.*, 2012)

2 What is polyphenol oxidase?

Polyphenol oxidase (PPO) is a trivial name for all copper metalloenzyme oxidoreductases which can oxidize colorless phenols to colored quinones (Bittner, 2006). The active site of the enzyme consists of two copper atoms each in the ligand field with three conserved histidine residues (Yoruk and Marshall, 2003). Distinction is generally made between tyrosinases and laccases (Zeeb *et al.*, 2014).

Tyrosinases are able to catalyze the o-hydroxylation of a monophenol (cresolase activity), as present in the amino acid tyrosine, followed by the oxidation of o-dihydroxyphenol to o-diquinone (catecholase activity) (Bittner, 2006). The latter compounds are highly reactive and are able to polymerize with other phenols and quinones or to bind with nucleophilic groups in amino acids, such as thiol and amino groups, through Michael-type addition reactions (Bittner, 2006; Thalmann and Lötzbeyer, 2002). This results in the formation of melanin-like protein-phenol complexes (Yoruk and Marshall, 2003) (Figure 1.2). As only the initial quinone formation is catalyzed, cross-linking is rather uncontrollable, which is a major disadvantage of such a oxidoreductase reaction (Heck *et al.*, 2013). Tyrosinases are present in almost any organism, including animals, plants, fungi and bacteria (Mayer, 2006) and are most commonly known for the damage-induced browning of fruits and vegetables. Browning is generally unwanted in fruits and vegetables because of its detrimental effect on product quality, which resulted in numerous browning prevention and inhibition techniques (Kim and Uyama, 2005; Pilizota and Subaric, 1998). In certain cases, however, polymerization of quinones is of primary interest. The common brown color in black tea (Stodt *et al.*, 2014; Harbowy and Balentine, 1997) and cocoa (Misnawi *et al.*, 2002), for example, is also the result of a tyrosinase-induced reaction. Plant tyrosinases, being part of the chloroplast thylakoid membranes, often have to be activated before they can catalyze their reactions (Lee *et al.*, 2009). Tyrosinase-induced browning in plants only occurs in wounded tissues, due to the separate subcellular compartmentation of the enzyme and its diphenolic

Figure 1.2 Reaction scheme of the tyrosinase-catalyzed formation of protein-phenol complexes (Thalmann and Lötzbeyer, 2002)

substrates, which reside in the vacuole (Lee *et al.*, 2009; Yoruk and Marshall, 2003). Further, the lack of a pronounced substrate specificity makes that, besides phenolic groups within proteins, also many small phenolic substrates have the potential to cross-link with proteins. Typical substrates of tyrosinase besides tyrosine are L-3,4-dihydroxyphenylalanine, 4-methylcatechol, caffeic acid and chlorogenic acid (Yoruk and Marshall, 2003). The affinity of tyrosinase differs substantially between several phenolic substrates (Paul and Gowda, 2000; Demian and Makris, 2015; Selinheimo *et al.*, 2009) and has been shown to be mainly determined by the unsaturation and length of the sidechain (Demian and Makris, 2015). Besides, unfolded 'soft' proteins are more readily cross-linked by tyrosinase than globular 'hard' proteins. This is due to a reduced accessibility of the tyrosine residues of the protein for the tyrosinase, but cross-links can be formed in the latter upon addition of small phenolics as mediator (Fairhead and Thony-Meyer, 2010) to overcome the absence of surface-exposed tyrosine residues on the target protein (Heck *et al.*, 2013).

Laccase differs from tyrosinase by its unique ability to catalyze the oxidation of p-diphenol substrates besides o-diphenols (Yoruk and Marshall, 2003) and that cross-linking is based on the formation of free radicals instead of quinone formation (Zeeb *et al.*, 2014). Laccases are also ubiquitously present in nature and are, amongst others, responsible for the lignin degradation by fungi and lignin biosynthesis in plants.

As PPO is mainly associated with tyrosinase activity, for the remainder of this thesis "PPO" will refer to tyrosinase activity, unless stated otherwise. "PPO" is preferred rather than

"tyrosinase" because the latter might be erroneously interpreted as limited to oxidation of the amino acid tyrosine.

3 The protective role of polyphenol oxidase

Fresh forages generally contain a high proportion of esterified PUFA, e.g. in fresh grasses C18:3 may represent more than 50 % of the total FA content (Boufaïed *et al.*, 2003a). However, especially in intensified dairy and ruminant meat systems, animal diets often consist of conserved forages, which are low in esterified lipids due to transformation of triacylglycerides into non-esterified FA and glycerol during wilting and ensiling (Dewhurst *et al.*, 2003; Van Ranst *et al.*, 2009a; Van Ranst *et al.*, 2010). Accordingly, these unesterified PUFA are readily available for BH. Nevertheless, several *in vivo* and *in vitro* experiments showed a reduction in BH of PUFA when conserved red clover was a substantial part (Halmemies-Beauchet-Filleau *et al.*, 2013; Van Ranst *et al.*, 2013) or the sole part (Lee *et al.*, 2014) of the herbivore's ration. Lee *et al.* (2003) partially attributed the reduced BH in ensiled red clover to a protective role against hydrolysis of FA during wilting and ensiling by PPO. Indeed, red clover has been shown to exhibit both a high PPO activity as well as to contain a high content of PPO substrates, i.e. diphenolic compounds such as clovamide and phaselic acid (Lee, 2014). Lee *et al.* (2004) were the first to demonstrate the link between PPO-activity of red clover species and both plant-mediated proteolysis and lipolysis. The link between quinones, generated because of PPO-activity, and reduction in lipolysis and BH of PUFA in the rumen was evidenced shortly afterwards (Lee *et al.*, 2007). As a result, it has been suggested that PPO in red clover is both responsible for increased nitrogen use efficiency (Lee, 2014) as well as the protection of lipids against microbial degradation (Van Ranst *et al.*, 2011).

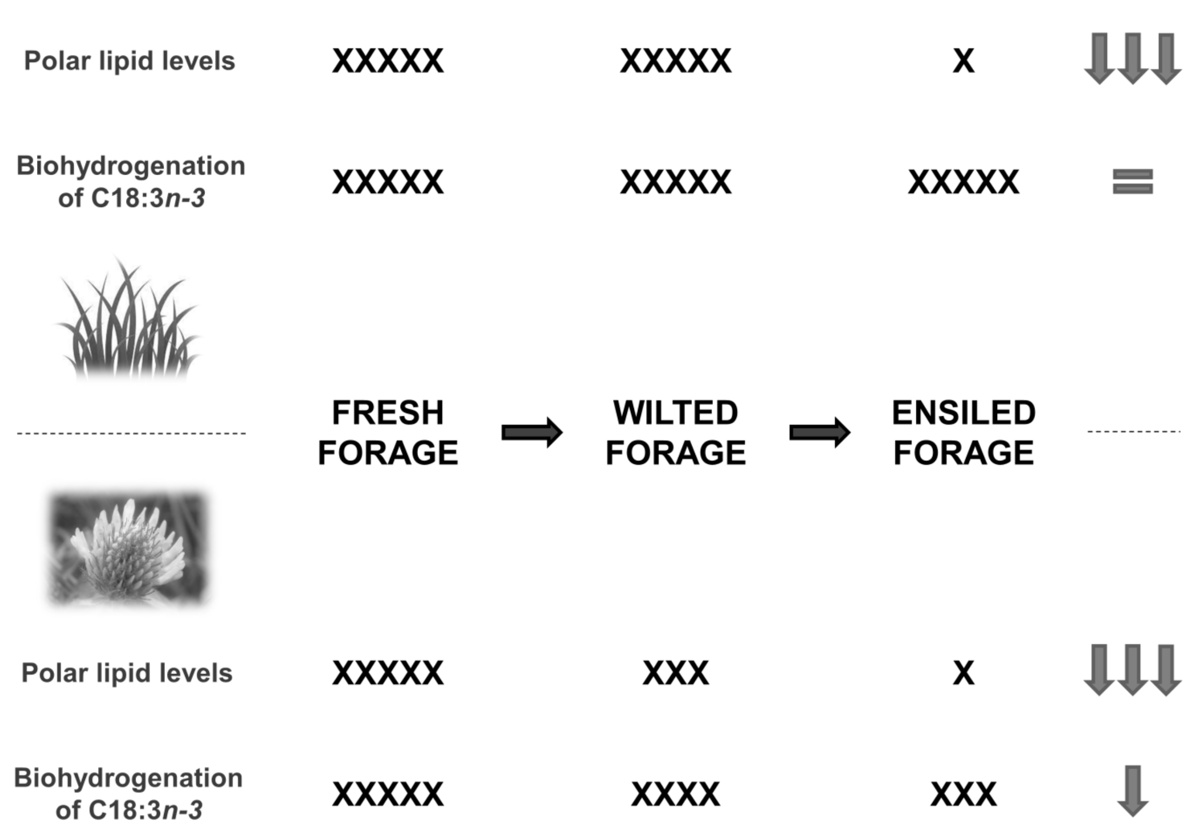
A summary of postulated mechanisms and current thinking on red clover's increased flow of PUFA across the rumen has been published by Lee (2014). Three potential working mechanisms have been proposed involving PPO-catalyzed quinone formation (Van Ranst *et*

al., 2011): quinones are hypothesized to bind to plant lipases (1) or polar lipids (PL) (2), or could be involved in the formation of a network of protein-bound phenol entrapping thylakoid lipids ('encapsulation') (3) (Lee *et al.*, 2010). Others have put forward the possibility of changes in microbial (biohydrogenating) ecology (Huws *et al.*, 2010) or ruminal digestion kinetics to explain reduced BH of red clover FA with increasing rumen outflow of PL (Halmemies-Beauchet-Filleau *et al.*, 2013). The increased duodenal flow of esterified FA seems a common feature in most of the proposed mechanisms, but most evidence indicates lipid is protected against ruminal BH by encapsulation in a matrix of cross-linked protein-phenol molecules (Lee *et al.*, 2010; Van Ranst *et al.*, 2011). Indeed, differences in ruminal outflow rates cannot explain enhanced amounts of PUFA remaining after *in vitro* simulation of rumen metabolism.

In the following chapter of PART I (***Chapter 2***), the objective is to further extend the current knowledge on protection of PUFA in roughages. The aim is to assess whether BH of red clover FA is associated with esterified polar lipid levels of the wilted or ensiled starting material, to clarify the possible role of *in silo* microbial activity on PL disappearance and to improve the current understanding of the exact role of PPO in reducing BH of conserved red clover.

Chapter 2

Effect of changes in lipid classes during wilting and ensiling of red clover using two silage additives on in vitro ruminal biohydrogenation



Redrafted after Gadeyne F., De Ruyck K., Van Ranst G., De Neve N., Vlaeminck B. and Fievez V., 2016, Effect of changes in lipid classes during wilting and ensiling of red clover using two silage additives on *in vitro* ruminal biohydrogenation, Journal of Agricultural Science, 154, 553-566.

Chapter 2

Effect of changes in lipid classes during wilting and ensiling of red clover using two silage additives on in vitro ruminal biohydrogenation

Abstract. Although forage lipid is generally rich in polyunsaturated fatty acids (PUFA), recovery of these fatty acids (FA) in milk and meat of ruminant origin is generally low, due to microbial biohydrogenation (BH) taking place in the rumen. As lipolysis is a prerequisite for BH, the latter process is expected to be enhanced when (conserved) forages contain lower levels of esterified FA (particularly polar lipids; PL). However, this wasn't observed in previous studies with red clover. Furthermore, red clover inclusion in the herbivore's diet was associated with decreased rumen BH as compared with other forages. Differences in plant lipase activity during wilting and ensiling has been attributed to changes in disappearance from the PL fraction, but a potential role of microbial lipases *in silo* has not yet been elucidated. Therefore, the aims of this study were to assess whether BH of red clover FA is linked with PL levels of the (conserved) starting material and to clarify the possible role of *in silo* microbial activity on PL disappearance. In order to obtain sufficient variation in forage PL and microbial activity, labscale silages were made by wilting and ensiling damaged or undamaged red clover using molasses or formic acid as ensiling additive, while perennial ryegrass was used as a control. Distribution of lipids within three lipid fractions (PL, free fatty acids and neutral lipids) in forages was determined and BH calculated after 24h *in vitro* rumen incubation. Results indicated microbial lipases in silages did not enhance FA disappearance from the PL fraction. A gradual decrease of FA in the PL fraction upon conservation was found, both in red clover and ryegrass, irrespective of the degree of damage. In red clover PL losses started from the wilting phase, while substantial PL

disappearance from ryegrass only started upon ensiling. Unexpectedly, proportions of PUFA in the PL fraction of red clover were positively correlated with PUFA BH, while this effect wasn't observed for ryegrass. PUFA in conserved red clover seemed to be partially protected against ruminal BH, while disappearance of FA from the PL fraction did not seem to be hampered. Results indicated the encapsulation mechanism as a consequence of protein-bound phenol (PBP) formation induced by polyphenol oxidase (PPO) is still the most probable hypothesis to explain red clover's increased flow of PUFA across the rumen.

1 Hypothesis and objective

PUFA in roughages are extensively lipolyzed and hydrogenated upon ruminal passage, but previous research has shown BH of PUFA is reduced when (conserved) red clover was part of the herbivore's ration. This has partly been attributed to a protective role of PPO. However, lipolysis also might take place prior to ingestion, in particular during preserving processes, such as wilting and ensiling, which dramatically reduce forage PL. As lipolysis is a prerequisite of the BH process, it could generally be assumed that BH of FA in the rumen is higher with decreasing levels of esterified PL remaining in the forage. In contrast to what would be expected, Van Ranst *et al.* (2010) observed a reduction of rumen BH of red clover PUFA upon longer ensiling, despite the lower levels of forage PL. However, poor silage quality might have contributed to these observations. Additionally, previous silage-based experiments particularly focused on plant enzymes (Van Ranst *et al.*, 2009a) liberated by cell damage whereas the role of microbial lipases during ensiling has been largely neglected.

Summarizing, ruminal BH of PUFA in the rumen was hypothesized to be stimulated when levels of PL remaining in the (conserved) forage were decreased, whereby microbial lipases could enhance lipolysis *in silo*. Therefore, the aim of this study was twofold: first, assessing whether reduction in red clover PUFA BH is associated with an enhanced conservation of forage PL, and second, clarifying the possible role of microbial activity during ensiling on PL disappearance. For this purpose, several treatments were considered, including fresh, wilted

and ensiled material which had been exposed to various post-harvest treatments. The experimental set-up was similar to the study by Van Ranst *et al.* (2010) and aimed to induce variation in forage PL content. Furthermore, molasses or formic acid were used as silage additives to stimulate or impair microbial activity, respectively. Perennial ryegrass was used as a control. The current experiment further aimed at improving the understanding of the role of PPO in reducing BH, particularly observed in conserved red clover products.

2 Materials and methods

2.1 Plant material

Red clover (*Trifolium pratense* L. cv Lemmon) was sown in May 2010 (50°59'07.7"N/3°47'22.0"E) in three fields at the Institute for Agricultural and Fisheries Research (ILVO, Belgium). These fields were kept as replicates throughout the whole experiment. The forage was harvested at the early blooming stage 10 cm above ground level using a Haldrup harvester (J. Haldrup s/a, Løgstør, Denmark) twice in 2010 and 2012 and four times in 2011 before harvesting for this experiment on 10 September 2012. Each year, mineral fertilizer was applied in March, after the first and second cut of the red clover (6 kg N/ha, 20 kg P_2O_5/ha and 140 kg K_2O/ha). The average dry matter content of red clover was 234 g/kg. The cultivar Lemmon was used, as this cultivar is known for its high PPO activity (Van Ranst *et al.*, 2009b).

Perennial ryegrass (*Lolium perenne* L.) was used in this experiment as a negative control, since ryegrass hardly shows any PPO activity and subsequent quinone forming ability in comparison with red clover (Van Ranst *et al.*, 2009b). Ryegrass was sown on 20 April 2010 (50°59'01.7"N/3°46'26.4"E) in three fields, kept as replicates throughout the whole experiment and harvested in the vegetative stage five times in 2011 and three times in 2012 before harvesting for this experiment using a Haldrup harvester on 16 August 2012. The

forage was fertilized yearly (296 kg N/ha, 14 kg P_2O_5/ha and 276 kg K_2O/ha). The average dry matter content of ryegrass was 248 g/kg.

2.2 Silages

Each of the three red clover replications was split into three equal parts (5 kg of fresh material each) for the different treatments (Figure 2.1). The first part was undamaged, the second part was crushed by hand (squeezing and turning) and the last part was frozen in liquid nitrogen immediately followed by thawing. The degree of cell damage as such was not measured, but slight discoloration for the crushed and frozen/thawed treatment was observed. Then, forages were wilted on the day of harvesting in a ventilated oven at 35°C until a dry matter content of at least 350 g/kg. When the targeted dry matter content was reached, the oven was switched off and the material left over night in the oven, if not, the oven was switched off over night and wilting was continued the next day. After wilting, a silage additive was added to all treatments (undamaged, crushed or frozen/thawed), either molasses (9 g/kg wilted material) or formic acid (3 ml/kg wilted material). The total time between harvesting and ensiling, including the period of artificial drying, was about 24h. About 250 g of wilted forage containing a silage additive was ensiled by vacuum packing in polyethylene bags (poly nylon vacuum bags 300 x 400 mm and 20 µm thick) and stored for 2, 4, 10 or 60 days in a dark place at a constant temperature of 18°C before opening the silage and sampling. Samples of about 75 g were taken on the field from the intact parent herbage and will be further referred to as “fresh material”. Samples taken before ensiling will be referred to as “wilted material”. Samples taken immediately after opening of the silage are referred to as “ensiled material”. Samples were vacuum packed and stored at -18°C until further analysis.

Ryegrass was wilted, ensiled and sampled in a similar way: 4- and 60-days silages were made using undamaged ryegrass and molasses (9 g/kg wilted material) for comparative reasons (Figure 2.1).

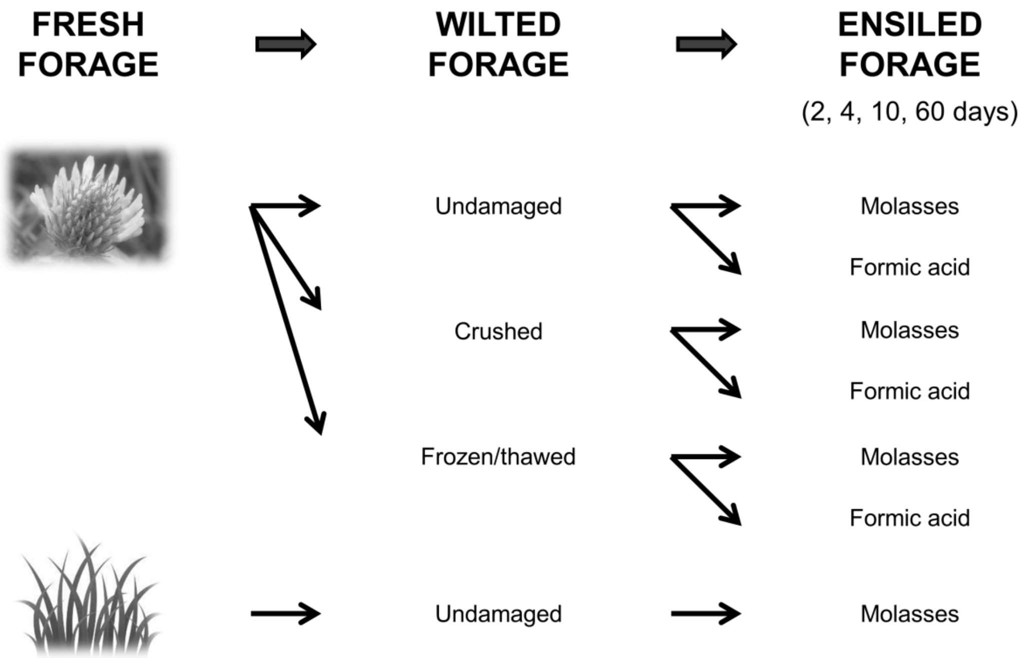


Figure 2.1 Overview experimental set-up

2.3 Incubations

Samples taken for *in vitro* incubation were lyophilized before grinding in a mill with a 1 mm sieve (Brabender Technology, Duisburg, Germany). Ruminal incubations were performed as described by Van Ranst *et al.* (2010). In brief, 250 mg of freeze-dried and ground fresh, wilted, 4-days or 60-days ensiled material was put into 125-ml incubation flasks together with 10 ml buffer solution, 10 ml distilled water and 5ml of rumen fluid. The buffer solution contained 7.16 g/l disodium phosphate dodecahydrate ($Na_2HPO_4.12H_2O$), 3.1 g/l monopotassium phosphate (KH_2PO_4), 0.248 g/l magnesium chloride hexahydrate ($MgCl_2.6H_20$), 17.48 g/l sodium bicarbonate ($NaHCO_3$) and 2 g/l ammonium bicarbonate (NH_4HCO_3). Fievez *et al.* (2007) demonstrated no *in vitro* adaptation period was required and the currently used inoculum/buffer ratio was appropriate to estimate BH of C18:2*n-6* or C18:3*n-3*. Rumen fluid was taken before morning feeding from three fistulated sheep, which were fed hay ad libitum and had free access to drinking water. Fistulation of the sheep was approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO), Belgium (Ethical Commision ILVO 2009, file number 114). Rumen contents from

sheep were combined (pH = 6.44) and filtered through a sieve with a pore size of 1 mm under continuous CO_2 flushing at 39°C. Incubation flasks were flushed with CO_2 before adding 1 ml of ethane (internal gas standard) followed by incubation under intermittent shaking at 39°C for 24h in a batch culture incubator (Edmund Bühler GmbH, Hechingen, Germany). After 24h, flasks were removed from the incubator and placed in ice water. Measurements of pH (Hanna Instruments, Temse, Belgium), gas (Hassim *et al.*, 2010) and volatile fatty acids (VFA; vide infra) were performed as a quality control of the incubations. Five ml of homogenized rumen fluid was taken before and after 24h incubation and freeze-dried before analysis of long-chain FA.

2.4 Analysis

2.4.1 Silage quality

The quality of the ensiled forages was assessed by analyzing VFA, lactic acid and ammonia after opening of the plastic bags.

<u>Volatile fatty acids</u> (acetic acid, propionic acid, butyric acid) were analyzed using a chromatographic method. Silage (5 g wet material) was mixed in 25 ml acidified water (20 ml 10/1 phosphoric acid/formic acid per liter distilled water) using an Ultra-Turrax (9500 rpm, T25, IKA-Labortechnik, Staufen, Germany) equipped with a S25N18 dispersing element (IKA-Labortechnik, Staufen, Germany) and shaken for 2h using a Multi-Tube Vortex (VX-2500, VWR International, Leuven, Belgium). Next, samples were filtered and centrifuged (22000 x g, 30 min, 4°C) before filtering again the remaining supernatants. Samples were analyzed using gas chromatography (HP 7890A, Agilent Technologies, Diegem, Belgium) equipped with a flame ionization detector and a Supelco Nukol capillary column (30 m x 0.25 mm x 0.25 µm, Sigma-Aldrich, Bornem, Belgium). The temperature program was as follows: 120°C at injection for 0.2 min; increased at 10°C/min until 180°C and remained at this temperature for 3 min; injector temperature: 250°C; detector temperature 255°C. For this temperature program, 0.3 µl was injected with a split/splitless ratio of 25:1 using H_2 as carrier

gas at 0.8 ml/min. VFA peaks were identified based on their retention times, compared to external standards. Ruminal VFA were analyzed using the same chromatographic protocol.

Lactic acid concentrations were determined using the remaining supernatants from the VFA analysis through oxidation to acetaldehyde using Conway microdiffusion chambers and spectrophotometry at 224 nm (Conway, 1957).

Ammonia was also determined using the supernatants remaining after preparation for VFA analysis, based on the method described by Chaney and Marbach (1962). In brief, 2 ml of supernatant was taken and acidified using 2 ml 0.2 N HCl. Samples were shaken, centrifuged (1700 x g, 20 min) and 1 ml supernatant collected in an experimental tube. Next, 4.5 ml of a solution, containing 10 g/l phenol (Merck, Darmstadt, Germany) and 0.05 g/l sodium nitroprusside dihydrate ($Na_2(Fe(CN)_5NO)\cdot 2H_2O$; Sigma-Aldrich, Bornem, Belgium), and 4.5 ml of a solution, containing 5 g/l sodium hydroxide (NaOH) and 4.2 ml/l 10 % sodium hypochlorite (NaClO; Sigma-Aldrich, Bornem, Belgium), were added, vortexed and left at room temperature for 1 h. Ammonia was quantified using an external standard (0 to 20 mg/l NH_4Cl, Sigma-Aldrich, Bornem, Belgium) and by measuring the absorbance at 625 nm.

2.4.2 Isolation of lipid fractions and fatty acid quantification

First, lipids from fresh, wilted or ensiled material were extracted. Therefore, 5 g of fresh or wilted material or 3 g of ensiled material was extracted as described by Lourenço *et al.* (2007) using chloroform/methanol (C/M) (2/1, v/v) and 4 ml C19:0 as an internal standard (2.5 mg/ml chloroform, Sigma-Aldrich, Bornem, Belgium) and brought to a final volume of 100 ml chloroform/methanol. Extracts were stored at -18°C until further analysis.

Next, lipid extracts were separated into three lipid fractions by solid phase extraction as described by Van Ranst *et al.* (2010): polar lipids (PL; mainly composed of glycolipids and phospholipids), free fatty acids (FFA) and neutral lipids (NL) (composed of triacylglycerides (TAG), diacylglyceridesand and monoacylglycerides). 0.25 ml TAG-C13:0 (0.25 mg/ml

chloroform, Nu-Chek Prep Inc., Elysia, Minnesota, USA) and 0.1 ml C21:0 (0.5 mg/ml chloroform, Sigma-Aldrich, Bornem, Belgium) were used as internal standard for the NL, FFA and PL fractions, whereby C21:0 was only added to the FFA and PL fraction after extraction and before methylation for quantification.

Finally, the different fractions were methylated and FA quantified using gas chromatography as described by Van Ranst *et al.* (2010). A gas chromatograph (HP 6890, Agilent Technologies, Diegem, Belgium) equipped with a Solgel-wax column (30 m x 0.25 mm x 0.25 µm, SGE Analytical Science, Ringwood, Victoria, Australia) was used. The temperature program was as follows: 150°C for 2 min; increased at 3°C/min until 250°C; injector temperature: 250°C; detector temperature 280°C. For this temperature program, 2 µl was injected using a split/splitless ratio of 50:1 and H_2 as carrier gas at a flow rate of 1 ml/min. FA peaks were identified based on their retention times, compared to external standards (Supelco 37, Supelco Analytical, Pennsylvania, USA; PUFA-3, Matreya LLC, Pleasant Gap, Pennsylvania, USA). Quantification of FA methyl esters was based on the area of the internal standards and on the conversion of peak areas to the weight of FA by a theoretical response factor for each FA (Ackman and Sipos, 1964; Wolff *et al.*, 1995).

2.4.3 Long-chain fatty acids and in vitro ruminal biohydrogenation

The total FA composition of fresh, wilted or ensiled material and ruminal incubation fluid was determined after lyophilization by direct transesterification according to the method described by Gadeyne *et al.* (2015) (see also section 2.5 of Chapter 4). An aliquot of 250 mg of lyophilized plant material or 5 ml of rumen fluid sampled before or after 24h incubation was used together with 2 ml C13:0 (1 mg per ml toluene, Sigma-Aldrich, Bornem, Belgium) as internal standard to quantify FA. Gas chromatography equipment and conditions were the same as described for the lipid fractions (vide supra). *In vitro* ruminal BH of C18:3*n-3* was calculated as [(proportion of C18:3*n-3* in total C_{18} $FA)_{0\,h}$ – (proportion of C18:3*n-3* in total C_{18} $FA)_{24h}$] / (proportion of C18:3*n-3* in total C_{18} $FA)_{0\,h}$, assuming no net synthesis of C_{18} fatty

acids in the rumen. BH of C18:2*n-6* was calculated similarly.

2.5 Statistics

All results were analyzed using the MIXED procedure of SAS (SAS Enterprise Guide 6, SAS Institute Inc., Cary, North Carolina, USA). Perennial ryegrass (undamaged material using molasses) was compared with red clover (frozen/thawed material using molasses) using a two-sample T-test (TTEST procedure).

Characteristics of differently treated wilted red clover were compared using the following model: $Y_i = \mu + T_i + \varepsilon$, where Y_i is the response, T_i the fixed effect of treatment (i = undamaged, crushed or frozen/thawed) and ε the residual error.

Treatments imposed on red clover silages were compared using the following model: $Y_{ijk} = \mu + S_i + A_j + T_k + S_i \times A_j + S_i \times T_k + A_j \times T_k + S_i \times A_j \times T_k + \varepsilon$, where Y_{ijk} is the response, S_i the fixed effect of ensiling period (i = 2, 4, 10 or 60 days), A_j the fixed effect of additive (j = molasses or formic acid), T_k the fixed effect of treatment (k = undamaged, crushed or frozen/thawed) and ε the residual error. Ensiling period (S_i) was considered as a repeated measurement and partitioned into linear and quadratic contrasts for both the main and interaction effects, unless ensiling period contained only two factors (i = 4 or 60 days; results of *in vitro* rumen incubations). Linear rate analysis for the loss of lipids from the PL fraction was done using the REG procedure.

The following model was used to compare treatments imposed on perennial ryegrass: $Y_i = \mu + S_i + \varepsilon$, where Y_i is the response, S_i the fixed effect of stage (i = fresh, wilted, 4- or 60-days silage) and ε the residual error.

Results for red clover total FA distributions and BH were analyzed using the following model: $Y_i = \mu + S_i + \varepsilon$, where Y_i is the response, S_i the fixed effect of stage (i = fresh, wilted, 2-, 4-, 10- or 60-days silage) and ε the residual error.

Differences were significant at $P < 0.05$, while tendencies were assigned at $P < 0.10$. Differences among least squares means were evaluated using Tukey's multiple comparison test.

Finally, relationships between varying PL levels, imposed by the different treatments, and the level of C18:3*n-3* BH were evaluated by linear regression analysis using the REG procedure of SAS.

3 Results

3.1 Silage quality

As one of the objectives was to assess the influence of *in silo* microbial activity, formic acid was added as a silage additive to prevent *in silo* microbial development. To check whether silages of satisfactory quality and variation in silage microbial activity was obtained with the two silage additives, end products of microbial activity during ensiling (lactic acid, NH_3-N, acetic acid and butyric acid) were measured. Results for red clover and perennial ryegrass silages are presented in **Fout! Verwijzingsbron niet gevonden.** and Table 2.2, respectively.

Lactic acid was detected in both red clover and perennial ryegrass silages. Lactic acid concentration in red clover silages increased linearly over time with molasses as silage additive, in contrast to formic acid where a much smaller increase over time was observed. When molasses was used, greater lactic acid concentrations were found in the more severely damaged material compared to the undamaged counterparts, in contrast to formic acid where only minor or no consistent effects of damage were observed.

NH_3-N concentrations in red clover and perennial ryegrass silages were generally low. Short-term red clover silages (2 and 4 days) with frozen/thawed forage and molasses contained much greater concentrations of NH_3-N compared to the undamaged and crushed silages, in contrast to the longer ensiled counterparts.

Table 2.1 Silage parameters (lactic acid, NH_3-N, acetic acid and butyric acid) of red clover which was undamaged, crushed or frozen/thawed before wilting and ensiled with molasses or formic acid as additive for 2, 4, 10 or 60 days (n=3)

Stage	Treatment	Lactic acid (mg/g DM)		NH_3-N (µg/g DM)		Acetic acid (mg/g DM)		Butyric acid (µg/g DM)	
		MOL	FOR	MOL	FOR	MOL	FOR	MOL	FOR
2-days silage	ND	18.0	12.0	0.100	3.18	6.73	3.21	13.8	10.6
	CR	15.9	11.0	0.070	7.03	5.38	3.62	14.8	0.000
	FT	29.5	10.4	61.4	0.187	7.32	3.16	36.2	0.000
4-days silage	ND	37.5	12.4	2.33	0.062	9.25	3.75	132	118
	CR	34.8	11.8	1.84	0.044	6.74	3.40	5.61	86.8
	FT	46.7	10.7	20.3	0.138	11.5	3.73	0.000	24.7
10-days silage	ND	48.6	12.2	0.120	0.114	12.7	4.01	20.1	0.000
	CR	44.5	16.1	0.103	0.074	10.8	3.64	18.3	0.000
	FT	49.6	14.5	0.314	0.223	13.0	4.61	59.1	22.5
60-days silage	ND	59.8	31.1	0.124	0.117	12.9	4.48	47.6	22.5
	CR	75.5	34.8	0.104	0.086	13.2	4.26	15.9	6.30
	FT	130	38.6	0.237	0.149	15.0	4.18	0.000	26.7
SEM (df = 48)		4.41		4.4703		0.559		35.873	
P-value									
Stage (LC)		<0.001		<0.001		<0.001		0.701	
Stage (QC)		<0.001		0.005		0.073		0.128	
Additive		<0.001		0.018		<0.001		0.783	
Treatment		<0.001		0.008		0.003		0.229	
Additive × Stage (LC)		<0.001		0.002		<0.001		0.923	
Additive × Stage (QC)		0.764		0.077		0.116		0.664	
Treatment × Stage (LC)		0.006		<0.001		0.808		0.879	
Treatment × Stage (QC)		0.002		0.008		0.011		0.649	
Additive × Treatment		0.001		0.004		0.008		0.746	
Additive × Treatment × Stage (LC)		0.047		<0.001		0.416		0.626	
Additive × Treatment × Stage (QC)		0.007		0.001		0.298		0.776	

DM, dry material; MOL, molasses; FOR, formic acid; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom; LC, linear contrast; QC, quadratic contrast

Table 2.2 Silage parameters (lactic acid, NH3-N, acetic acid and butyric acid) of perennial ryegrass which was undamaged and ensiled with molasses as additive for 4 or 60 days (n=3)

Stage	Lactic acid (mg/g DM)	NH_3-N (µg/g DM)	Acetic acid (mg/g DM)	Butyric acid (µg/g DM)
4-days silage	51.4	0.139	6.17	36.0
60-days silage	69.0	0.133	11.2	43.8
SEM (df = 4)	2.34	0.0074	1.046	25.53
P-value				
Stage	0.006	0.570	0.027	0.839

DM, dry material; SEM, standard error of the mean; df, degrees of freedom

Higher acetic acid concentrations were observed with increased ensiling duration when molasses was used, both for red clover as well as perennial ryegrass. Red clover silages with molasses showed higher acetic acid concentrations than silages with formic acid. Highest butyric acid concentrations were observed for non-damaged 4-days silages.

However, butyric acid concentrations were generally low in all silages and not affected by ensiling duration, additive or treatment.

3.2 Lipid fractions in fresh, wilted and ensiled forage

Total lipids in forage extracts were fractionated before methylation into three classes: PL, FFA and NL. The distribution of total lipids into these lipid fractions in fresh, wilted and ensiled red clover and perennial ryegrass is presented in Figure 2.2. The total amount of FA and the relative composition of the major FA C16:0, C18:0, C18:1*n-9*, C18:2*n-6* and C18:3*n-3* in fresh, wilted and ensiled red clover or perennial ryegrass are reported in Addendum A.1, Addendum A.2 and Addendum A.3. Numerical values and results of statistical analysis for data shown in Figure 2.2 are reported in Addendum A.4, Addendum A.5 and Addendum A.6.

Most lipids in fresh red clover were present as PL (80.9 g/100g FA), while only minor proportions were present as FFA (2.90 g/100g FA) or NL (16.2 g/100g FA) (Figure 2.2). The proportion of PL dropped markedly while FFA and NL increased during wilting of red clover. There was a tendency for less PL when wilted material was more severely damaged ($P=0.079$). As a result, lipolysis during wilting, i.e. the disappearance of FA from the PL fraction (as compared with the PL fraction of the fresh forage), varied on average between 22.5 and 52.7 g/100g FA for the undamaged and frozen/thawed wilted red clover, respectively. The PL fraction decreased further linearly upon ensiling. The longer red clover was ensiled, the lesser the PL fraction ($P<0.001$; linear contrast) and the higher the FFA fraction ($P<0.001$; linear contrast). Compared with the wilted material, the greatest changes from esterified to non-esterified FA were generally observed upon ensiling. Also NL increased upon wilting ($P<0.001$). Remarkably, a plateau or maximum value seemed to be reached for NL from the wilting phase onwards, as this fraction was unchanged during the later silage stages irrespective of the degree of damage and both for molasses and formic acid supplemented forages. The FFA fraction continued to increase until 60 days of ensiling. Despite high variation within type of damage (treatment) for both additives (Addendum A.5),

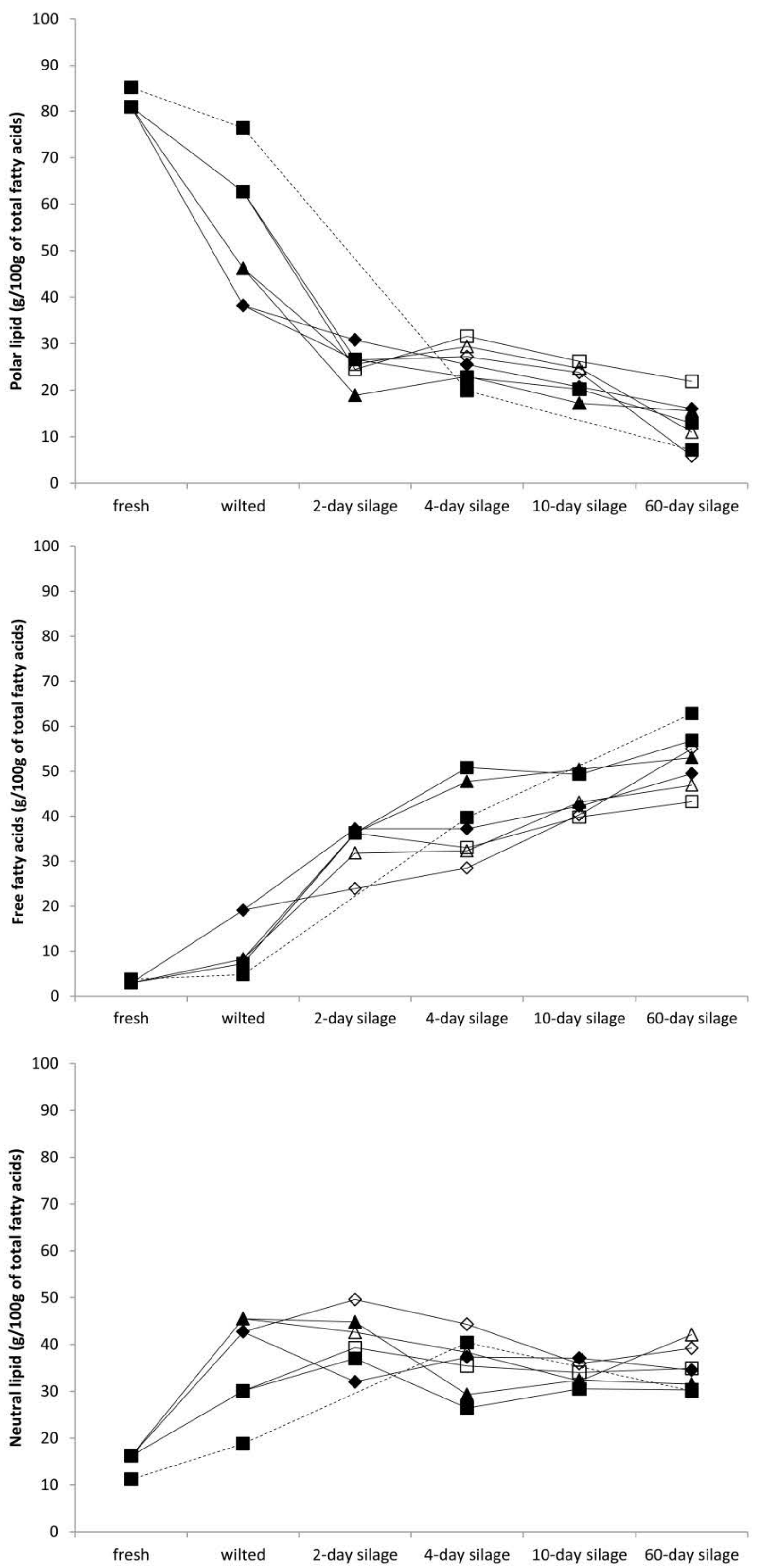


Figure 2.2 Distribution of total fatty acids in the polar lipid, free fatty acid or neutral lipid fraction (g/100g total fatty acids) of fresh, wilted and ensiled (2, 4, 10 or 60 days) red clover (data points connected by full lines) or perennial ryegrass (data points connected by dotted lines) which was undamaged (squares), crushed (triangles) or frozen/thawed (diamonds) before wilting and ensiled with molasses (full symbols) or formic acid (empty symbols) as additive (n=3)

a tendency towards lower PL (P=0.058) and higher FFA (P<0.001) proportions were found in red clover silages with molasses as compared with formic acid, but a significant interaction was found between the quadratic contrast of silage duration and additive (P=0.008 and P=0.043 for PL and FFA, respectively). Polar lipids were released at a slower rate in silages with molasses (-0.157 g/100g FA/day; P<0.001) than in silages with formic acid (-0.250 g/100g FA/day; P<0.001), whereby the PL fraction for formic acid silages reached the smallest level after 60 days of ensiling (only 5.83 g/100g FA remained in the PL fraction of frozen/thawed red clover silages). FFA did not increase in formic acid-supplemented silages when ensiled shortly (2 and 4 days; P>0.999), as opposed to silages with molasses (P=0.005). However, after a longer ensiling period, FFA levels in silages with both additives were similar (P=0.097 and P=0.369 for 10 and 60 days, respectively).

As for red clover, most lipids in perennial ryegrass were present as PL (85.1 g/100g FA) and only minor proportions as FFA (3.75 g/100g FA) or NL (11.2 g/100g FA). Lipids in the PL fraction of ryegrass gradually turned into FFA and NL over the wilting and ensiling stages. The sharpest decrease in PL was observed between the wilted forage and the 4-days ensiled material (P<0.001). Between 4 and 60 days of ensiling (P=0.001), NL lipids in ryegrass silages seemed to decrease in favor of FFA. Lipid distributions in perennial ryegrass and red clover were similar for the 4-days silages using molasses (frozen/thawed forage; P=0.222, P=0.668 and P=0.516 for PL, FFA and NL, respectively). However, PL was lower and FFA higher for red clover compared to ryegrass for the wilted material (frozen/thawed; P<0.001 and P=0.003 for PL and FFA respectively), but the opposite was found for the 60-days ensiled material (frozen/thawed; P<0.001 and P=0.007 for PL and FFA respectively). Higher levels of NL were observed for wilted red clover compared to grass (P<0.001), but no differences were found between the 60-days silages (P=0.111). Overall, disappearance of FA from the PL fraction was faster in red clover as compared to ryegrass as only limited decreases were observed from ryegrass PL during wilting. The opposite was

true for ensiled material where PL reached lower levels in ryegrass as compared with red clover.

3.3 In vitro incubation of fresh, wilted and ensiled forage

Treatments as described in the previous section resulted in different proportions of FA in the PL and FFA fraction. FA in the esterified PL and NL fraction need to be hydrolyzed prior to ruminal BH. Hence, differences in lipolysis could result in differences in BH by microbial hydrogenases in the rumen. Therefore, fresh, wilted, 4- and 60-days ensiled red clover and perennial ryegrass were subjected to 24h *in vitro* rumen incubations. Results for BH of C18:2*n-6* and C18:3*n-3* (the major unsaturated FA in the forages) in fresh, wilted and ensiled red clover and perennial ryegrass are shown in Figure 2.3. Numerical values and results of statistical analysis for data shown in Figure 2.3 are reported in Addendum A.4, Addendum A.6 and Addendum A.7. To monitor the quality of the incubations, both VFA (acetic acid, propionic and butyric acid) and gas (H_2 and CH_4) produced by the micro-organisms from the forages were measured after 24h. In this way, it was possible to exclude that differences in BH would have been caused by major changes in microbial activity (indicated by the total VFA production) or shifts in the microbial population (indicated by changes in VFA or CH_4 proportions). Fermentation characteristics for fresh, wilted and ensiled red clover or perennial ryegrass are presented in Addendum A.8, Addendum A.9 and Addendum A.10. Overall, differences in fermentation characteristics between treatments for both red clover and perennial ryegrass were small.

Fresh red clover showed BH levels of 0.875 and 0.802 for C18:2*n-6* and C18:3*n-3* respectively (Figure 2.3). No differences in BH of C18:2*n-6* were found between damage-levels of the wilted material ($P=0.247$), while BH of C18:3*n-3* in frozen/thawed red clover was lower as compared with the less damaged treatments (undamaged = crushed > frozen/thawed; $P=0.002$). BH of both PUFA was more extensive for red clover material which had been ensiled for a longer period ($P<0.001$), although differences were largest for

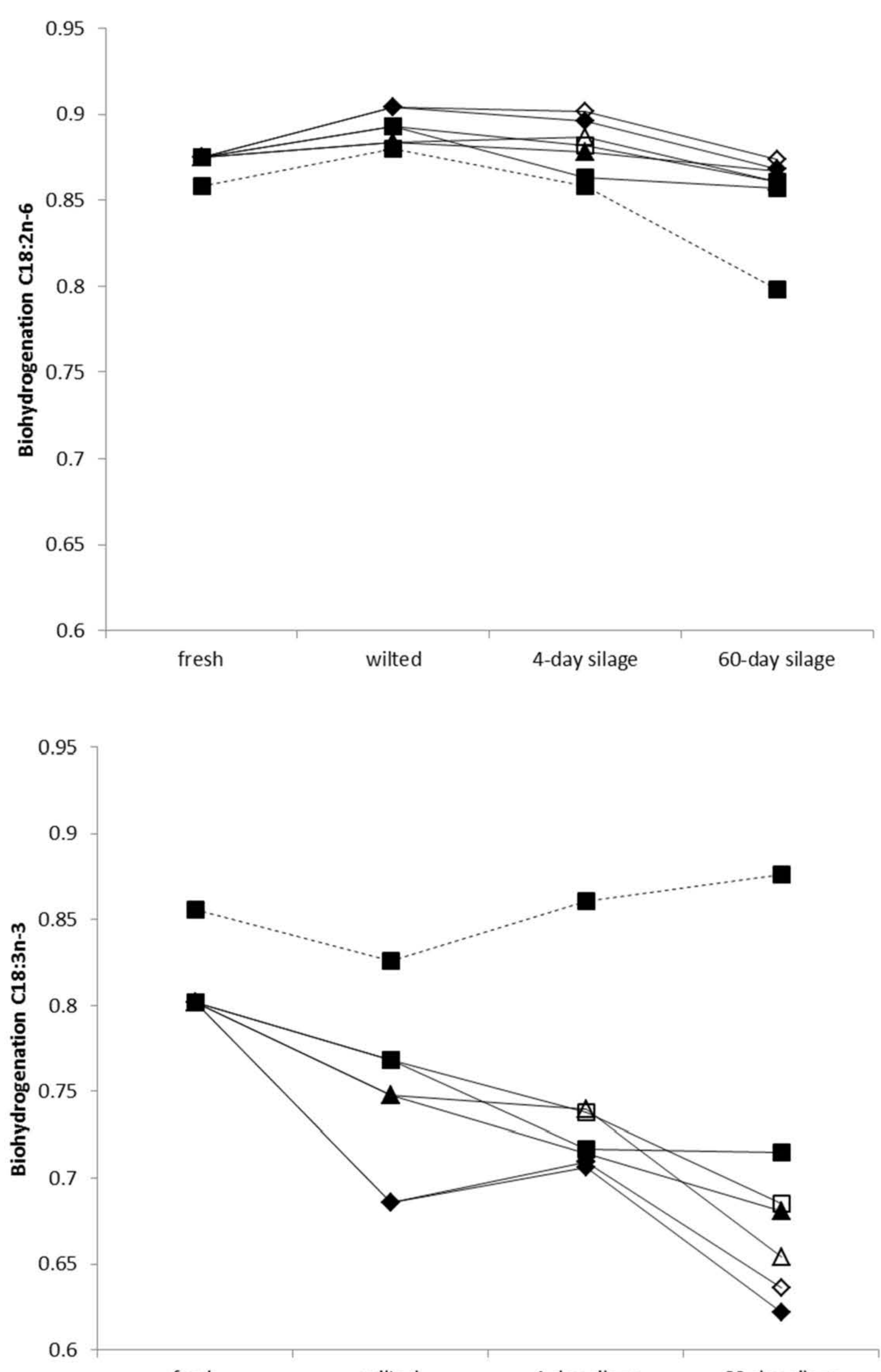


Figure 2.3 Biohydrogenation of C18:2*n-6* and C18:3*n-3* after 24 *in vitro* rumen incubation of fresh, wilted and ensiled (4 or 60 days) red clover (data points connected by full lines) or perennial ryegrass (data points connected by dotted lines) which was undamaged (squares), crushed (triangles) or frozen/thawed (diamonds) before wilting and ensiled with molasses (full symbols) or formic acid (empty symbols) as additive (n=3)

C18:3*n-3*. There was no effect of silage additive on BH (P=0.101 and P=0.855 for C18:2*n-6* and C18:3*n-3* BH, respectively). Damaging affected C18:2*n-6* and C18:3*n-3* BH oppositely: while BH of C18:2*n-6* gradually increased when red clover was damaged more (undamaged = crushed < frozen/thawed; P<0.05), a decrease in C18:3*n-3* BH was observed upon increased damage (undamaged > crushed > frozen/thawed; P<0.05). In contrast to C18:2*n-6* (P=0.325), interaction effects were found for C18:3*n-3* (P=0.003): BH of 4-days red clover silages and undamaged 60-days silages did not differ, but BH of C18:3*n*-3 in 60-days ensiled red clover gradually decreased upon damage (undamaged > crushed > frozen/thawed). No or only small differences occurred between fresh, wilted, 4- and 60-days ensiled red clover of C18:2*n-6* BH, whereas C18:3*n-3* BH was lowest in 60-days silages containing red clover which had been most severely damaged before wilting (freezing and thawing). A reduction in BH up to 18 % for frozen/thawed silages with molasses as compared with the fresh forage was observed.

On the other hand, fresh perennial ryegrass showed BH levels of 0.858 and 0.856 for C18:2*n-6* and C18:3*n-3*, respectively. No decrease in BH of C18:3*n-3* upon wilting and ensiling of fresh perennial ryegrass was observed (P=0.061), while only a slight decrease in BH of C18:2*n-6* was seen after 60-days of ensiling (P=0.001). As a result, frozen-thawed red clover using molasses resulted in lower levels of BH for C18:3*n-3* as compared to perennial ryegrass (P=0.002, P=0.002 and P<0.001 for wilted, 4- and 60-days silages, respectively). The opposite was true for BH of C18:2*n-6* for 4- and 60-days ensiled forage (P=0.034 and P<0.001, respectively), while similar levels were found for BH of C18:2*n-6* in both wilted forages (P=0.153).

4 Discussion

As forages are often wilted and ensiled for preservation during storage, their FA gradually disappear from the PL fraction, leading to an increase of FFA (Dewhurst *et al.*, 2003; Van Ranst *et al.*, 2010; Van Ranst *et al.*, 2013). Results from this study confirm the gradual

turnover of FA originating from the PL fraction to FFA and NL upon conservation, both in red clover and perennial ryegrass. However, it remained unclear whether this disappearance of FA from the PL fraction is primarily caused by plant enzymes, liberated during harvesting, wilting or ensiling, or if there is any contribution in this turnover of enzymes from microbes occurring during ensiling. Second, the unexpected observation by Van Ranst *et al.* (2010), indicating a decreased rumen microbial BH of linolenic acid upon longer ensiling of red clover when smaller amounts of PL remained, deserved further investigation. Both aspects will be discussed in the next sections, followed by a paragraph to support the understanding of which mechanism is more likely to explain red clover's increased flow of PUFA across the rumen, based on the current results.

4.1 Contribution of microbial development during ensiling on lipid metabolism

Previous research has mainly focused on plant enzymes being responsible for FA conversions during wilting and ensiling (Ding *et al.*, 2013; Lee *et al.*, 2004; Van Ranst *et al.*, 2009c). One of the most characteristic features of membrane deterioration in stressed plants is the progressive decline in phospholipid and galactolipid levels, accompanied by relative enrichment of FFA and NL by stress-induced degradation of membrane lipids, e.g. by wounding of the plant material, by enzymes such as galactolipase (Kaniuga, 2008). However, the contribution of microbes could not be excluded. Therefore, it was evaluated whether microbial activity could play a role during ensiling in this turnover of FA from PL, by comparing silages with molasses or formic acid, assuming the former to stimulate microbial activity (in particular Lactobacilli) *in silo* (Lattemae *et al.*, 1996), while formic acid dropped pH in the latter silages to such an extent that fermentation was restricted (Dewhurst and King, 1998; Lattemae *et al.*, 1996). Progressively increasing lactic acid concentrations were found upon longer ensiling using molasses as an additive, while low levels were observed for silages containing formic acid. Further, short-term silages with frozen/thawed forage and molasses contained high concentrations of NH_3-N, suggesting more extensive proteolysis,

but the disappearance in the long term silages could indicate NH_3-N was then used for microbial growth. Microbial lipases, which become more abundant with increasing microbial activity (Van Ranst *et al.*, 2010), were hypothesized to enhance lipolysis *in silo*. Lipolytic activity by Lactobacilli has been shown before, although lipase activities of extracts obtained from lactic acid bacteria were substantially lower than those reported for other micro-organisms (Meyers *et al.*, 1996). However, no consistent results over the different silage additives were found, suggesting microbial lipases *in silo* did not contribute to a large extent in PL disappearance. Accordingly, the current results confirm observations by Ding *et al.* (2013), suggesting plant enzymes play the most prominent role in silage lipolysis.

4.2 Effect of variation in forage polar lipid levels on in vitro ruminal biohydrogenation

In order to study the effect of varying levels of PL on *in vitro* ruminal BH, an experimental set-up similar to the one described by Van Ranst *et al.* (2010) was used. Wilting and ensiling over different time intervals of red clover forage, damaged to varying extent, allowed to create substantial variation in proportions of FA remaining in the PL fraction, while the use of molasses and formic acid as silage additive ensured sufficient silage quality. The following subsections will focus on the variation in PL and BH, imposed by the different treatments, whereafter the link between both is discussed.

4.2.1 Variation in fatty acids disappearing from the polar lipid fraction in red clover and perennial ryegrass

It has been suggested before that activation of PPO in red clover, by damaging the crop, could lead to protection against disappearance of FA from the PL fraction (Lee *et al.*, 2009). High PPO activity has been reported in different red clover cultivars (Van Ranst *et al.*, 2009b). Activation of PPO is needed due to the separate subcellular compartmentalization of the enzyme, residing in the chloroplast, and its diphenolic substrates, which are presumably present in the vacuole (Lee *et al.*, 2010). It is known that higher PPO activity leads to higher

production of PBP and decreased lipase activity (Van Ranst *et al.*, 2009c; Lee *et al.*, 2004). For the current experimental set-up, it was assumed that exposing red clover to liquid nitrogen, followed by immediate thawing, leads to a larger degree of PPO activation than crushing the forage or leaving it unharmed (Lee *et al.*, 2009). Van Ranst *et al.* (2009c) also found the highest induction of PPO activity for frozen/thawed red clover in comparison with undamaged material. However, results from the latter as well as the current study were inconclusive concerning the disappearance of FA from the PL fraction when ensiled red clover was more severely damaged. Nevertheless, both in the current as well as the study of Van Ranst *et al.* (2010), fermentation progressed during ensiling of red clover and differences in PL proportions were found compared to the original forage, resulting in similar and relatively small levels of FA remaining in the PL fraction after wilting or ensiling, irrespective of the level of damage. These observations suggest there was no effect of damage, and hence PPO activation, on the disappearance of FA from the PL fraction. The fact that the degree of damage didn't result in the expected variation in protection might be related to the suicidal inactivation properties of PPO (Munoz-Munoz *et al.*, 2010), which may limit the extent of PPO oxidation, protein-bound phenol production and ultimately FA protection (Lee *et al.*, 2013). Furthermore, lower PL proportions were observed in silages with molasses than with formic acid, similar to the findings of Koivunen *et al.* (2015), which suggested formic acid addition during ensiling of red clover reduced *in silo* lipolysis. Notably, the lowest PL levels were observed for frozen/thawed red clover ensiled for 60-days with formic acid, but differences with other treatments were not significant (Addendum A.5).

Besides different levels of damage imposed on red clover forage, treatments with undamaged perennial ryegrass were included for comparison. PPO in ryegrass and red clover are not the same, as they show different PPO activities (Winters *et al.*, 2003) and have varying affinity for different substrates (Parveen *et al.*, 2010). Still, comparisons between both were made, in which ryegrass was considered as control, due to its low PPO activity compared with red clover. As a result, both forages were expected to have different abilities

to create quinones, PBP and presumed protection levels. Therefore, statistical comparisons were made between undamaged ryegrass and frozen/thawed red clover, as these were considered to be the most extremes. The pattern of PL disappearance in grass and red clover differed. Although a faster turnover in red clover was found during the wilting phase, substantial disappearance from the PL fraction of ryegrass only started upon ensiling. These differences might be explained by an unintentional more severe cell damage in red clover or might be related to different release patterns of lipases and diacylglycerol transferase (DGAT) induced by wilting and damage stress. Indeed, DGAT liberation might explain the increases in NL (Kaup *et al.*, 2002). Finally, most ryegrass PL disappeared in favor of FFA and NL after ensiling for a longer period, whereas PL levels in red clover (12.9 to 16.0 g/100g FA) remained slightly higher ($P<0.001$) than in their ryegrass counterparts (7.1 g/100g FA). This effect was also observed recently by Koivunen *et al.* (2015), showing lower lipolysis in silages of red clover ensiled with formic acid than a mixture of timothy (*Phleum pretense* L.) and meadow fescue (*Festuca pratensis* Huds.) grasses.

4.2.2 Variation in biohydrogenation in red clover and perennial ryegrass

Esterified forage FA are largely transformed to FFA by microbial lipases, galactosidases or phospholipases produced in the rumen (Jenkins, 1993), followed by a rapid hydrogenation of unsaturated FFA by ruminal microbes (Buccioni *et al.*, 2012). In the current study, lower *in vitro* C18:3*n-3* BH was observed when wilted or ensiled red clover was damaged to a higher extent. These results are in contrast with the findings of Van Ranst *et al.* (2010), where a reduction in BH was observed upon wilting and ensiling of fresh red clover, irrespective of damage. As it was hypothesized before, FA in damaged wilted red clover seemed to be protected better against ruminal hydrogenases, most probably by stimulation of PPO and the presumed consequent formation of PBP (Lee *et al.*, 2010). Although PBP as such were not measured, a severe browning reaction in the frozen/thawed forage was observed, which is an indication of quinone formation and polymerization (Lee, 2014). In contrast to red clover, no differences were found in BH of C18:3*n*-3 for perennial ryegrass between the different

wilted and ensiled stages. Differences in C18:3*n*-3 BH between both forages became larger as red clover was wilted and ensiled, possibly because PBP complexes in frozen/thawed wilted and ensiled PPO-rich red clover could develop better than in undamaged and PPO-poor ryegrass. Van Ranst *et al.* (2010) also showed a decrease in BH was achieved by intensively damaging red clover, but only after 60 days of ensiling, which is suggested to be due to high PPO activity. However, one would not expect quinones to be formed in the absence of oxygen in good quality silages. Possibly, small levels of oxygen were present or leaked into the poly-ethylene bags during ensiling. This could explain why prolonged ensiling of red clover, in contrast to perennial ryegrass, resulted in better protection: possibly small but adequate levels of oxygen in the labscale silages were enough to result in the necessary quinone formation to obtain protection. Prior damage was an essential pre-treatment to activate PPO during the wilting stage, while further contact between PPO and the diphenolic substrate during the ensiling stage might have ameliorated protection.

BH of C18:2*n-6* in red clover, however, didn't follow the trend of C18:3*n-3* BH. Unexpectedly, BH of C18:3*n-3* was lower than for C18:2*n-6* in fresh red clover, although BH for both FA still fell within the ranges of the meta-analysis based on *in vivo* data by Glasser *et al.* (2008). Differences between C18:2*n*-6 and C18:3*n-3* BH after the various treatments of the fresh herbage might be related to the different physical location of both FA: C18:3*n-3* is mainly found in chloroplast membranes, where PPO is also present (Lee *et al.*, 2010), while C18:2*n-6* is found in many plant cell organelles (Hawke, 1973).

4.2.3 Association between proportions of polar lipids and linolenic acid biohydrogenation in red clover and perennial ryegrass

Lipolysis of esterified FA by either plant or microbial lipases is a prerequisite before BH can take place in the rumen (Buccioni *et al.*, 2012; Lourenço *et al.*, 2010). Also in the current study, most FA of the original PL fraction of the fresh forage gradually hydrolyzed upon wilting and prolonged ensiling. Generally, it could be assumed that BH of FA in the rumen of

animals fed with wilted or ensiled forage would be higher, as more FA are already present in a non-esterified form, facilitating the BH of these FA. The relation between FA remaining in the PL fraction of red clover and perennial ryegrass forage and *in vitro* rumen C18:3*n-3* BH is shown in Figure 2.4, combining all different red clover and ryegrass treatments of the current study. It is clearly shown that reduced proportions of FA in the PL fraction were associated with reduced BH of C18:3*n-3*, an effect which was observed before in red clover (Van Ranst *et al.*, 2010). Previous studies with timothy (*Phleum pratense* L.) (Boufaïed *et al.*, 2003b) also showed a decreased *in vitro* rate of C18:3*n-3* BH upon wilting. However, this effect wasn't observed for perennial ryegrass, where C18:3*n-3* BH was relatively constant and unaffected by the proportion of PL remaining in the forage. Apparently, red clover FA seemed to be protected against ruminal hydrogenation to some extent, but not against disappearance from the PL fraction. These findings are in contrast with the results from Halmemies-Beauchet-

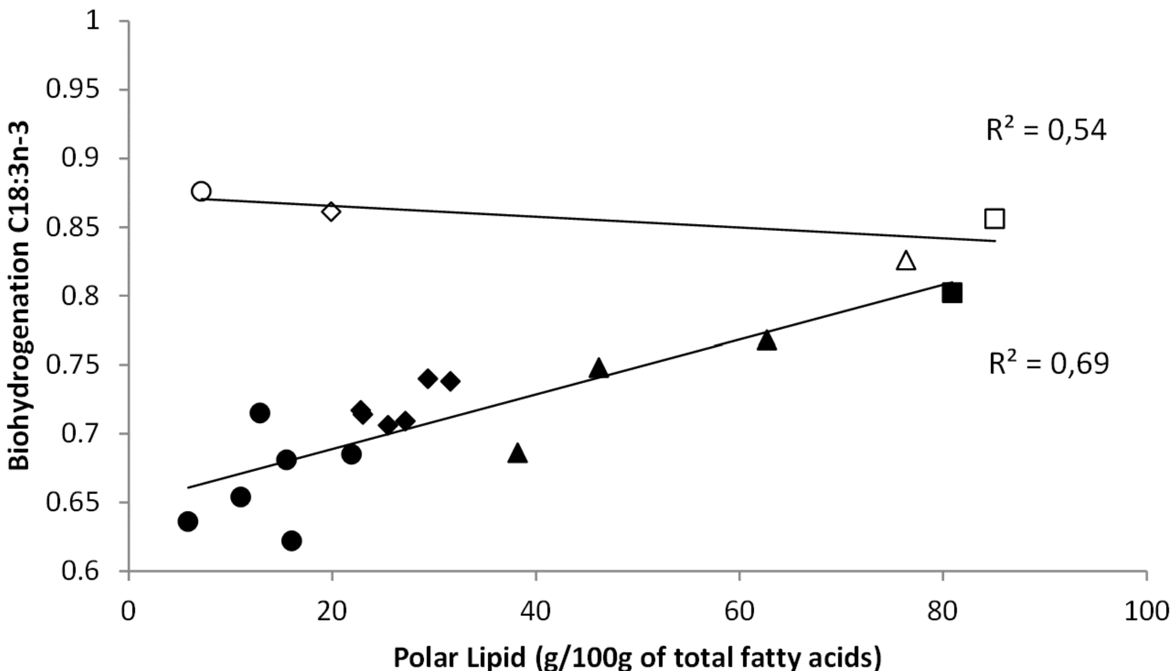


Figure 2.4 Effect of increasing proportions of total fatty acids in the polar lipid fraction on biohydrogenation of linolenic acid after 24h *in vitro* rumen incubation of red clover (full data points; undamaged, crushed or frozen/thawed before wilting and ensiled with molasses or formic acid; n=3) and perennial ryegrass (empty data points; undamaged and ensiled with molasses; n=3). Variation in polar lipid proportions was imposed by combining fresh (squares), wilted (triangles) and ensiled (4 days, diamonds; 60days, circles) treatments

Filleau *et al.* (2013), which suggested increased PUFA leaving the rumen arises principally from dietary lipid escaping lipolysis rather than escaping rumen BH. It may be possible that even though PL is reduced by lipolytic activity, PPO-induced complexing by PBP formation still progressed after the wilting phase, resulting in decreased BH, as Lee *et al.* (2013) reported the continuation of oxidation by non-enzymatic processes in the extended wilt, showing the importance of o-diphenolic substrate concentration besides PPO activity to protect forage C18:3*n-3* against rumen BH.

4.3 Evidence for mechanisms of red clover PUFA protection across the rumen

Recently, Lee (2014) summarized the potential mechanisms explaining red clover's increased flow of PUFA across the rumen: first, deactivation of plant or microbial lipases by quinone binding; second, quinone binding to PL, reducing lipolysis; third, changes in microbial (biohydrogenating) ecology; fourth, altered digestion kinetics with increased flow rate of red clover lipids through the rumen and so reduced microbial processing; fifth, entrapment of thylakoid lipid within PBP reducing access to microbial lipases. The last two mechanisms has been postulated as the most probable ones. However, alterations in rumen digestion kinetics or forage particle size distribution to explain differences in *in vivo* PUFA escape from the rumen (Halmemies-Beauchet-Filleau *et al.*, 2013) could not have played a role in the current *in vitro* set-up. Additionally, disappearance of lipid from the PL fraction still progressed upon ensiling, which make direct lipase inhibition by PPO-created quinones or quinone-lipid binding (Van Ranst *et al.*, 2011) unlikely to be the main reasons of protection of FA against BH. The possibility of altered biohydrogenating communities by incubating red clover or perennial ryegrass (Huws *et al.*, 2010) couldn't be ruled out with the current set-up. Finally, also the encapsulation hypothesis as a consequence of PBP formation induced by PPO still stands. Differences observed in this study between C18:2*n-6* and C18:3*n*-3 BH, related to the different physical location of both FA, were used before to suggest that

protection of PUFA against ruminal BH most likely is obtained by thylakoid lipid entrapment within PBP matrices (Lee *et al.*, 2010; Van Ranst *et al.*, 2011).

5 Conclusions

Both in red clover and ryegrass and irrespective of the degree of damage, a gradual decrease of FA in the PL fraction upon conservation was found. In red clover, PL losses started from the wilting phase, while substantial PL disappearance from ryegrass only started upon ensiling. Proportions of PUFA remaining in the PL fraction after wilting and ensiling of red clover were positively correlated with PUFA BH, while this wasn't the case for ryegrass. Red clover PUFA seemed to be partially protected against ruminal BH, while disappearance of FA from the PL fraction did not seem to be hampered. Further, microbial lipases in silages did not enhance FA disappearance from the PL fraction. Finally, the encapsulation mechanism as a consequence of PBP formation induced by PPO still seems the be most probable hypothesis to explain red clover's increased flow of PUFA across the rumen.

However, reductions in ruminal BH of red clover PUFA because of this PPO-based protection remain limited, which is illustrated by the rather small reductions in BH after 24h *in vitro* incubation in rumen fluid of high PPO red clover in this chapter and previous studies (Van Ranst *et al.*, 2011). Moreover, only the protection against ruminal BH of red clover lipids was addressed. Protection also did not seem to be transferable to lipids of co-ensiled forages with low PPO-activity, e.g. grasses (Van Ranst *et al.*, 2013). As red clover is not a popular fodder and has a low fat content, transfer of this natural mechanism to protect PUFA-rich sources such as linseed or fish oil against ruminal BH is of interest. Therefore, the objective of the remainder of this thesis is to assess the protection of emulsified fatty acids as a new means to protect non-roughage PUFA against BH through the action of PPO.

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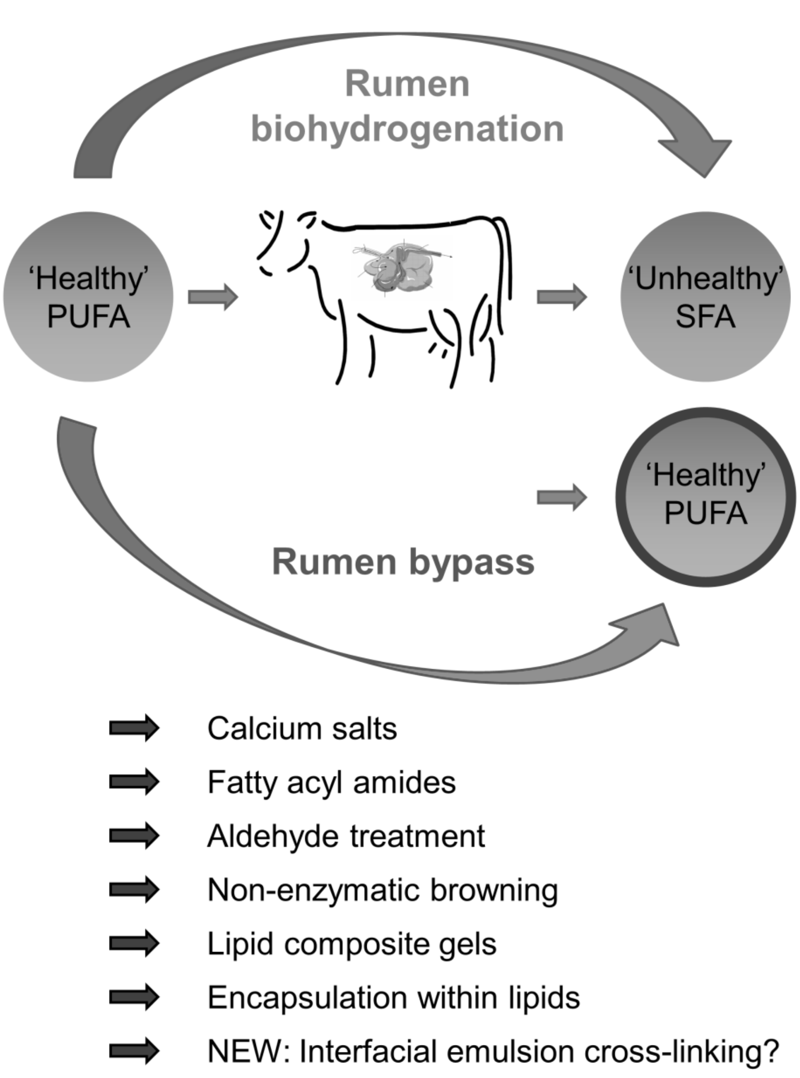
PART II

PROTECTION OF EMULSIFIED FATTY ACIDS

Chapter 3

Introduction part II - State of the art in rumen lipid protection technologies and emerging interfacial protein cross-linking methods



Redrafted after Gadeyne F., De Neve N., Vlaeminck B. and Fievez V., 2016, State of the art in rumen lipid protection technologies and emerging interfacial protein cross-linking methods, European Journal of Lipid Science and Technology, 118, *[published online]*.

Chapter 3

Introduction part II - State of the art in rumen lipid protection technologies and emerging interfacial protein cross-linking methods

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Abstract. Polyunsaturated fatty acids (PUFA) given to ruminants are to a large extent hydrogenated to more saturated forms by microbial metabolism. Numerous protection technologies have been developed to overcome this hydrogenation process in order to increase the amount of unsaturated fatty acids bypassing the rumen and resulting in an effective transfer to the peripheral tissues. This review gives an overview of the current state of the art in rumen lipid bypass technologies, with the focus on both patent-described protection mechanisms, possible advantages or drawbacks of the technologies and protection results being described in recent scientific literature. Lipid bypass techniques which are dealt with include calcium salts, fatty acyl amides, aldehyde treatment, non-enzymatic browning, lipid composite gels and encapsulation within lipids. Further, the potential of a novel rumen lipid protection technology, based on interfacial cross-linking of emulsions, is explored. Therefore, an overview is given on current knowledge of different types of enzymatically induced cross-linking of protein at emulsion interfaces, both for existing food and possible ruminal bypass applications.

1 Introduction

Consumers are increasingly aware of healthy food (Hoefkens *et al.*, 2011). PUFA constitute an important part of a healthy diet, as numerous studies have proven their beneficial effects (Belury, 2002; Horrocks and Yeo, 1999; Simopoulos, 1991). However, due to microbial saturation in the rumen, i.e. biohydrogenation (BH), the majority of dietary PUFA are

converted into more hydrogenated products, leading to the loss of their health features, before absorption in the small intestine (Figure 3.1). Accordingly, meat or milk from cattle supplemented with unprotected *n-3* PUFA sources do not reach concentrations defined by the European Food Safety Authority (European Food Safety Authority NDA Panel, 2009) to permit labeling as “source of” or “rich in” *n-3* PUFA (Doreau *et al.*, 2015). Moreover, such labels only can be granted to products which are enriched in a natural way, i.e. through ruminant feeding. Furthermore, PUFA are also related to increased fertility and better reproductive performances of ruminants (Staples *et al.*, 1998; Gulliver *et al.*, 2012). Therefore, both with respect to human and animal health care as well as from a resource-saving and economic perspective, protection of PUFA from ruminal BH is of interest. Furthermore, it is important that the bypassing lipid allows post-ruminal release once it reaches the small intestine and absorption of PUFA into the lymphatic system, resulting in an effective transfer to peripheral tissues.

Numerous protection technologies have been developed to overcome this undesirable BH. Although first attempts date from several decades ago, research is still ongoing to develop new ways of rumen protection of dietary lipids and to extend the plurality of rumen bypass methods that currently exist, as many of these technologies use harmful products, are not cost-effective or are lacking consistency regarding rumen protection efficiency (Jenkins and Bridges, 2007). Therefore, the aim of this review is to give an overview of the current state of the art in rumen lipid bypass technologies, based on patent publications and recent scientific literature. Finally, the potential of a novel emerging rumen lipid protection technology, based on interfacial cross-linking of emulsions, will be explored. In the following sections, emulsions as well as protein cross-linking are frequently mentioned as one of the primary features needed to obtain rumen bypass. For the sake of a better understanding of this review, a short description about emulsions, emulsifiers and protein cross-linking is given first.

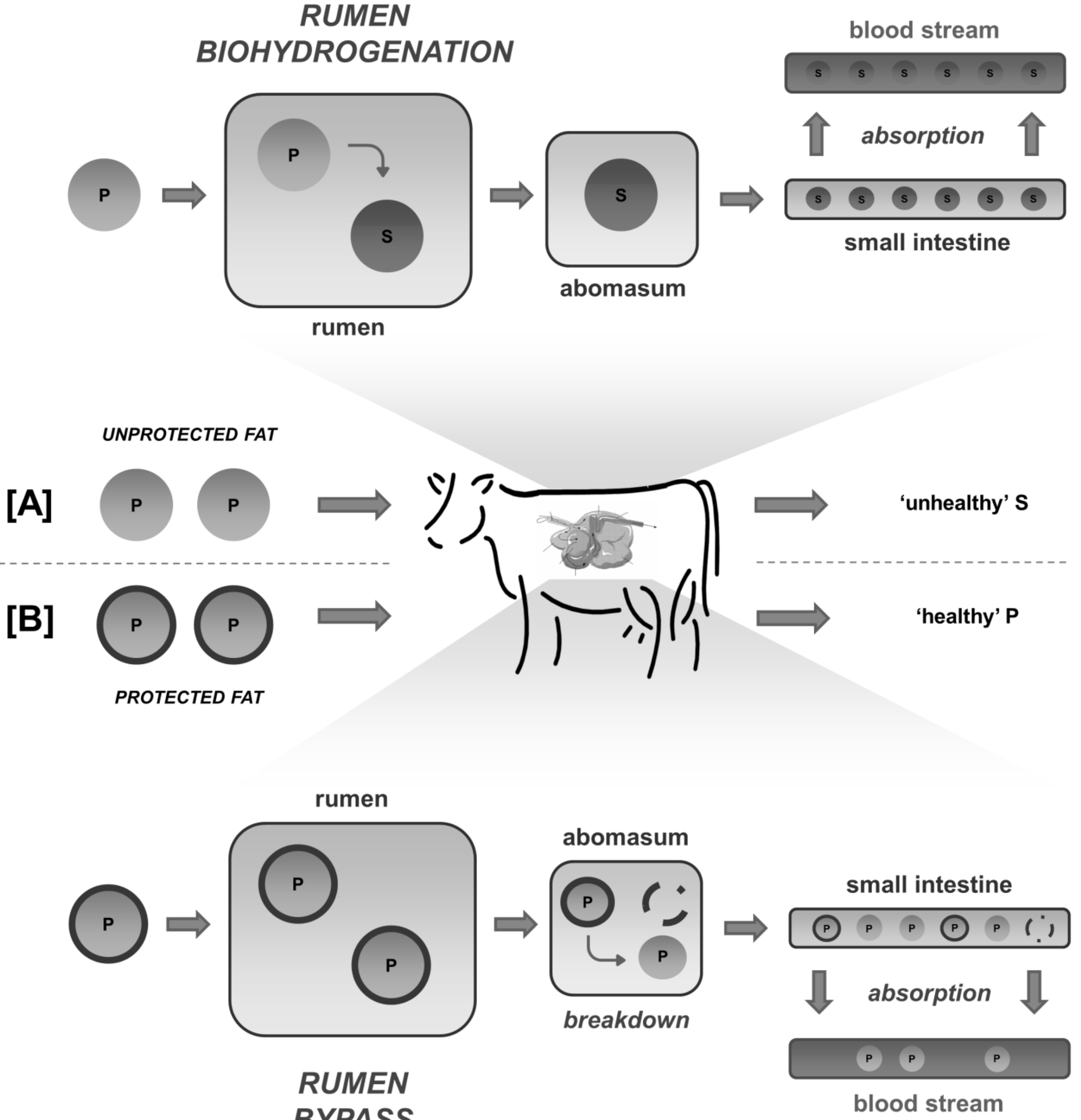

Figure 3.1 Protection, release and absorption principle of a lipophilic rumen bypass product: as polyunsaturated fatty acids (P) are hydrogenated to more saturated fatty acids (S) upon ruminal passage (panel A), P sources administered to ruminants have to be protected against microbial turnover to transfer them to the peripheral tissues (panel B)

2 Emulsions and protein cross-linking

2.1 What are emulsions?

A plethora of standard works has been published on colloids and more in particular emulsions, which can be consulted for more detailed information (Becher, 2001; Dickinson and Miller, 2001; Hiemenz and Rajagopalan, 1997). Only a brief summary of the basic and most relevant concepts is given here.

An emulsion is a dispersion of two immiscible liquids, e.g. oil and water. Such a system is thermodynamically unstable, so the liquid components will generally not spontaneously mix and form a colloidal dispersion. Dispersion of one liquid in another demands a large energy input. Accordingly, emulsification most often requires very intense stirring, e.g. using a simple blender, a colloid mill or a rotor-stator stirrer such as an Ultra-Turrax. Alternatively, high pressure might be applied, e.g. using a microfluidizer or a valve homogenizer. Emulsions can be obtained when oil is mixed in water (O/W), whereby a fine dispersion of oil droplets in water is formed. Similarly, water can be dispersed in oil (W/O). Also multiple emulsions exist, for example when water is first dispersed in oil and this mixture is then emulsified in water (W/O/W). However, due to the thermodynamically unstable behavior of emulsions, the oil and water will demix in the long run. Therefore, emulsions which are called 'stable' refer to the kinetic stability of one liquid into another, meaning the transition to the lowest energy state, i.e. the complete demixing of both liquids, is so slow it can hardly be observed within a considered period. Typical examples of emulsions in food applications are milk and mayonnaise (O/W) and butter (W/O).

Besides the equipment used, energy-input, rheological characteristics of both phases, emulsified volume fraction, temperature during and duration of emulsification, an important aspect of emulsions are the surfactants (or emulsifiers) used to obtain kinetic stability. These surfactants adsorb at the oil/water interface, lowering the interfacial tension between the two

phases and thereby preventing recoalescence of the droplets. Surfactants are amphiphilic compounds which render them surface-active in nature, meaning they contain a hydrophobic and hydrophilic part. In other words, they contain parts which are soluble in both polar and nonpolar solvents. Frequently used emulsifiers are proteins, which are surface-active as they contain both hydrophobic as well as hydrophilic functional groups. Their surface-activity depends on many factors such as size, charge, amino acid sequence, solubility and unfolding speed (Magdassi, 1996). Adsorption of proteins at the interface is dynamic as molecules reaching the surface first normally are initially occupying the interface, but these molecules can be displaced by other more surface-active ones (Dickinson, 1999). Indeed, proteins typically unfold upon adsorption, exposing differing areas of the protein chain (Phillips *et al.*, 1994), which may lead to another adsorption equilibrium. Generally, soft and hard proteins are distinguished as emulsifiers. Soft proteins have a random structure, are flexible and display large conformational changes upon adsorption, whereas hard proteins show a fixed geometry and undergo limited or no interfacial conformational changes (Malmsten, 1998). Examples of soft and hard proteins are casein and globular proteins such as β-lactoglobulin or enzymes, respectively. Protein adsorption at an oil-water interface is generally irreversible, but adsorbed proteins can be displaced by smaller, more surface-active surfactants such as sodium dodecyl sulphate (Mackie *et al.*, 2000; Gunning *et al.*, 2004; Mackie, 2004; De Feijter *et al.*, 1987). For the remainder of this paper, only proteins will further be considered as emulsifiers. Please refer to other works for more information on protein-stabilized emulsions (Dickinson, 1998; McClements, 2004; Nik *et al.*, 2010; McClements and Gumus, 2016).

2.2 What is protein cross-linking?

Cross-linking is simply the process whereby one polymer chain binds to another. In the context of this review, cross-linking refers to the covalent binding whereby at least one protein is involved. Both non-enzymatic as well as enzymatic cross-linking occurs, which has been thoroughly reviewed by Buchert *et al.* (2010), Zeeb *et al.* (2014) and Heck *et al.* (2013).

The most commonly occurring types of cross-linking reactions with proteins will be summarized in this section.

2.2.1 Non-enzymatic cross-linking

One of the most common, temperature-sensitive (Buchert *et al.*, 2010), cross-linking reactions is the 'browning' Maillard reaction, which is responsible for the typical brown or dark color of many food products. The Maillard reaction is a collective name for several non-enzymatic reactions which can yield many types of reaction products (Friedman, 1996), which can potentially be toxic. One example of such a toxic compound is acrylamide, which can be generated from food compounds during heat treatment (Mottram *et al.*, 2002). The most important reaction related with this non-enzymatic browning is the non-enzymatic glycation of reducing sugars to amino groups of amino acids and proteins to form glycated proteins. These can further rearrange to more stable ketoamines (also called Amadori products) and potentially form cross-links between adjacent proteins or with other amino groups (Friedman, 1996) (Figure 3.2a).

Proteins can also be covalently bonded by adding chemical cross-linkers, such as formaldehyde, which is one of the oldest cross-linking agents. The reaction of formaldehyde with proteins involves a series of reactions, starting with the formation of methylol derivatives through reaction of formaldehyde with the amino and thiol groups of amino acids. Afterwards, partial condensation to an imine occurs, which can cross-link with amino acids such as glutamine, asparagine, tryptophan, histidine, arginine, cysteine, and tyrosine residues (Metz *et al.*, 2004) (Figure 3.2b). Due to its small size, formaldehyde can easily penetrate cell walls and membranes, resulting in cross-linking between proteins (Sinz, 2010). Formaldehyde is considered a carcinogenic compound due to its ability to form DNA-protein cross-links (Szende and Tyihak, 2010). Also glutaraldehyde is often used as cross-linking agent (Migneault *et al.*, 2004). Other examples of chemical protein cross-linkers are amine-reactive

Figure 3.2 Reaction schemes of the most common protein cross-linking reactions: a/ initial stages of the Maillard reaction (Friedman, 1996); b/ formaldehyde-induced (Metz *et al.*, 2004); c/ transglutaminase-catalyzed (Heck *et al.*, 2013); d/ tyrosinase-catalyzed (Thalmann and Lötzbeyer, 2002); e/ laccase- or peroxidase-catalyzed (Heck *et al.*, 2013; Zeeb *et al.*, 2014)

N-hydroxysuccinimide esters, which can create stable amide or imide bonds with the free N-terminus of proteins or the amino groups of lysine, and carbodiimides (Sinz, 2010).

Several other non-enzymatic cross-linking reactions also exist, including disulfide cross-linking of thiol groups of cysteine upon protein folding, isopeptide cross-linking, di- or trityrosine cross-linking or lipid peroxidation (Buchert *et al.*, 2010). Also genipin, has been suggested as cross-linking agent, with lower cytotoxicity in comparison with aldehyde cross-linking agents (Sung *et al.*, 1998), as it reacts spontaneously with amines in proteins.

2.2.2 Enzyme-catalyzed cross-linking

Distinction can be made between two types of enzymatic protein cross-linking: i/ direct covalent binding of proteins induced by the action of transferases or hydrolases, and ii/ enzyme-mediated covalent binding via reactive species that are enzymatically generated by oxidoreductases and spontaneously react further with proteins to form protein networks (Heck *et al.*, 2013). Examples of enzymes involved in the first type of reaction include transglutaminase (EC 2.3.2.13) and sortase A (EC 3.4.22.70), while tyrosinase (EC 1.14.18.1), laccase (EC 1.10.3.2), peroxidase (EC 1.11.1.x), sulfhydryl oxidase (EC 1.8.3.2), lysyl oxidase (EC 1.4.3.13) and glucose oxidase (EC 1.1.3.4) induce the second type of reactions.

The most often described cross-linking enzyme is transglutaminase. This is the more common name of protein-glutamine γ-glutamyltransferase, as it catalyzes the formation of an isopeptide bond between the group of γ-carboxamides of glutamine residues and the first-order ε-amine groups of different compounds such as proteins (Kieliszek and Misiewicz, 2014). Cross-linking of proteins occurs if glutamine and lysine residues of proteins or peptides are linked with each other (Figure 3.2c). Transglutaminase has been identified in various mammals, fish and plants, but the only commercially available food-grade protein cross-linking enzymes nowadays are of microbial origin. The most distinct application of this enzyme is the restructuring of meat or fish in order to add market value to products of poorer

quality (Buchert *et al.*, 2010) because of which it is commonly known as 'meat glue'. One example of a large-scale produced and relatively low-cost commercially available microbial transglutaminase is Activa® from Ajinomoto (Kyobashi, Japan).

Another commonly known cross-linking enzyme is polyphenol oxidase (PPO). This oxidoreductase which can oxidize colorless phenols to colored quinones (Bittner, 2006) has already extensively been reviewed in section 2 of Chapter 1. For completeness and comparative reasons, the reaction schemes of tyrosinase and laccase are also reported in Figure 3.2d and Figure 3.2e, respectively.

Alike PPO, peroxidases are also oxidoreductases, but ideally use H_2O_2 as electron acceptor. As a result of the peroxidase-induced oxidation reaction, a radical is formed which can further react with substrates such as phenols, thiols and amino acids (Buchert *et al.*, 2010). Quinones can be produced by this peroxidase-catalyzed reaction which can further undergo non-enzymatic polymerization reactions (Zeeb *et al.*, 2014) (Figure 3.2e). Peroxidases are haem-proteins which are widely present in nature, but the most studied one is extracted from horseradish (Zeeb *et al.*, 2014).

3 Overview of existing rumen lipid protection technologies

To overcome ruminal BH of dietary PUFA, a number of protection technologies have been developed. In the following sections, an overview of the current art is given. A distinction is made between two major types of rumen lipid protection: first, the alteration of the FA structure by blocking the free carboxyl end of FA for bacterial isomerases, and second, the encapsulation in a shell to completely prevent the hydrogenating bacteria to access the FA (Jenkins and Bridges, 2007). In each section, a brief description of the method is given, with a focus on both patent and scientific literature dealing with this technique. Table 3.1 gives an overview of the most common rumen lipid bypass technologies, indicating the main principle of the technology as well as possible disadvantages. Transfer efficiencies of PUFA from the diet to the milk were calculated from scientific literature and are presented to give an

Table 3.1 Protective mechanism, possible disadvantages and literature-extracted transfer of polyunsaturated fatty acids (PUFA) from intake to dairy cow's milk for the most described or promising rumen lipid bypass technologies

Protection technique	Protective mechanism (+) Disadvantages (-)	Lipid source	Evaluated PUFA[a]	Transfer[b] (%) PROT	Transfer[b] (%) UPROT	Reference
Calcium salt	+ Blocking free FA	Linseed oil	C18:3*n-3*	0.67	-	(Chouinard *et al.*, 1998)
	carboxyl end	Linseed oil	C18:3*n-3*	1.2	-	(Sultana *et al.*, 2008)
	- Protection impaired	Linseed oil	C18:3*n-3*	1.9	1.5[c]	(Cortes *et al.*, 2010)
	by dissociation	Fish oil	C22:6*n-3*	6.0	3.3[d]	(Castaneda-Gutierrez *et al.*, 2007b)
	- Limited amount of	palm oil	C18:2*n-6*	13.2	-	(Theurer *et al.*, 2009)
	protectable PUFA	CLA oil	t10c12	1.9-7.2	-	(de Veth *et al.*, 2005)[e]
	- Free FA needed	CLA oil	t10c12	3.2	nd[f]	(de Veth *et al.*, 2005)
		Soybean oil	C18:2*n-6*	6.5	6.9	(Lundy *et al.*, 2004)
Fatty acyl amide	+ Blocking free FA	Canola oil	C18:2*n-6*	17	18	(Loor *et al.*, 2002)
	carboxyl end	Soybean oil	C18:2*n-6*	5.5	6.9	(Lundy *et al.*, 2004)
	- Free FA needed	CLA oil	t10c12	7.1	nd[f]	(Perfield *et al.*, 2004)
Formaldehyde	+ Encapsulation in	Canola/soybean	C18:2*n-6*	25-44	-	(Gulati *et al.*, 2005)[e]
	formaldehyde-	Cottonseed	C18:2*n-6*	43	-	(Gulati *et al.*, 2005)[e]
	protein matrix	S/L	C18:3*n-3*	19-24	-	(Gulati *et al.*, 2005)[e]
	- Toxic	Soybean/fish oil	C22:6*n-3*	10-14	-	(Gulati *et al.*, 2005)[e]
	- Untargeted	Linseed oil	C18:3*n-3*	13	3.0[g]	(Sterk *et al.*, 2012b)
	reaction	CLA oil	t10c12	7.0	nd[f]	(de Veth *et al.*, 2005)
	- Expensive	CLA oil	t10c12	6.9-8.6	-	(Gulati *et al.*, 2006a)
Non-enzymatic browning[h]	+ Encapsulation in sugar-protein matrix - Potentially toxic - Expensive - Oxidation of PUFA					
Lipid composite gels	+ Encapsulation in	Soybean oil	C18:2*n-6*	46-69[i]	22-37	(Carroll *et al.*, 2006)
	gelled matrix	S/L oil	C18:3*n-3*	81-225[ij]	21	(Heguy *et al.*, 2006)
	- large H_2O volumes	S/L oil	C18:3*n-3*	13-19	-	(van Vuuren *et al.*, 2010)
Encapsulation within lipid	+ Encapsulation in	CLA oil	t10c12	7.9	nd[f]	(Perfield *et al.*, 2004)
	high-melting point	CLA oil	t10c12	5.1	nd[f]	(Castaneda-Gutierrez *et al.*, 2007a)
	lipid matrix	CLA oil	t10c12	4.8	-	(Moallem *et al.*, 2010)
	- low payloads	CLA oil	t10c12	6.3	nd[f]	(Odens *et al.*, 2007)
	- low post-ruminal	CLA oil	t10c12	2.4-5.8	-	(Pappritz *et al.*, 2011)
	release	CLA oil	t10c12	4.9	-	(Schwarz *et al.*, 2009)
		Algal oil	C22:6*n-3*	1.0	-	(Stamey *et al.*, 2012)
		Algal biomass	C22:6*n-3*	2.0-3.4	-	(Stamey *et al.*, 2012)
		Echium oil	C18:4*n-3*	3.2-3.4	-	(Bainbridge *et al.*, 2015)

PROT, protected supplement; UPROT, unprotected supplement (control); t10c12, *trans*-10, *cis*-12 C18:2; S/L, soybean/linseed

[a] the most prominent PUFA within the oil was used for evaluation; [b] transfer was calculated as [g PUFA in milk]/[g PUFA in diet]×100, whereby fat was assumed to contain 90 % (w/w) FA; [c] whole linseed; [d] ruminal infusion of fish oil, no statistical difference with the treatment; [e] summary of earlier studies; [f] no t10c12 C18:2 measured in milk of control treatment (nd); [g] extruded whole linseed; [h] no scientific references describing *in vivo* milk data available; [i] net transfer efficiency as reported in reference: 16-30 (Carroll *et al.*, 2006) and 9-43 (Heguy *et al.*, 2006); [j] calculation with data from reference results in an unrealistically high transfer

indication of the protection potential for each technique. This index is given rather than *in vivo* rumen bypass assessments based on abomasal or duodenal flows, as the number of (recent) studies reporting these fluxes is rather limited. Moreover, diet to milk transfer efficiencies integrate both effectiveness against ruminal breakdown as well as post-ruminal release (and hence, limitations of duodenal overprotection) and differences in partitioning of FA between different tissues. By comparison, transfer efficiencies from the small intestine to the milk reported in post-ruminal infusion studies maximally reach 49 % for C18:2*n-6* and C18:3*n-3* (Shingfield *et al.*, 2013), 22 % for *trans*-10*, cis*-12 C18:2 (de Veth *et al.*, 2004) and 25 % for C22:6*n-3* (Shingfield *et al.*, 2013).

3.1 Alteration of the fatty acid structure

3.1.1 Calcium salts

Calcium salts of long chain FA are soaps formed by creation of an ionic bond between the free carboxyl group of the FA and Ca ions (Figure 3.3). The possibility to protect Ca salts of FA against ruminal BH was first proposed by Palmquist and Jenkins (1987) and believed to be caused by the insoluble character of the Ca salts, permitting an efficient bypass across the rumen without disturbing the rumen microorganisms. As dissociation of a range of different Ca salts varied between pH levels of 4.5 and 6 (Sukhija and Palmquist, 1990), salts dissociate again in the acid environment of the abomasum, which makes the FA available for absorption in the small intestine. The preparation of Ca salts is typically performed by adding a suspension of CaO in water to a liquid source of FA and then stirring the reaction mixture rapidly, leading to hardening of the mixture as the salt is formed (Morgan and Blagdon, 2005) and resulting in a dry and easily friable product (McAskie, 1989). Metals other than Ca, such as magnesium and other Group II elements, are also suitable (Morgan and Blagdon, 2005).

However, a major disadvantage of this technology is that dissociation might already occur in the rumen, if the pH decreases beneath 6.3 (Chalupa *et al.*, 1986; Van Nevel and Demeyer, 1996), making the FA accessible to bacterial isomerases. Further, there is a maximum of

Figure 3.3 Example of a calcium salt with alpha-linolenic acid, i.e. Ca(octadeca-9,12,15-trienoic acid)$_2$

unsaturated FA which might be protected. Indeed, it was shown before dissociation of Ca salts depends on the unsaturation of the FA in the soaps (Sukhija and Palmquist, 1990). Dissociation constants are generally lower for more unsaturated FA (Kanicky and Shah, 2002), which means more dissociation will occur for Ca salts with high concentrations of unsaturated FA at a given rumen pH. Additionally, salt formation generally does not go to completion, leaving unreacted unsaturated free FA in the product. This can potentially lead to processing difficulties due to the development of a congealed product mass because of the relatively low melting point of unsaturated free FA (Strohmaier *et al.*, 2005). Accordingly, rumen bypass Ca salt supplements typically have an unsaturation level of less than about 50 % (Morgan and Blagdon, 2005). Methods have been developed to overcome problems with unreacted unsaturated free FA, e.g. by using elevated levels of CaO or by reducing the amount of unsaturated FA (Strohmaier *et al.*, 2005). Also other claims have been made to incorporate FA such as conjugated linoleic acids (CLA) (Luchini *et al.*, 2006), C20:5*n-3* or C22:6*n-3* (Strohmaier *et al.*, 2004) or other (water soluble) nutrients such as choline (Morgan and Blagdon, 2005). Finally, a major (economic) disadvantage is that the production of Ca salts requires free FA as precursors. As FA in most oils are present as triacylglycerides, hydrolysis from its glycerol backbone is required before protection can be created. One example of a commercialized Ca salt is Essentiom® from Church & Dwight (Ewing, New Jersey, USA), a product that contains high levels of protected *n-6* and *n-3* PUFA.

Several studies have been reported on the use of Ca salts of unsaturated FA. In the studies of Chouinard *et al.* (1998) and Sultana *et al.* (2008), C18:3*n-3* in milk only slightly increased upon administration of Ca soaps of linseed oil-derived FA, compared with non-supplemented treatments or the addition of Ca soaps with more saturated FA. Similar results were reported by Cortes *et al.* (2010) who compared Ca salts of linseed oil with whole flaxseed. Castenada-Gutierrez *et al.* (2007b) found Ca salts of fish oil offered no additional protection against the BH of C20:5*n-3* and C22:6*n-3* as compared with untreated fish oil. Compared with the milk of non-supplemented animals, Theurer *et al.* (2009) found limited increases in C18:2*n-6* concentrations in milk fat when Ca salts of palm FA high in PUFA were fed. Despite some slight augmentation of protected FA in milk fat in several studies, calculated transfer efficiencies of the respective FA from the diet to the milk remained low (Table 3.1). De Veth *et al.* (2005) summarized studies from before 2005 in which Ca salts of CLA have been fed to lactating dairy cows. There, transfer efficiencies of *trans*-10*, cis*-12 C18:2 varied considerably between 1.9 and 7.4 %, irrespective of dietary doses. Besides variation in experimental conditions, the large variation in transfer efficiencies between experiments also might be related to differences in protection efficiency, probably induced by variation in production procedures of the Ca salts. Generally, very inconsistent results are found in literature dealing with Ca salts of unsaturated FA, but most of them reported an incomplete protection, supported by the many BH intermediates which were found in milk and/or rumen fluid (Huang *et al.*, 2009; Sippel *et al.*, 2009; Kliem *et al.*, 2013; Fiorentini *et al.*, 2015; Gallardo *et al.*, 2014; Chouinard *et al.*, 1998; Sultana *et al.*, 2008).

3.1.2 Fatty acyl amides

Fatty acyl amides consist of a FA chemically linked through an amide bound to an amine (Figure 3.4). Originally, this protection technology was used to create rumen bypass methionine as the amide bond between the carboxyl group of stearic acid (Langar *et al.*, 1978) or other acyl groups (Fahnenstich *et al.*, 1978) and the amine of methionine resists rumen bacterial breakdown. Later, this approach was proven potentially useful to protect

Figure 3.4 Example of a fatty acyl amide with alpha-linolenic acid, i.e. N-butyl-octadeca-9,12,15-trienamide

unsaturated FA against ruminal BH, using either amino acids (Fotouhi and Jenkins, 1992), non-acidic primary amines, such as aliphatic amines containing 1 to 30 carbon atoms (Jenkins, 1996), or ammonia (Cummings and Forrest, 1997).

Microbial degradation of fatty acyl amides, resulting in a free carboxyl group, seems limited as the bulky fatty acyl and amine moieties provide steric hindrance around the amide bond which impairs access of microbial enzymes (Jenkins and Bridges, 2007). As for the Ca salts, the production process of simple amide-protected supplements requires free FA as precursor.

Alike the Ca salt application, several studies reported a partial protection of amide linked FA against ruminal BH (Reeves *et al.*, 1998; Jenkins, 1995; Loor *et al.*, 2002). Lundy *et al.* (2004) suggested fatty acyl amides were more effective than Ca salts to increase the post-ruminal flow of FA. Perfield *et al.* (2004) reported an equally effective transfer of *trans*-10*, cis*-12 C18:2 to the milk in amide-protected (7.1 %) or lipid encapsulated (vide infra) supplements. However, fatty acyl amides do not seem to be more effective in transferring dietary PUFA to milk than pure oil (Table 3.1).

3.2 Encapsulation in a microbe-resistant shell

3.2.1 Aldehyde treatment

In the seventies, Scott and Hills (1975) proposed a method to protect unsaturated FA by encapsulation within a protein aldehyde reaction product. Prior to aldehyde addition, lipids first need to be emulsified using proteins such as casein, gelatin or other plant, fish, meat or

oilseed proteins to ensure a homogeneous distribution of the lipid within the protein, and can further be processed using spray-drying to obtain a coated particulate solid (Figure 3.5). The cross-linked protein matrix protects the lipid from degradation in the rumen but can be degraded in the abomasum, allowing digestion in the lower gut and making rumen protected dietary lipid available for absorption by the ruminant. Further, the spray-drying process results in entrapped air voids within the particles, which helps reducing the density of the lipid supplement to obtain a quick separation from other material in the rumen and a fast passage to the abomasum. The most commonly applied aldehyde is formaldehyde, but also glutaraldehyde or glyoxal can be used (Scott and Hills, 1975). Besides emulsions, lipid body organelles in oil seeds were also claimed to be (partially) protected against ruminal degradation by aldehyde cross-linking (Rawlings and Rawlings, 1975; Lebo and Winowiski, 2013), whereby the oilseed initially needs to be comminuted, in order to ensure the cross-

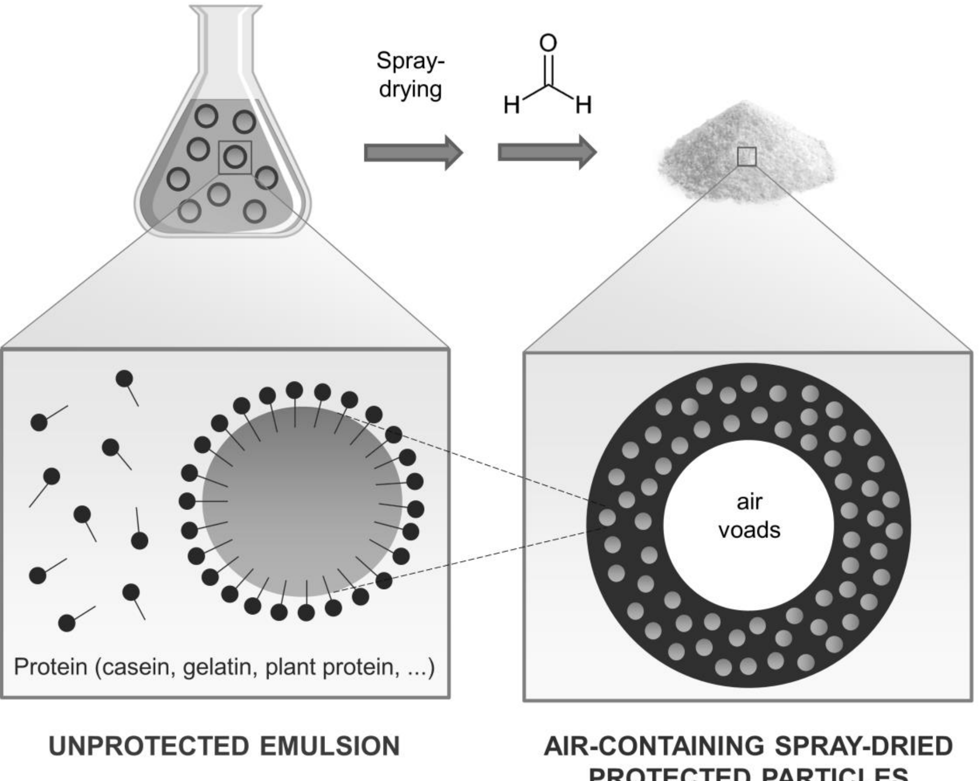

Figure 3.5 Principle of formaldehyde-treated spray-dried rumen bypass particles, containing lipid embedded within a cross-linked protein matrix, according to the method described by Scott and Hills (1975)

linked protein matrix completely surrounds the lipid within the oilseed. The oil seed protein is often activated with a hydroxide, urea or ammonia prior to aldehyde treatment, in order to promote protein-aldehyde complexation around the lipid globules (Rawlings and Maher, 1976).

However, formaldehyde is a noxious product and its use in the European Union is subject to strict regulations (2011/391/EU). Formaldehyde is currently only authorized in the European Union for use as silage additive, preservative for skimmed milk intended for use in pigs up to six months of age and as preservative in cosmetics (European Food Safety Authority FEEDAP panel, 2014). In the United States, formaldehyde is approved for use as feed additive under certain conditions (FDA Section 573.460). The use of formaldehyde for encapsulation purposes might lead to toxicity and regulatory problems, since it is possible that some unreacted formaldehyde remains within the coating. More recent methods have been claimed to overcome these problems by preventing the contact between the aldehyde and the content of preformed gelatin protein capsules, followed by a washing and drying step to remove any unreacted aldehyde (Jenkins, 2009). It was shown that such formaldehyde-treated protein capsules substantially reduced BH of CLA *in vitro* (Myers *et al.*, 2016). The application of formaldehyde to emulsions in order to create cross-linking of protein is also non-targeted, meaning it is hard to prevent formaldehyde from reacting with other nutrients besides proteins such as e.g. unsaturated FA (Jones, 1972). Although formaldehyde treatment is considered to be the most effective technique so far, its application remains limited nowadays due to its high cost, the bad image of chemical treatments of feedstuffs and possible residues in the final animal products (Doreau *et al.*, 2015).

The method proposed by Scott and Hills (1975) effectively prevented ruminal BH, both *in vitro* as well as *in vivo* (Scott *et al.*, 1971). Since then, many researchers have reported about formaldehyde-treated fats (Doreau *et al.*, 2015; Jenkins and Bridges, 2007). Gutati *et al.* (2005) summarized transfer efficiencies from the diet to cow's milk for several formaldehyde-treated rumen-protected FA (Table 3.1). Larger transfers were reported for C18 PUFA (19 -

44 %) than C22 PUFA (10 - 14 %). More recently, Sterk *et al.* (2010) showed *in vitro* BH of C18:3*n-3* in formaldehyde treated linseed was significantly lower than in unprotected linseed oil (24.3 % versus 43.5 %, respectively). The same researchers also reported higher levels of C18:3*n-3* in plasma triacylglycerides and milk fat of lactating dairy cows when formaldehyde treated linseed oil was fed compared with crushed and extruded whole linseed and a larger transfer of C18:3*n-3* from the diet to the milk (Sterk *et al.*, 2012b). Further, Sinclair *et al.* (2005) observed *in vitro* BH of C18:3*n-3* in linseed was significantly lower when formaldehyde treatment was accompanied with a sodium hydroxide or formic acid pre-treatment. Transfer efficiencies from the diet to the milk of *trans*-10*, cis*-12 C18:2 were larger when the CLA was formaldehyde protected (7.0 %) as compared with protection as Ca salt (3.2 %) (de Veth *et al.*, 2005). A similar transfer efficiency (6.9 or 8.6 %) for formaldehyde protected *trans*-10*, cis*-12 C18:2 was found in the study of Gulati *et al.* (2006b).

3.2.2 Non-enzymatic browning

Besides the former aldehyde method, encapsulation of oil can also be obtained by other cross-linking techniques. Richardson (1992) claimed a method to protect PUFA oils within protein capsules by cross-linking the proteins with reducing sugars. This procedure involves three steps: first, an aqueous emulsion of oil in a solution of protein and reducing sugars such as lactose is made, second, emulsions are freeze dried to yield a dry powder, and third, the powder is browned in an oven to produce rumen protected granules (Figure 3.6). Similar methods have been proposed to protect FA within oilseeds (rather than oils) against ruminal BH. Klopfenstein *et al.* (1998) described a procedure for ruminally inert fat, whereby the browning reactions render the protein within the oil seeds resistant to ruminal degradation, resulting in the encapsulation of the oil in a protective matrix. Hereby, oilseeds have to be cracked prior to browning to ensure penetration of the reducing sugars into the interior of the oilseed. Other claims also proposed to encapsulate PUFA using an outer coating of Maillard reaction products for non-ruminant applications (Subramanian *et al.*, 2012). Further, extrusion can be related with this non-enzymatic browning technique too. Extrusion basically

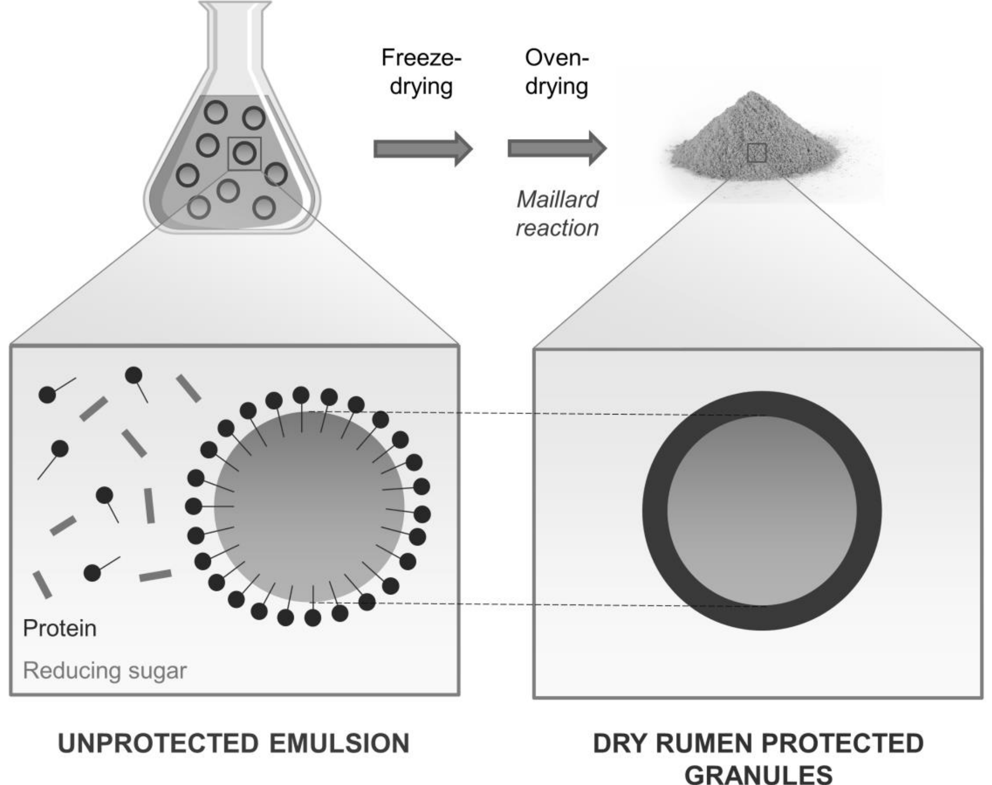

Figure 3.6 Principle of encapsulating oil by cross-linking proteins and reducing sugars by a Maillard reaction to create dry rumen bypass particles, according to the method described by Richardson (1992)

is the process of pushing a deformable material through a narrow section whereby frictional heat is generated. This process is often applied on oilseeds to ensure bioavailability by rupturing the protective organelles that contain the lipids, which inherently also would imply an increased exposure of PUFA to rumen BH. Recently, a method has been claimed by extrusion of flaxseeds in the presence of at least one reducing sugar to increase the PUFA content of milk (Vanvolsem, 2016). Maillard reaction products were also shown to limit oxidation of e.g. fish oil (Augustin *et al.*, 2006). In conclusion, heat treatment and a reducing sugar are the most important elements in this case to obtain protection, however, Kaleem *et al.* (2013) recently suggested that the partial protection of PUFA against BH in heated soybeans is at least to some extent related to their content of aldehydes, which are generated as lipid oxidation products during the heating step.

A plus of this protection technology is the fact that no additional hazardous chemicals are used. However, browning does not appear to be as effective as other methods (Richardson, 1992). The process can also be quite expensive due to the requirement of reducing sugars and the heating steps (Rosenberg and DePeters, 2010). Further, exposure at temperatures required for an effective Maillard-type cross-linking can oxidize the unsaturated FA of the oils, which may lead to the formation of toxic compounds (Rosenberg and DePeters, 2010), besides the potential formation of toxic molecules by the Maillard reaction itself.

Not many scientific papers deal with the protection of PUFA against ruminal BH by non-enzymatic browning. An *in vitro* study demonstrated xylose-treated oils offered only limited protection against ruminal BH (Sinclair *et al.*, 2005). Recently, Alvarado-Gilis *et al.* (2016) studied the possibility of non-enzymatic browning of a blend of ground flaxseed, soybean meal, molasses, and baker's yeast, but failed to improve resistance of PUFA against BH. Instead, ruminal degradability of crude protein in soybean meal has been shown to be reduced after non-enzymatic browning (Borucki Castro *et al.*, 2007; Can *et al.*, 2011).

3.2.3 Lipid composite gels

A more recent method describes the potential of composite gels containing amino acids and lipids to bypass the rumen. Rosenberg and DePeters (Rosenberg and DePeters, 2010) claimed dispersions of lipid droplets in an aqueous protein phase can be protected against ruminal degradation by heat-induced gelatinization. The formation of composite gels, i.e. the combination of gelled protein and lipid, basically consists of two steps: first, an emulsion is formed by emulsifying lipids in a matrix of dissolved and/or suspended proteins, and second, emulsions are heated at a temperature of 80°C to 125°C to produce a composite gel that is protected from degradation in the rumen (Figure 3.7). Accordingly, dispersed phase droplets become embedded within the gelled protein matrix in which the lipids are protected against degradation, modification, or removal from the gel during passage through the rumen. In contrast with the aldehyde treatment, the cross-linking of protein is not induced by a divalent

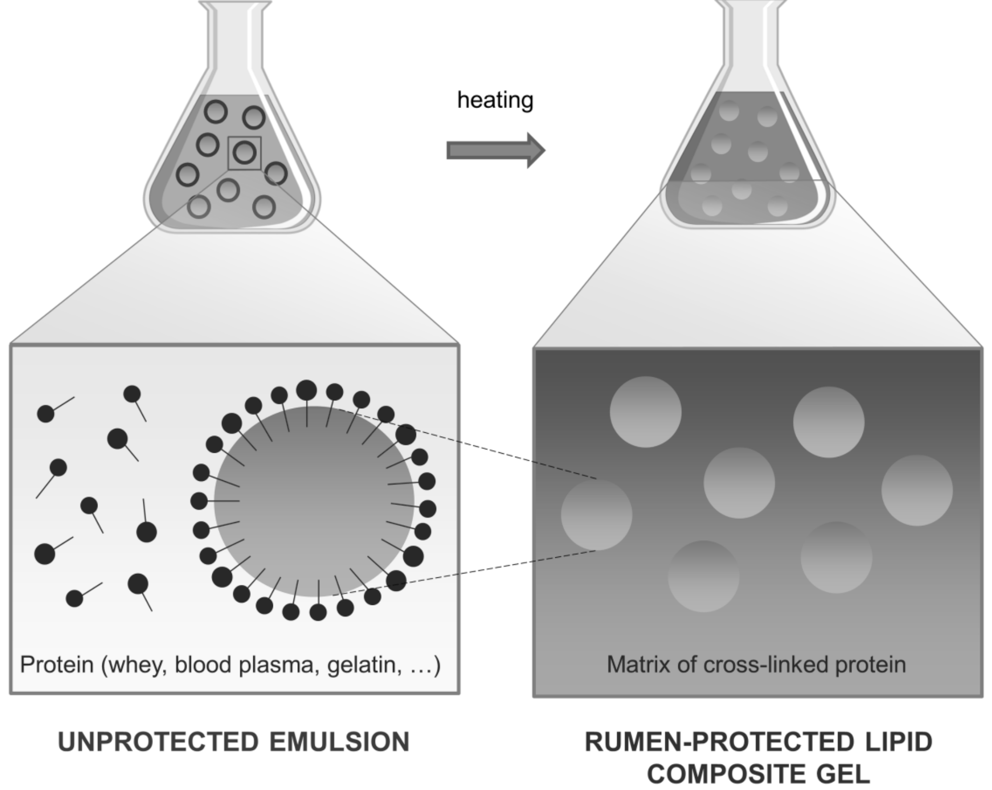

Figure 3.7 Principle of rumen bypass lipid composite gels, containing lipid embedded within a gelled cross-linked protein matrix, according to the method described by Rosenberg and DePeters (2010)

linker such as formaldehyde, but by gelation of proteins such as whey protein, blood serum proteins, gelatin or peanut, cereal, fish or soy proteins, whereby the proteins are predominantly cross-linked by disulfide bonds, hydrophobic interactions, ionic interactions, or hydrogen bonding. Reducing sugars, such as glucose, lactose, fructose, mannose, maltose, ribose and galactose can be present in the matrix to additionally cross-link the proteins by a Maillard reaction. The same authors also claimed a method to prepare dry protected composites (Rosenberg and DePeters, 2005). Others also used the same gelling principles to stabilize lipid emulsions for non-ruminant applications (Kyogoku and Harada, 1992). Similar claims were made to encapsulate lipids in gels using albumin (Freeman, 1988; Rawlings and Procter, 1980).

Embedding lipids in a protein matrix of whey or blood proteins has the advantage of upgrading such side streams, while creating rumen bypass lipid. However, it could be assumed that gelled emulsions have a low shelf-life as they contain generally large volumes

of water, which may cause deterioration of the gels and the enclosed lipids during storage and could limit the large scale practical production and application of the gels (van Vuuren *et al.*, 2010). Nevertheless, as gels are prepared at elevated temperatures, others consider composite gels shelf-stable (Weinstein *et al.*, 2016).

Carrol *et al.* (2006) were the first to report on the efficacy of whey protein gel complexes to increase the PUFA content of bovine milk fat. Indeed, whey protein emulsion gels of soybean oil resulted in an increase in the C18:2*n-6* content of milk fat after a 1-week administration, without increasing *trans* C18:1 intermediates, resulting in net transfer efficiencies (compared with unprotected oil) of dietary C18:2*n-6* to the milk of up to 30.1 % (Table 3.1). Similarly, Heguy *et al.* (2006) found that feeding whey protein isolate gel complexes of soybean/linseed oil successfully increased the PUFA content and decreased *trans* FA of plasma and milk lipids. The largest net transfer efficiencies of dietary C18:3*n-3* to the milk occurred when gel complexes were formed using whey protein isolate (43 %), compared with the use of gelled whey protein concentrate (9 %) or calcium salts (9 %) (Heguy *et al.*, 2006). More recent research demonstrated the persistent effect of long-term administration (10 weeks) to dairy cows of whey protein concentrate gel composites of soybean and linseed oil to increase PUFA levels in milk fat, showing a transfer efficiency of C18:3*n-3* from the diet to the milk up to 19 % (van Vuuren *et al.*, 2010). A study with dairy goats also demonstrated the efficacy to increase the PUFA content of milk using whey protein isolate gels which were produced at a much lower temperature, i.e. prepared at 85 to 90°C in a steam tunnel, in comparison with the former studies in which gels were prepared at 120°C (Weinstein *et al.*, 2016).

3.2.4 Encapsulation within lipids

Technologies described in the previous sections relied on some kind of cross-linking with protein to achieve rumen protection. In other formulations, active compounds are protected in a microcapsule of lipids, formulated according to either one of the two basic concepts: active compounds are either embedded in a lipid matrix or are formulated in small spheres, which

then are coated with lipid (Desai and Jin Park, 2005; Wu and Papas, 1997). Originally, most methods related to lipid encapsulation dealt with the protection of hydrophilic compounds such as choline, amino acid derivatives, proteins, vitamins, enzymes, carbohydrates, drugs and hormones. For example, a method to encapsulate bioactive substances was proposed in the nineties by Klose (Klose, 1993), whereby particles comprise a core with the bioactive substance and a hydrophobic coating, which completely encapsulates the core. This hydrophobic coating is composed of hydrogenated fats and could be surrounded with a surfactant to ensure that the particles do not float on the rumen contents. Several methods have also been claimed to incorporate hydrophobic compounds as such, including unsaturated FA, and on their protection against ruminal BH (Ando *et al.*, 1987; Baalsrud *et al.*, 1976; Lorenzon, 2015; Maruyama *et al.*, 1985; Meade *et al.*, 1999). Generally, coatings are comprised of FA with a high melting point, i.e. at least higher than the matrix it envelops (Jobe *et al.*, 2003; Lorenzon, 2015) (Figure 3.8). Despite this common overall principle, the composition of the outer coating particularly differs between described methods, which results in varying protection efficiencies (Table 3.1): e.g. a coating comprising mainly saturated FA, wherein the fraction of C18 FA is greater than 85 % (Lorenzon, 2015), the use of highly saturated glyceride-free FA (Meade *et al.*, 1999), inclusion in the coating of chitosan

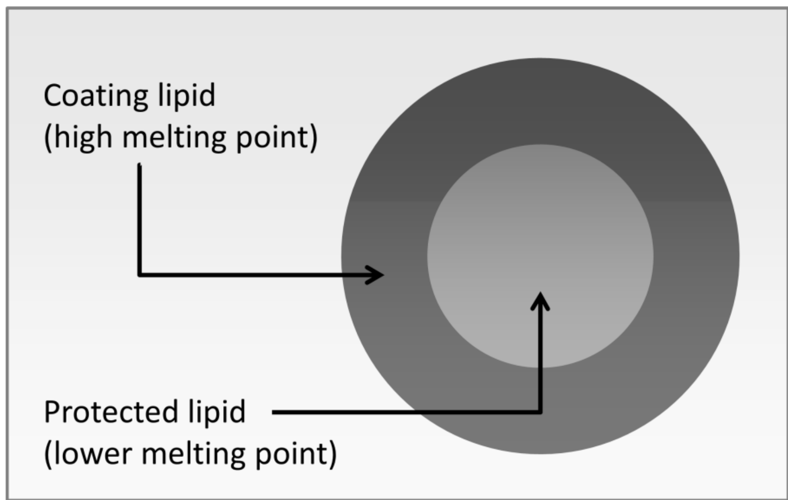

Figure 3.8 Principle of rumen bypass lipid particles by encapsulation within other lipids

(Maruyama *et al.*, 1985) or acid-sensitive substances such as cellulose derivatives, polyvinyl derivatives or nitrogen-containing polysaccharides (Ando *et al.*, 1987). Also the inner core characteristics differ among products, e.g. in the patent described by Lorenzon (Lorenzon, 2015), CLA in the inner core is adsorbed on a solid silica substrate.

Embedding substances in a lipid matrix or the process of applying a lipid coating has the advantage of using low cost food-grade materials compared to formulated polymeric coatings (Wu and Papas, 1997). However, disadvantages of these protection methods are the low payloads of the active material and its limited post-ruminal release and absorption which is generally inversely related to the degree of rumen protection (Wu and Papas, 1997). Because of these low payloads, only expensive lipids, which can be administered in low doses to have an *in vivo* effect, are encapsulated. One example of a commercialized lipid encapsulated product is Lutrell® from BASF (Ludwigshafen, Germany), containing the bioactive isomer *trans*-10*, cis*-12 C18:2.

Most research of the last decade dealing with lipid encapsulated oils reported on the protection of CLA against ruminal BH. Perfield *et al.* (2004) found the largest transfer of dietary CLA to the milk (7.9 %) in case of lipid encapsulated CLA compared with protection technologies such as amide, formaldehyde and Ca-salts of CLA (Table 3.1). Other more recent studies also used lipid encapsulated CLA and reported transfer efficiencies from the diet to the milk in dairy cows between 2.4 % and 6.3 % (Castaneda-Gutierrez *et al.*, 2007a; Moallem *et al.*, 2010; Odens *et al.*, 2007; Pappritz *et al.*, 2011; Schwarz *et al.*, 2009; Hutchinson *et al.*, 2011). Besides differences in characteristics of the outer coating and inner core, differences between studies also might be related to processing of the supplement, which can result in degradation of the lipid capsule (Hutchinson *et al.*, 2011). In studies with lactating goats (Lock *et al.*, 2008) and sheep (Lock *et al.*, 2006; Sinclair *et al.*, 2007) maximum transfer efficiencies upon dietary administration of lipid encapsulated CLA only reached 1.85 % and 2.5 to 3.8 %, respectively. Other researchers have reported on the lipid encapsulation of algal biomass or oil but increased milk fat yield of *trans* C18:1 isomers

indicated that supplements were at least partially released in the rumen (Stamey *et al.*, 2012). Transfer efficiencies to the milk of C18:3*n-3*, C18:3*n-6* and C18:4*n-3* of dietary lipid encapsulated Echium oil were also maximally 3.9 %, to some extent due to a partial ruminal BH of the FA (Bainbridge *et al.*, 2015). This illustrates the rather limited potential of this protection technology to (partially) protect PUFA against ruminal BH.

3.2.5 Other encapsulation techniques

Jenkins *et al.* (2009) claimed a method to encapsulate feed supplements using polymeric microspheres. In this procedure, the protective coating is a biocompatible polymer such as poly-lactide or another aliphatic polyester and is biodegradable by passage through the acidic abomasum and enzymatic hydrolysis in the intestine, but resistant to degradation in the rumen. They claimed that at least 50 % of the encapsulated PUFA remained unhydrogenated after exposure to ruminal conditions for 24h. Jay *et al.* (2006) reported on the use of such microspheres to protect PUFA against ruminal BH. In this study, poly(D,L-lactide) and poly(L-lactide-co-caprolactone,75:25) were used to produce microspheres by using a single emulsion-solvent evaporation technique. Hereby, polymers are first dissolved in dichloromethane together with unesterified C18:2*n-6* and emulsified with polyvinyl alcohol as surfactant, resulting in the creation of microspheres after 12h agitation, which then can be filtered and dried. The latter procedure showed to be effective to prevent ruminal BH, but the polymer only poorly degraded in the abomasum.

More recently, another claim has been made to protect PUFA oils against ruminal degradation using a polymeric coating (Akashe *et al.*, 2014). In this method, the functional ingredient is coated with multiple layers: first, an inner coating of enteric material such as zein or caseinate, and second, an outer coating consisting of a delayed-release material such as gum arabic, gelatin, ethylcellulose or hydroxypropyl methylcellulose.

Further, Gawad *et al.* (2015) encapsulated linseed oil in alginate/carrageenan Ca beads to protect against rumen BH. Hereby, linseed oil was emulsified using Tween 80 in a gel

solution of alginate and κ-carrageenan. Linseed oil beads were formed by injection of the emulsion in a $CaCl_2$ solution as cross-linking agent to obtain hard gel beads. They demonstrated the potential of this method as a decreased BH of PUFA in alginate/carrageenan Ca beads was found upon *in vitro* incubation compared with unprotected linseed oil.

Also nano-encapsulation has been proposed to protect FA. Heo *et al.* (2016a) observed nano-encapsulated CLA, as free FA, was effectively protected against *in vitro* ruminal BH. Nano-emulsions of CLA could be created by passing a pre-emulsion of CLA in water with lecithin and glycerol as emulsifier through a high-pressure homogenizer (Heo *et al.*, 2016b), although it is unclear whether extra steps are needed to obtain rumen-protected nano-encapsulated CLA.

3.3 Techniques not falling under the aforementioned categories

Several other rumen lipid protection technologies have been developed or described in literature. Techniques not falling within the previous categories are briefly addressed below. Most of these methods were either abandoned for further research, did not result in sufficient protection and/or were only recently published.

In the late seventies, a method was proposed by Lyon *et al.* (1981) to prevent lipids from ruminal degradation by coagulate formation. Hereby, leafy green vegetable material, e.g. from alfalfa, was mixed with lipids, after which the mixture was coagulated by heating and/or dropping the pH between 3 and 4. Coagulates were separated from the mixture and heated above 80°C to obtain protein-lipid complex particles resistant to microbial degradation.

A new approach to prevent ruminal BH of PUFA, based on the application of esterase inhibitors, has been suggested by Sargolzehi *et al.* (2015). They demonstrated the potential of pyridostigmine bromide, a acetylcholine esterase inhibitor, to reduce rumen lipolytic activity, as no BH of C18:2*n-6* was observed after six hours *in vitro* incubation. Indeed,

lipolysis of triacylglycerides is a prerequisite for BH of PUFA. Antibiotics such as ionophores and amoxicillin (Van Nevel and Demeyer, 1995) or bacteria-specific IgY-antibodies (Krueger *et al.*, 2009) limited the rate of *in vitro* lipolysis only to a limited extent, i.e. up to 20 or 50 %, respectively.

Finally, other methods to reduce rumen BH of PUFA-rich sources such as treatment of grounded linseed with condensed quebracho tannins in the presence or absence of casein (Kronberg *et al.*, 2007) or the use of clays as oil adsorbents (Oliveira *et al.*, 2016) were also investigated, but were not effective in providing rumen protection.

4 Potential of a novel rumen bypass technology based on interfacial cross-linking of emulsions

In this section, the possibility of a new rumen lipid protection technology is explored, i.e. by cross-linking interfacial emulsifying proteins in order to protect unsaturated FA against ruminal BH. Techniques requiring harmful chemical reagents, e.g. glutaraldehyde (Romoscanu and Mezzenga, 2005), to cross-link adsorbed protein layers are excluded from this review, which particularly focuses on enzymatic approaches to cross-link interfacial layers of emulsions. Advantages of this technology include the rather small amounts of protein which are needed to emulsify oil, as protein is only needed to cover lipid emulsions, in the micrometer diameter range, with a thin layer of a few nanometers. Moreover, few enzyme is necessary too, as they are only needed as catalyst to induce cross-linking of the interfacial protein layer. The following sections will particularly deal with current knowledge of enzymatically induced cross-linking of emulsion interfaces, both for existing food and possible ruminant bypass applications.

4.1 Interfacial protein cross-linking of emulsions in food applications

4.1.1 Transglutaminase

One way of reinforcing adsorbed protein interfaces of emulsions is by adding transglutaminase. Most studies evaluate the effect on lipid oxidation, colloid stability and behavior in the human gastro-intestinal tract, but no ruminal applications of transglutaminase cross-linking of emulsions are reported yet.

Proteins such as bovine serum albumin (Chanyongvorakul *et al.*, 1997), β-lactoglobulin (Chanyongvorakul *et al.*, 1997; Faergemand *et al.*, 1997) and caseins (Kellerby *et al.*, 2006; Faergemand *et al.*, 1997) have been demonstrated to be good substrates for the transglutaminase reaction on proteins adsorbed at oil-water interfaces. It has been shown that transglutaminase-treated adsorbed β-casein layers are thinner than untreated ones due to an increase in layer density by the catalytic activity of the enzyme (Partanen *et al.*, 2013). This higher layer density, mainly by intramolecular cross-links, might reduce the contact between phases in an emulsion system, resulting in a kind of encapsulated physical structure (Partanen *et al.*, 2013). As such, cross-linked interfacial material forms a physical barrier for oxygen transfer which slows down oil oxidation (Ma *et al.*, 2012; Phoon *et al.*, 2014). In the study of Kellerby *et al.* (2006), a cohesive interfacial protein layer was formed after addition of transglutaminase to fish oil-in-water emulsions by cross-linking between the adsorbed sodium caseinate proteins. However, oxidative stability was not increased compared with untreated emulsions. Further, transglutaminase cross-linking of adsorbed interfacial protein was found to reduce coalescence and Ostwald ripening in oil-in-water emulsions (Faergemand *et al.*, 1998). However, cross-linking protein prior to emulsification has the disadvantage of leading to poorer emulsion stability than post-emulsification cross-linking (Sharma *et al.*, 2002). In relation to the physical stability of emulsions, claims have also been made about solid oil-based products whereby transglutaminase was used to cross-link proteins at the interface of oil droplets containing unsaturated FA (Romoscanu and

Mezzenga, 2012). Recently, scanning electron micrographs illustrated spray-dried soy protein particles containing α-tocopherol had a dense non-porous wall structure due to the formation of a compact protein network after cross-linking with transglutaminase (Nesterenko *et al.*, 2014), which might have an effect on its release. Similarly, the *in vitro* study of Macierzanka *et al.* (2012) demonstrated transglutaminase cross-linked interfaces of sodium caseinate emulsions were less susceptible to pepsinolytic hydrolysis. However, no complete prevention of hydrolysis occurred as even the thickest cross-linked layers were susceptible to proteolysis by trypsin and chymotrypsin and displacement by bile salts under duodenal conditions. A recent *in vivo* study with similar cross-linked emulsions showed that modification of the interfacial layer of an emulsion can alter the early postprandial profiles of glucose, insulin, cholecystokinin, appetite and satiety through decreased protein digestion, but without significantly affecting gastric emptying or overall digestion of lipids (Juvonen *et al.*, 2015). Overall, important factors in the extent of protein cross-linking are the time of transglutaminase addition (i.e. prior, during or after emulsification), doses of enzyme and protein, and type or flexibility of the proteins, as the reaction requires the availability of certain amino acid side chains (Macierzanka *et al.*, 2011; Ridout *et al.*, 2015).

4.1.2 Tyrosinase

Similar to transglutaminase, tyrosinase-induced cross-linking could be hypothesized to strengthen the interfacial protein layers. However, contradictory results have been reported so far. The study of Ercili-Cura *et al.* (2012) showed the interfacial film strength of β-lactoglobulin at the air-water interface was lowered by cross-linking using tyrosinase from *Trichoderma reesei*. This could be due to the low amount of tyrosine residues in this protein. On the other hand, heat treatment could improve the cross-linking through a decrease in the ordered secondary structure of the protein, liberating more reactive sites for the cross-linking enzyme (Ercili-Cura *et al.*, 2012). Isaschar-Ovdat *et al.* (2015) recently demonstrated cross-linking of soy glycinin using tyrosinase from *Bacillus megaterium* after emulsification led to the formation of cold-set gel-like structures of small droplets linked by covalent bonds without

the need for an external cross-linker, which they link to the increased amounts of tyrosine residues being exposed upon protein adsorption. Also whey proteins, cross-linked using tyrosinase extracted from *Agaricus bisporus* showed higher emulsifying activity, foam capacity and foam stability compared with their non-cross-linked counterparts (Wu *et al.*, 2013). Alternatively, a cross-linking mediator such as small phenolics might be needed to make tyrosinase a real effective cross-linker. Ali *et al.* (2013) and Reinkensmeier *et al.* (2016) showed an improved stability of emulsified lipids when cross-linking proteins and phenols. Digestion of tyrosinase cross-linked interfacial protein layers of emulsions are not reported yet. However, β-casein which was cross-linked by *Trichoderma reesei* tyrosinase has been shown to be broken down in many small protein fragments during *in vitro* gastric digestion (Monogioudi *et al.*, 2011) and was more resistant to proteolytic digestion compared to native β-casein (Monogioudi *et al.*, 2011; Stanic *et al.*, 2010). In conclusion, the use of tyrosinase to cross-link interfacial protein layers is far less studied as compared with transglutaminase cross-linking, presumably due to the lack of a commercial, economic and large-scale enzyme production.

4.1.3 Laccase and peroxidase

Unlike the tyrosinase application whereby quinones are generated, another technique is to cross-link interfacial layers of emulsions through radical coupling (Figure 3.2e). Laccase acts on phenolic compounds resulting in the formation of free radicals. Up till now, this mechanism is particularly applied to stabilize oil-in-water emulsions with multiple layers by laccase or peroxidase-induced cross-linking. Hereby, a primary protein-stabilized emulsion is coated with a polysaccharide by electrostatic deposition, which is essentially the only mechanism to hold together the different layers of these multiple emulsions. Accordingly, colloidal properties were hypothesized to be improved by laccase- or peroxidase-induced cross-linking. Littoz and McClements (2008) were the first to demonstrate this using β-lactoglobulin and beet pectin, whereby the ferulic acid groups within the beet pectin can be covalently cross-linked with protein by laccase, resulting in beet pectin layers that remain

attached to the droplet surface upon changes in pH. Similarly, enzymatic cross-linking has been applied to enhance the physical stability of emulsions stabilized with ferulic acid containing compounds and proteins (Zeeb *et al.*, 2012; Zaidel *et al.*, 2013; Sato *et al.*, 2015; Zeeb *et al.*, 2011) or soybean oil bodies (Chen *et al.*, 2010). Storage-stable protein emulsions could also be obtained when whey protein isolate was chemically modified by vanillic acid in order to enhance its cross-link ability due to the creation of extra ferulic acid-like reactive sites for the laccase enzyme (Ma *et al.*, 2011). Also peroxidase can be used to cross-link the ferulic groups of the pectin coating of multilayer emulsions to improve emulsion stability (Li *et al.*, 2012; Zaidel *et al.*, 2013). However, Beicht *et al.* (2013) stated formation of covalent cross-links between single pectin molecules might not be sufficient to modify the porosity or permeability of the interfacial membrane, as laccase-induced cross-linking of adsorbed sugar beet pectin did not lower the release of lutein in comparison to non-cross-linked emulsions. Zeeb *et al.* (2015b) confirmed this observation, as the rate and extent of *in vitro* gastro-intestinal lipid digestion of fish gelatin emulsions electrostatically deposited with sugar beet pectin did not differ whether or not they were cross-linked with laccase. This means multilayer laccase cross-linked coatings might only be of interest to improve the stability of lipid droplets against aggregation and not/less for encapsulation purposes.

4.1.4 Non-enzymatic approaches

Besides enzymatic approaches, alternatives exist to obtain cross-linked emulsion interfaces, without the use of harmful chemical cross-linkers. Several studies reported on the cross-linking of interfacial proteins by applying heat. Thermal cross-linking of a protein-stabilized interface was suggested as an efficient method to provide elasticity to the interface (Mezzenga and Ulrich, 2010) and to improve the functional properties of spray-dried emulsions (Wang *et al.*, 2016; Mezzenga and Ulrich, 2010). However, results of thermal treatment of protein-stabilized emulsions on lipid oxidation are conflicting (Berton-Carabin *et al.*, 2014). Also Sandra *et al.* (2008) suggested adsorbed β-lactoglobulin proteins cross-linked when subjecting triacylglyceride emulsions to heat treatment. However, this interfacial

cross-linking did not affect digestion by pancreatic lipase, suggesting lipase could adsorb to the droplet surface and gain access to the emulsified triacylglycerides irrespective of the nature of the interfacial layer surrounding the droplets.

Further, claims have been made on the creation of microcapsules by interfacial cross-linking of plant polyphenols (Levy and Andry, 1998). However, creation of impermeable capsules requires treatment of polyphenols, e.g. flavonoids, with a cross-linking agent such as diacid halides.

Recently, the use of genipin as cross-linking agent of emulsions has been proposed. Hu *et al.* (2015) demonstrated that the cross-linking of the interfacial casein layer using genipin led to enhanced stability of nanoemulsions and delayed the digestion of lipid in the droplets under simulated conditions. Genipin-induced cross-linking of legume protein isolates also resulted in an increased creaming stability of the emulsions (Johnston *et al.*, 2015). Here, it was thought a strengthening of the interface occurred by covalent cross-links between the exposed lysine groups of proteins at the oil-water interface, whereby further crosslinking with neighboring proteins remaining in the bulk solution increased the continuous phase viscosity which lead to enhanced emulsion stability.

4.2 Rumen lipid protection, emulsions and PPO-induced cross-linking: the way forward?

As demonstrated in the previous section, several cross-linking techniques already have been applied to obtain a reinforced interfacial protein barrier. Most of them aimed at optimizing emulsion stability and limiting lipid oxidation during storage. Only a limited number of studies investigated the digestion of cross-linked protein interfaces of emulsions and the encapsulated lipid content in the gastro-intestinal tract and fewer succeeded in limiting digestion of cross-linked protein. To our knowledge, none of such interfacial cross-linking studies yet has demonstrated an effective protection against lipid digestion in the gastro-intestinal tract (of humans or other monogastrics).

The possible use of PPO-induced cross-linked protein to provide an effective rumen protection of lipid in roughages has been postulated before (see section 3 of Chapter 1). Indeed, Van Ranst *et al.* (2011) hypothesized PPO-catalyzed quinone formation may be involved in the formation of a network of protein-bound phenol entrapping thylakoid lipid, resulting in some kind of encapsulation which prevents thylakoid PUFA from ruminal BH. Based on this forage-focused work, this naturally occurring mechanism is now proposed to be the main driving force of a new method to protect non-roughage PUFA against BH. The hypothesis of this new protection mechanism is outlined in Figure 3.9. It is postulated that lipophilic nutrients, such as linseed oil, could be encapsulated and protected against degradation by ruminal microbes by emulsifying a PUFA-rich oil with a PPO-containing extract and treating this with a diphenol, in order to form a protective interfacial barrier that withholds ruminal bacteria to hydrolyze and hydrogenate the emulsified lipids.

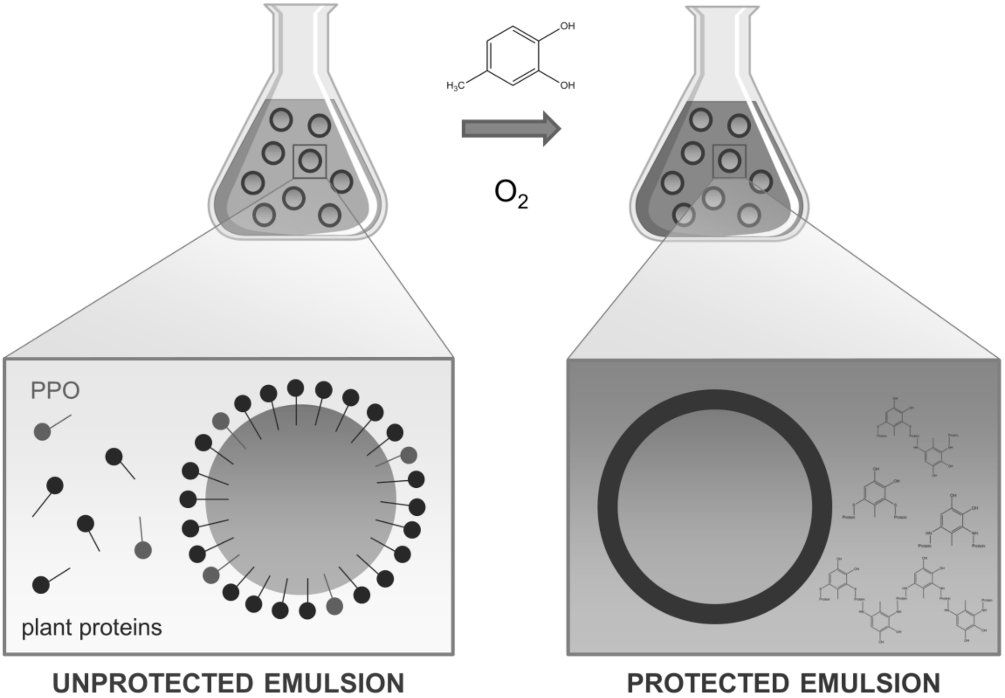

Figure 3.9 Principle of the polyphenol oxidase (PPO) protection mechanism of oil-in-extract emulsions by encapsulating oil within a cross-linked interfacial barrier of protein-bound phenols

5 Conclusions

Several technologies aiming to protect PUFA from ruminal BH have been described in patents and scientific literature. Up till now, it remains unclear and debatable which technique is the best due to the large variation in transfer efficiencies of dietary PUFA to milk, both between and within protection techniques. The highest range of transfer efficiencies was observed for the formaldehyde and lipid composite gel treatments. Additionally, the toxicity of formaldehyde is another major concern which limits the use of formaldehyde-treated fats in practice. Practical application of other techniques such as Ca salts, fatty acyl amides and encapsulation within lipids are better in this respect, but is hampered because they are rather expensive or are facing lower PUFA transfers from the diet to the milk. Therefore, lipid composite gels seem to be the best alternative at present. Nevertheless, alternative mechanisms are still being developed. Enzymatic approaches to cross-link interfacial protein layers of PUFA-rich emulsions to prevent their hydrogenation might be a new promising concept. The use of the natural and ubiquitously present PPO enzyme in this technology could open a new range of possibilities for further improvements in rumen lipid bypass technologies.

In the following parts of PART II, the objective is to explore the concept of this new PPO-based rumen bypass technology. In PART II A, red clover (***Chapter 4***) or other protein sources (***Chapter 5***) are used to protect emulsified PUFA against ruminal BH or to protect lipophilic nutrients against oxidation during storage (***Chapter 6)***. Further the post-ruminal availability of such rumen protected PPO-based emulsions is examined in PART II B, either by means of a mice trial (***Chapter 7***), an *in vitro* method (***Chapter 8***) as well as an *in vivo* trial with dairy cows (***Chapter 9***). Finally, in PART II C, the first steps were put towards upscaling of the protection technology (***Chapter 10***).

6 Acknowledgements

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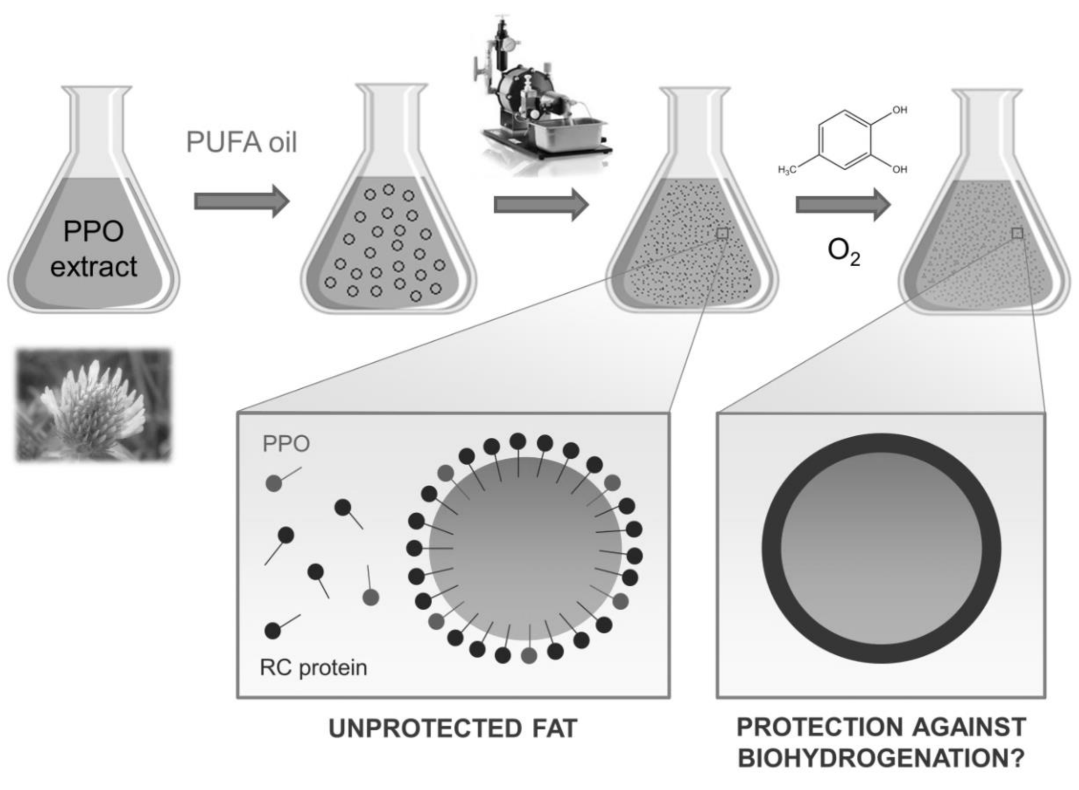
PART II A

PROTECTION OF EMULSIFIED FATTY ACIDS:

RUMEN BIOHYDROGENATION

Chapter 4

Protection of polyunsaturated oils against ruminal biohydrogenation using a polyphenol oxidase containing extract of red clover



Largely redrafted after Gadeyne F., Van Ranst G., Vlaeminck B., Vossen E., Van der Meeren P. and Fievez V., 2015, Protection of polyunsaturated oils against ruminal biohydrogenation and oxidation during storage using a polyphenol oxidase containing extract from red clover, Food Chemistry, 171, 241-250.

Partially redrafted after Fievez V., Gadeyne F. and Van Ranst G., 2015, Method to protect lipophilic nutrients against ruminal degradation, International Publication Number: WO 2015091840 A1

Chapter 4

Protection of polyunsaturated oils against ruminal biohydrogenation using a polyphenol oxidase containing extract of red clover

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Abstract. Polyunsaturated fatty acid (PUFA) are to a large extent prone to biohydrogenation (BH) in a ruminal environment, resulting in the loss of its healthy features when being fed to ruminants. Therefore, it was tested whether emulsions could be protected against *in vitro* ruminal BH by using protein extracts rich in polyphenol oxidase (PPO), an enzyme responsible for browning of plant tissues. PUFA rich emulsions were made with a protein extract of red clover before adding a synthetic diphenol (4-methylcatechol; 4-MC) to induce protection. Results after *in vitro* incubation confirmed the hypothesis and indicated the potential to prevent PUFA in linseed or fish oil from ruminal BH through addition of 4-MC to the emulsions. Protection depended on the amount of oil present and protein concentrations in the emulsions. Protection efficiency increased with increasing amounts of diphenol present in the emulsion per unit interfacial surface area. It is suggested that protection is caused by an effective encapsulation by cross-linking of the protein layer at the emulsion interface. For the first time, a method is described to protect PUFA using an enzyme abundantly available in nature, PPO, in combination with 4-MC.

1 Hypothesis and objective

The goal of this study was to evaluate, for the first time, the concept of protecting PUFA against rumen BH with PPO from a red clover extract. The protection is hypothesized to be obtained through encapsulation of emulsified lipids within protein-phenol complexes created by the action of PPO, an enzyme abundantly present in nature, including red clover (Mayer,

2006). Further, several factors which might determine the protection efficiency were addressed. To achieve protection, emulsions were prepared using protein extracts of red clover, which served as a source of both PPO as well as emulsifier. Afterwards, the efficacy of these red clover stabilized emulsions to protect PUFA against ruminal BH was assessed both with and without the supplementation of a diphenol.

2 Materials and Methods

2.1 Materials

Red clover (*Trifolium pratense* L. cv. Lemmon) was used as plant source in all experiments. It was sown in May 2010 (location: 50°59'4"N/3°47'6"E) and harvested 10 cm above ground level on different occasions during the growing season. Red clover received three fertilizer applications (in March and after the first and second harvest of the year; 6 kg N/ha, 20 kg P_2O_5/ha, 140 kg K_2O/ha). At harvest, red clover was at the early blooming stage. Plant material was frozen at -80°C immediately after harvest. The cultivar Lemmon was used, as this cultivar is known for its high PPO activity (Van Ranst *et al.*, 2009b).

Crude linseed oil (40.2, 194, 169 and 586 mg C18:0, C18:1*n-9*, C18:2*n-6* and C18:3*n-3* respectively per g total fatty acids) and fish oil (68.3, 179, 92.8, 38.6, 99.0, 187 and 112 mg C14:0, C16:0, C16:1*n-7*, C18:0, C18:1*n-9*, C20:5*n-3* and C22:6*n-3* respectively per g total fatty acids) were delivered by Dumoulin (Kortrijk, Belgium) and Nuscience (Drongen, Belgium), respectively. Casein was added to some (see further) emulsions as casein acid hydrolysate (Sigma, Diegem, Belgium). 4-MC, caffeic acid and chlorogenic acid were also purchased from Sigma-Aldrich (Bornem, Belgium). All other chemicals were of analytical grade and were purchased from either Sigma-Aldrich (Bornem, Belgium), Merck (Darmstadt, Germany), Carl-Roth (Karlsruhe, Germany) or VWR (Heverlee, Belgium), unless stated otherwise.

2.2 Preparation and characterization of protected fatty acid emulsions: general procedure

A three-step process was performed: first, proteins were extracted from red clover, second, a PUFA rich oil was emulsified with this protein extract and third, creation of protein-phenol complexes was induced by adding a synthetic diphenol. It was chosen to use PPO from an extract of plant origin instead of pure PPO, as purchased PPO is generally of fungal origin and protein concentrations would be too low to deliver enough surfactant to obtain stable emulsions. As the hypothesis for this work originated from previous studies with red clover (summarised by Van Ranst *et al.* (2011)), the latter was used as a vegetal source of PPO. A summary of emulsion characteristics in the different experiments is given in Table 4.1. Adjustments to the general procedure in the various experiments are mentioned in the Results section.

Proteins were extracted according to Van Ranst *et al.* (2009a) with adaptations. In short, 150 g of frozen red clover, 500 ml of 0.1 M sodium phosphate buffer with 30 mM ascorbic acid (pH=7.0), 0.5 g Triton X-100 and 1 g polyvinylpolypirrolidone were mixed in a blender for 1 min. After filtration and centrifugation (10000×g, 15 min, 4°C), acetone was added to the supernatant until a concentration of 800 ml acetone per liter total volume and kept at -18°C for 35 min. After centrifugation (5000×g, 5 min, 4°C) the pellet was re-dissolved in 400 ml of a 10 mM sodium phosphate buffer (pH=7.0) without ascorbic acid. This concentrated protein extract was used to measure PPO activity and protein content as described by Van Ranst *et al.* (2009a). To determine PPO activity, the absorbance (A) was measured at 400 nm 20, 30 and 40 seconds after addition of 4-MC. PPO activity was expressed as ΔA/min/mg protein. Folin-Ciocalteau reagent was added to the protein extract with copper and NaK-tartrate and the absorption was measured at 750 nm to determine the protein content, which was expressed as g protein per liter extract. PPO activity and protein content analysis was done in duplicate.

Table 4.1 Emulsion characteristics for the different experiments assessing protection of fatty acids against biohydrogenation. After their preparation and characterization, these emulsions were either or not treated with 4-methylcatechol[a] to create protein-bound phenol complexes, attempting to achieve rumen bypass products

	Emulsion treatment			D[4,3]	D[3,2]	D[v,0.5]	D[v,0.9]	SSA
	Cycles[b]	Oil[c]	CAS[d]	(µm)	(µm)	(µm)	(µm)	(m²/g oil)
Experiment 1	2	20	0	1.63	0.76	1.25	3.51	8.49
	4	20	0	1.44	0.96	1.22	2.70	6.72
	6	20	0	1.45	1.05	1.20	2.54	6.14
Experiment 2	8	20	0	1.43	1.21	1.31	2.24	5.33
	8	20	1	0.60	0.47	0.54	1.00	13.7
	8	20	2	0.56	0.47	0.52	0.88	13.7
Experiment 3[a]	5	20	0	0.78	0.56	0.64	1.21	11.4
Experiment 4	5	20	0	12.6	1.73	3.69	23.4	3.72
	5	40	0	9.88	2.61	5.38	20.4	2.47
	5	60	0	12.1	2.73	5.65	33.3	2.37
	5	80	0	23.0	3.62	13.7	57.2	1.79
	5	100	0	38.4	4.79	24.9	96.0	1.48
	5	120	0	25.0	4.64	22.2	54.8	1.41
Experiment 5	5	20	0	12.6	1.73	3.69	23.4	3.72
Experiment 6	5	10	0	0.85	0.67	0.79	1.45	9.63
	5	10	1	0.61	0.47	0.53	1.06	13.7
	5	10	2	0.56	0.44	0.49	0.95	14.7
	5	20	0	1.23	1.04	1.17	1.90	6.20
	5	20	1	1.08	0.77	0.94	1.88	8.38
	5	20	2	0.71	0.52	0.60	1.28	12.4
	5	30	0	1.62	1.34	1.50	2.58	4.81
	5	30	1	1.43	1.04	1.25	2.50	6.20
	5	30	2	1.15	0.56	0.86	2.54	11.5
	5	40	0	1.93	1.61	1.76	3.11	4.01
	5	40	1	2.24	1.54	1.82	3.95	4.19
	5	40	2	1.87	1.05	1.65	3.63	6.14
Experiment 7	5	20	0	2.67	0.87	1.20	4.42	7.48
Experiment 8	5	15	0	1.43	1.16	1.26	2.34	5.56

CAS, casein; D[4,3], volume-weighted mean diameter; D[3,2], surface-weighted mean diameter; D[v,0.5], 50 % median volume distribution diameter, meaning 50 % of the population shows droplet sizes with a diameter below the value mentioned; D[v,0.9], 90 % volume distribution diameter, meaning 90 % of the population shows droplet sizes with a diameter below the value mentioned; SSA, specific surface area (m²/g oil)

[a] besides 4-methylcatechol, caffeic acid or chlorogenic acid was used in experiment 3

[b] number of cycles emulsions passed through a microfluidizer at 25 MPa

[c] amount of linseed oil emulsified in g per liter protein extract, except for experiment 2 where fish oil was used

[d] amount of casein in g per liter protein extract, added to the continuous phase of the emulsion before emulsification

The concentrated protein extract, containing PPO, was further used to emulsify linseed or fish oil. First, coarse emulsions were created with a high speed Ultraturrax (T25 Basic, Ika Werke, Staufen, Germany), containing 20 g linseed oil per liter red clover protein extract, unless stated otherwise. These emulsions were put into a microfluidizer (M110S,

Microfluidics Corporation, Newton, Massachusetts, USA) and passed five times to create stable emulsions with small droplet sizes, unless stated otherwise, at a compressed air pressure of 0.18 MPa, which corresponds to a liquid pressure of 25 MPa. Applying pressures higher than 25 MPa resulted in rather unstable emulsions which easily started to cream after passing through the microfluidizer. During processing, emulsions were cooled by passing through a heat exchanger coil immersed into an ice-water bath. Particle size distributions were checked immediately after preparation with a Mastersizer S (Malvern Instruments, Malvern, UK) equipped with a 300RF lens. The automated sample dispersion unit MS-17 (Malvern Instruments, Malvern, UK) was used. Data were analyzed with the polydisperse model. Hereby, the real refractive index of the oil was fixed at 1.5295, whereas the imaginary refractive index was assumed to be 0.1000. Droplet sizes were characterized in terms of volume-weighted mean diameter (D_{43}), Sauter surface-weighted mean diameter (D_{32}), median volume-weighted distribution value D[v,0.5] and 90 % percentile of the volume-weighted distribution D[v,0.9] using the available software (Malvern Instruments, Malvern, UK). Specific surface areas (in m^2/g oil) were calculated based on an assumed oil density ρ of 930 kg/m^3 from the Sauter mean diameter: $SSA = 6 / [D_{32} \times \rho]$.

Finally, to induce protein-phenol complexing, 10 % (v/v) of a synthetic diphenol solution in distilled water was added to the emulsions to reach a concentration of 9/1 emulsion/diphenol solution. In all experiments, 4-MC was used, unless stated otherwise. Test emulsions had a final concentration of 20 mM 4-MC, unless stated otherwise. Emulsion mixtures were continuously shaken on a Unimax 2010 platform shaker (Heidolph, Schwabach, Germany) for 24h, unless stated otherwise, at room temperature (between 20 and 25°C) in order to allow PPO activity. Until further analysis, protected emulsions were stored in a refrigerator at 4°C, unless stated otherwise.

2.3 Assessment of reaction time upon addition of diphenol

To assess how long it takes to protect an emulsion against ruminal BH upon addition of a synthetic diphenol, time series were considered. Protection of emulsions was created as described before. However, emulsion mixtures were continuously shaken for 0, 0.5, 1, 2, 4, 8 or 24h at room temperature (between 20 and 25°C) before addition of 4-hexylresorcinol (4-HR; 50 % ethanol solution) and storage in a refrigerator at 4°C to obtain different levels of protection. It has been shown before that 4-HR is an effective inhibitor of PPO (Arias *et al.*, 2007). Emulsions had a final concentration of 3 mM 4-HR.

2.4 Assessment of protection against ruminal biohydrogenation

In vitro batch incubations were performed to evaluate the protection of PUFA in the various emulsions against ruminal BH. Therefore, 0.5 or 1 ml emulsion, 250 mg hay, 20 ml buffer solution (containing 3.58 g $Na_2HPO_4.12H_20$, 1.55 g KH_2PO_4, 0.124 g $MgCl_2.6H_20$, 8.74 g $NaHCO_3$ and 1 g NH_4HCO_3 per liter of distilled water) and 5 ml of rumen fluid were added to 125-ml incubation flasks. Rumen fluid was collected before the morning feeding from three rumen fistulated sheep, which were fed hay ad libitum and had free access to drinking water. Fistulation of the sheep was approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO), Belgium (EC 2009, 114). Rumen contents from the three sheep were combined and filtered through a sieve with a pore size of 1 mm under continuous CO_2 flushing at 39°C. Incubation flasks were thoroughly flushed with CO_2 to obtain anaerobic conditions and incubated under intermittent shaking at 39°C for 24h in a batch culture incubator (Edmund Bühler GmbH, Hechingen, Germany). After 24h of *in vitro* rumen incubation, flasks were removed from the incubator and placed in ice water to stop the microbial activity. After gas analysis (Hassim *et al.*, 2010), pH was measured (Hanna Instruments, Temse, Belgium) and culture contents were sampled for analysis of volatile fatty acids (Castro-Montoya *et al.*, 2012). Results for pH, gas and volatile fatty acids are not shown, as no differences were observed between treatments within the different experiments

and the control treatments, indicating that changes in extent of BH were not due to changes in microbial activity. For analysis of long chain fatty acids (FA), 5 ml of incubation fluid was taken and freeze-dried. Each emulsion was incubated in triplicate. Also, each incubation contained a non-protected control emulsion, which is an emulsion without added synthetic diphenol. Protection efficiencies were assessed through comparison with this control.

2.5 Fatty acid analysis

Lipids of freeze-dried culture content and emulsions were extracted and methylated using a direct transesterification reaction by a base-catalyzed followed by an acid-catalyzed step, based on the method described by Sukhija and Palmquist (1988), with modifications as described further. Toluene (2 ml), containing 0.2 mg/ml tridecanoic acid (Sigma, Diegem, Belgium) as an internal standard, was added. Extraction tubes were thoroughly vortexed with a Multi-Tube Vortex (VX-2500, VWR International, Leuven, Belgium) after which methanolic sodium hydroxide was added (2 ml, 0.5 M NaOH dissolved in methanol). Tubes were vortexed, incubated in a warm water bath at 70°C for 1 h and cooled again in an ice bath for 5 min. After addition of methanolic hydrochloric acid (3 ml), prepared by dissolving 10 ml acetyl chloride in 50 ml ice cold methanol, tubes were vortexed and incubated in an air oven at 50°C for 30 min. After cooling down in an ice bath for 5 min, hexane (3 ml) and water saturated with $NaHCO_3$ (4 ml) was added, vortexed and centrifuged (5 min, 1111×g). The supernatants, containing the methylated fatty acids, were taken off with a Pasteur pipet and filtered over a column containing glass wool, silica gel and active coal before pre-washing with hexane. Filtered solvents were evaporated using N_2, fatty acids re-dissolved in hexane (1 ml) and transferred to GC vials before gas chromatography analysis.

Fatty acids were analyzed with a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Brussels, Belgium) with a Solgel-wax column (30m x 0.25mm x 0.25µm; SGE Analytical Science, Victoria, Australia). The temperature program was as follows: 150°C for 2 min; increased at 3°C/min until 250°C; injector temperature: 250°C; detector temperature

280°C. For this temperature program, 2 µl was injected in which the split/splitless ratio was 50:1. Fatty acid peaks were identified based on their retention times, compared to external standards (BR2 and BR3, Larodan Fine Chemicals AB, Malmö, Sweden; Supelco 37, Supelco Analytical, Pennsylvania, USA; PUFA-3, Matreya LLC, Pleasant Gap, Pennsylvania, USA). Quantification of FA methyl esters was based on the area of the internal standard and on the conversion of peak areas to the weight of FA by a theoretical response factor for each FA (Wolff *et al.*, 1995; Ackman and Sipos, 1964).

In vitro rumen BH was calculated as described by Van Ranst *et al.* (2010): BH of C18:3*n-3* was calculated as [(proportion of 18:3 in total C18 FA)$_{0h}$ – (proportion of C18:3 in total C18 FA)$_{24h}$] / (proportion of C18:3 in total C18 FA)$_{0h}$. Efficiency of protection of C18:3*n-3* was calculated as [(BH of C18:3*n-3*)$_{non\text{-}protected}$ – (BH of C18:3*n-3*)$_{protected}$] / (BH of C18:3*n-3*)$_{non\text{-}protected}$. BH and protection efficiencies for C18:2*n-6*, C20:5*n-3* and C22:6*n-3* were calculated similarly. For C20:5*n-3* and C22:6*n-3*, proportions are based on total C20 and C22 FA, respectively.

2.6 Statistics

Results were analyzed by the MIXED procedure of SAS (SAS Enterprise Guide 5.1, SAS Institute Inc., Cary, North Carolina, USA). Prior to statistical analysis, technical replicates were averaged. Hence, the design didn't allow assessment of all interactions in experiment 1, 2 and 6, but did allow assessment of all (n-1)-way interactions (with n the number of factors tested).

Experiment 1 and 2 were analyzed by the following model: $Y_{ij} = \mu + T_i + D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, T_i the effect of treatment (i = 2 x 25 MPa, 4 x 25 MPa or 6 x 25 MPa for experiment 1 and i = 0, 1 or 2 g extra casein per liter emulsion for experiment 2), D_j the effect of diphenol concentration in the emulsion (j = 0 or 20 mM 4-MC) and ε the residual error.

Experiment 6 was analyzed by the following model: $Y_{ijk} = \mu + O_i + P_j + D_k + O_i \times P_j + O_i \times D_k + P_j \times D_k + \varepsilon_{ij}$, with Y_{ij} the variable of interest, O_i the effect of oil concentration (i = 10, 20, 30 or 40 g per liter red clover protein extract), P_j the effect of extra protein (j = 0, 1 or 2 g extra casein per liter emulsion), D_k the effect of diphenol concentration in the emulsion (k = 0, 12.5, 25 or 50 mM 4-MC) and ε the residual error.

The following model was used in experiment 7: $Y_{ij} = \mu + T_i + D_j + T_i \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, T_i the fixed effect of treatment (i = original or washed emulsions), D_j the fixed effect of diphenol concentration (j = 0, 12.5, 25 or 50 mM 4-MC) and ε_{ij} the residual error.

All mentioned differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

3 Results

3.1 Experiment 1: Effect of diphenol addition

In a first experiment, it was examined whether BH could be decreased and whether increasing the number of cycles for emulsions to pass through the microfluidizer influenced the protection efficiency. Emulsions were passed through the microfluidizer at 25 MPa for 2, 4 or 6 cycles (n=3) and stored at -80°C until incubation. The red clover extract (n=1) of experiment 1 contained 1.47 g protein per liter and had a specific PPO activity of 1825 ΔA/min/mg protein. Results are shown in Figure 4.1 (left). Adding 4-MC to the emulsions resulted in a significant decrease in BH for C18:2*n-6* and C18:3*n-3* (P=0.001). Although no differences in BH for the different treatments were found for C18:2*n*-6 (P=0.630) and C18:3*n-3* (P=0.793), it was decided to keep on working with emulsions passed more than twice through the microfluidizer, because this resulted in smaller emulsion droplet sizes as well as more narrow distributions, as seen from the reduction in 90th percentile with increasing number of cycles (Table 4.1), yielding more stable emulsions. Preliminary experiments

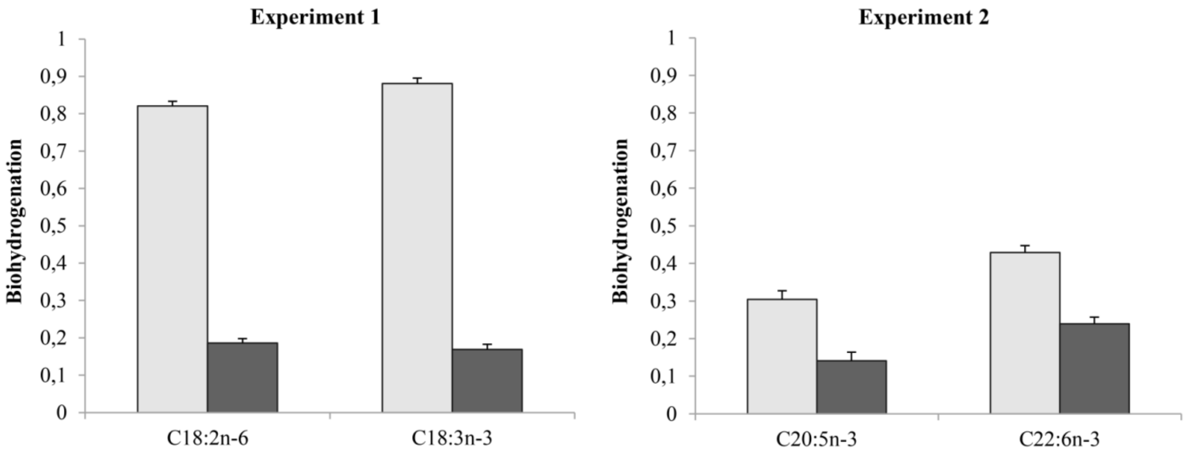

Figure 4.1 Addition of 4-methylcatechol reduces 24h *in vitro* biohydrogenation of both C18:2*n-6* and C18:3*n-3* from linseed oil-in-extract emulsions (experiment 1; prepared by varying the number of cycles emulsions passed through a microfluidizer at 25 MPa) and C20:5*n-3* and C22:6*n-3* from fish oil-in water emulsions (experiment 2; prepared by passing 8 times through a microfluidizer at 25 MPa, with 0, 1 or 2 g/l extra casein). Grey bars represent no addition of 4-methylcatechol, black bars represent emulsions having a final concentration of 20mM 4-methylcatechol. Error bars represent the standard error of the mean (3 original emulsions)

showed that stable emulsions were a prerequisite to obtain some level of protection (results not shown).

3.2 Experiment 2: Effect of oil type

Fish oil was used in experiment 2 (Figure 4.1, right). The red clover extract (n=1) used in this experiment contained 1.70 g protein per liter and had a specific PPO activity of 2486 ΔA/min/mg protein. Additionally, 0, 1 or 2 g casein hydrolysate per liter was added and emulsions (n=3) were stored at -80°C until incubation. As C20:5*n-3* and C22:6*n-3* were the major compounds of interest in fish oil, BH was calculated based on the latter FA. Adding 4-MC similarly resulted in decreased BH for C20:5*n*-3 (P=0.038) and C22:6*n-3* (P=0.017). Casein hydrolysate as extra emulsifier reduced the emulsion droplet sizes with concomitant larger specific surface areas (Table 4.1). Extra casein did not affect BH (P=0.172 for C20:5*n-3* and P=0.850 for C22:6*n-3*). BH values of 4-MC-free emulsions were lower compared to linseed oil emulsions, which is often observed *in vitro* for C20:5*n-3* and C22:6*n-3*, most notably because of the toxic effect on ruminal metabolism of these long chain PUFA (Maia *et al.*, 2007). For comparative reasons, linseed oil emulsions were also made by adding only 2

g of casein per liter as emulsifier, so without 4-MC or red clover protein extract, but this resulted in no differences in BH as compared with the 4-MC-free emulsions with or without red clover (results not shown). This indicated that both red clover protein extract, containing PPO, and 4-MC as diphenolic substrate were required to obtain a reduction in BH.

3.3 Experiment 3: Effect of diphenol type

In the third experiment, caffeic acid and chlorogenic acid were tested as alternatives for 4-MC in order to create protection. Emulsions (n=1) contained final diphenol concentrations of 0 or 15 mM. In comparison to 4-MC which was solubilized in water, both caffeic acid and chlorogenic acid were solubilized in ethanol due to their poor solubility in water at the desired concentration. The red clover extract (n=1) used in this experiment contained 1.50 g protein per liter and had a specific PPO activity of 996 ΔA/min/mg protein. Results are shown in Figure 4.2. A large reduction in BH of C18:3*n-3* after 24h *in vitro* incubation was observed when 15 mM of 4-MC was used compared with no diphenol. When the same concentrations of caffeic acid or chlorogenic acid were applied, only a very minor or no reduction in *in vitro*

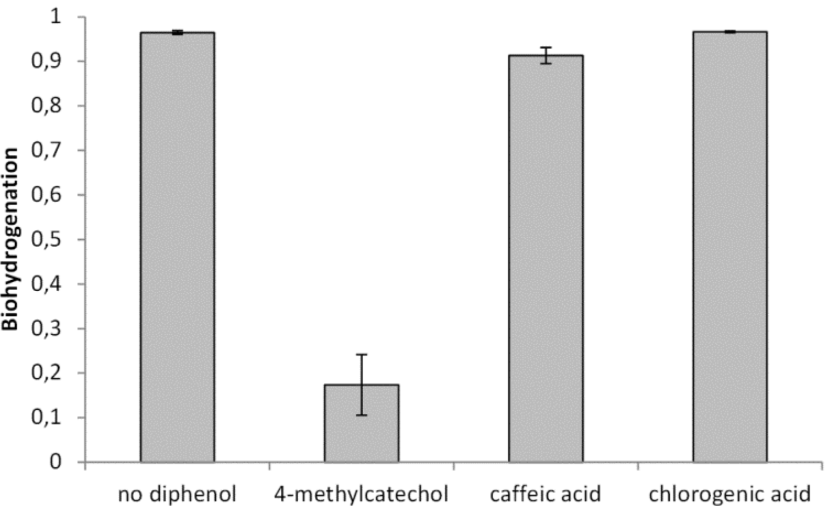

Figure 4.2 Addition of 4-methylcatechol reduced 24h *in vitro* biohydrogenation of C18:3*n-3* from linseed oil-in-extract emulsions, but very minor or no reduction was observed when caffeic acid or chlorogenic acid, respectively, was applied (experiment 3). Emulsions with diphenol contained a final diphenol concentrations of 15 mM. Error bars represent the standard deviation over three analytical replicates (1 original emulsion)

BH was observed. Similar results were obtained when a dispersion of caffeic acid or chlorogenic acid in water was used, instead of solubilization in ethanol (results not shown). Based on these results it was decided to continue working with 4-MC because of the large reductions in BH of C18:3*n-3* and the good solubility in water. Besides, 4-MC could be considered as a safe technical aid, as it has been found in beaver castoreum (Müller-schwarze and Houlihan, 1991) and is produced during intestinal digestion (Morita et al., 2003) or ruminal metabolism (Berger et al., 2015) of quercetin, a natural compound found in various fruits and plants (Morita et al., 2003). At cellular level, 4-MC has been considered cytotoxic as well as protective, depending on the context (Payton et al., 2011).

3.4 Experiment 4: Effect of oil concentration

In experiment 4, increasing concentrations of oil were tested. Indeed, in the previous experiments only low oil percentages were protected against BH. Six concentrations of oil were tested: 20, 40, 60, 80, 100 and 120 g of linseed oil per liter red clover protein extract. The red clover extract (n=1) used in this experiment contained 1.94 g protein per liter and had a specific PPO activity of 1144 ΔA/min/mg protein. Results are depicted in Figure 4.3. As observed before, the absence of 4-MC during the 24h shaking of the emulsions (n=1) resulted in unprotected emulsions, as BH levels for C18:3*n-3* of about 0.95 were found. Addition of 4-MC to reach a final concentration of 20 mM in the emulsions, reduced BH with the smallest concentrations of oil resulting in the largest reduction of BH, and hence largest protection efficiency. Also, more oil resulted in more unstable emulsions, reflected by the higher amount of oiling of (results not shown). Undispersed oil was (most probably) not successfully protected. Therefore, the protection of higher percentages of oil using the current protocol seemed challenging.

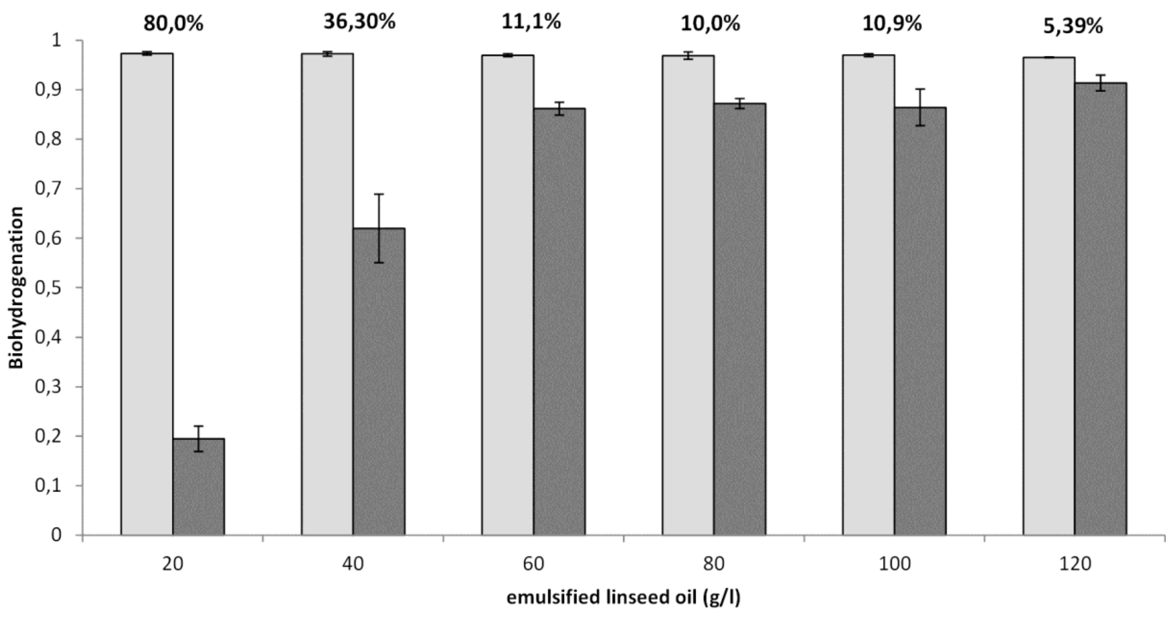

Figure 4.3 Higher percentages of C18:3*n-3* in emulsified linseed oil are more hydrogenated after 24h *in vitro* rumen incubation when a red clover protein extract and 20 mM 4-methylcatechol is used (experiment 4). Light grey bars represent no addition of 4-methylcatechol, dark grey bars represent emulsions having a final concentration of 20mM 4-methylcatechol. Percentages above columns represent the corresponding protection efficiencies and error bars represent the standard deviation over three analytical replicates (1 original emulsion)

3.5 Experiment 5: Effect of diphenol concentration

A dose-response relation was assessed in the fifth experiment to elucidate the relation between the 4-MC dose and the degree of protection. Six concentrations of 4-MC were applied to aliquots of one single emulsion (n=1). The final diphenol concentrations of these emulsion aliquots were 1.25, 2.5, 5, 10, 20 and 40 mM 4-MC. The red clover extract (n=1) used in this experiment contained 1.94 g protein per liter and had a specific PPO activity of 1144 ΔA/min/mg protein. Increasing concentrations of 4-MC resulted in a decrease in BH of C18:3*n-3* and a concomitant increase in protection efficiency (Figure 4.4), according to a logistic type of response: low concentrations of 4-MC (< 5 mM) only had a minor effect on reduction in BH, whereafter a steep decrease in BH of C18:3*n-3* was observed upon increasing 4-MC concentration (10 to 20 mM), whereas further increasing 4-MC concentrations (≥ 20 mM) only resulted in minor decreases in BH.

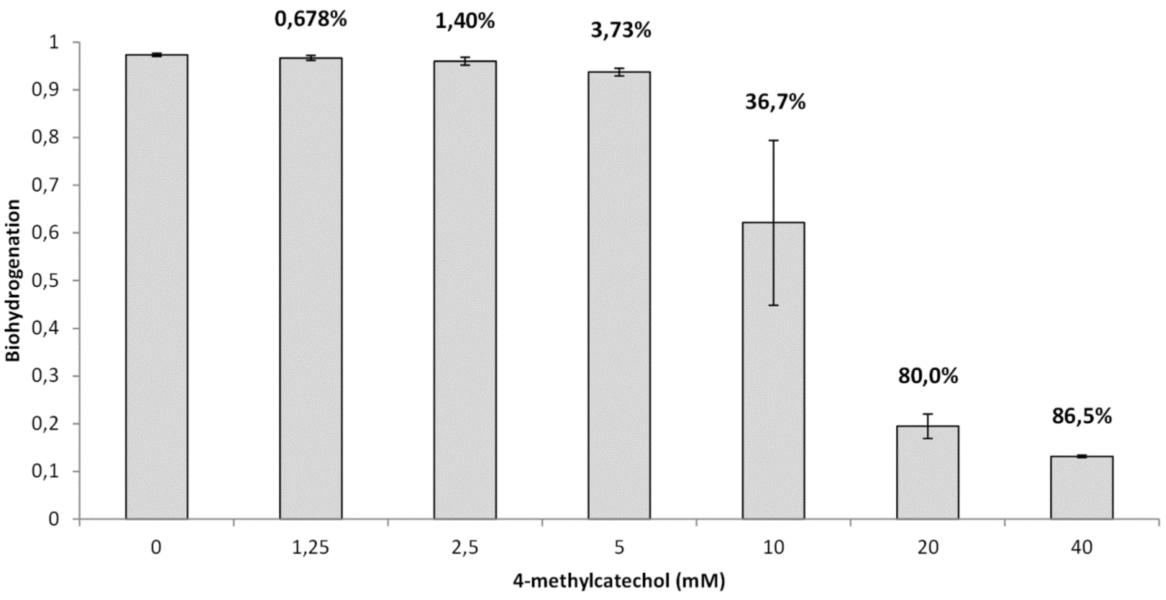

Figure 4.4 Concentrations of 4-methylcatechol of at least 10 mM result in less hydrogenation of C18:3*n-3* after 24h *in vitro* rumen incubation when a red clover protein extract and 20 g oil per liter protein extract are used (experiment 5). Percentages above columns represent the corresponding protection efficiencies and error bars represent the standard deviation over three analytical replicates (1 original emulsion)

3.6 Experiment 6: Combination set-up

In the next experiment, a combination set-up was performed to assess the combined effects of oil concentration, extra casein addition and diphenolic substrate concentration. A 4x3x4 factorial design was used to test 4 concentrations of oil (10, 20, 30 and 40 g of linseed oil per liter red clover protein extract), 3 protein concentrations (0, 1 or 2 g extra casein hydrolysate per liter emulsion) and 4 diphenol concentrations (to achieve test emulsions with a final concentration of 0, 12.5, 25 or 50 mM 4-MC). The red clover extract (n=1) in this experiment contained 4.87 g protein per liter and had a specific PPO activity of 403.9 ΔA/min/mg protein. Figure 4.5 gives an overview of the results. Again, adding 4-MC to the emulsions resulted in a decrease in BH of C18:3*n-3* after 24h rumen incubation, with increasing concentrations of 4-MC resulting in a further reduction of BH ($P<0.001$). PUFA were more extensively hydrogenated when more oil was included in the oil-in-extract emulsions ($P<0.001$). Noteworthy, this increase in BH with higher oil concentrations was found within each level of

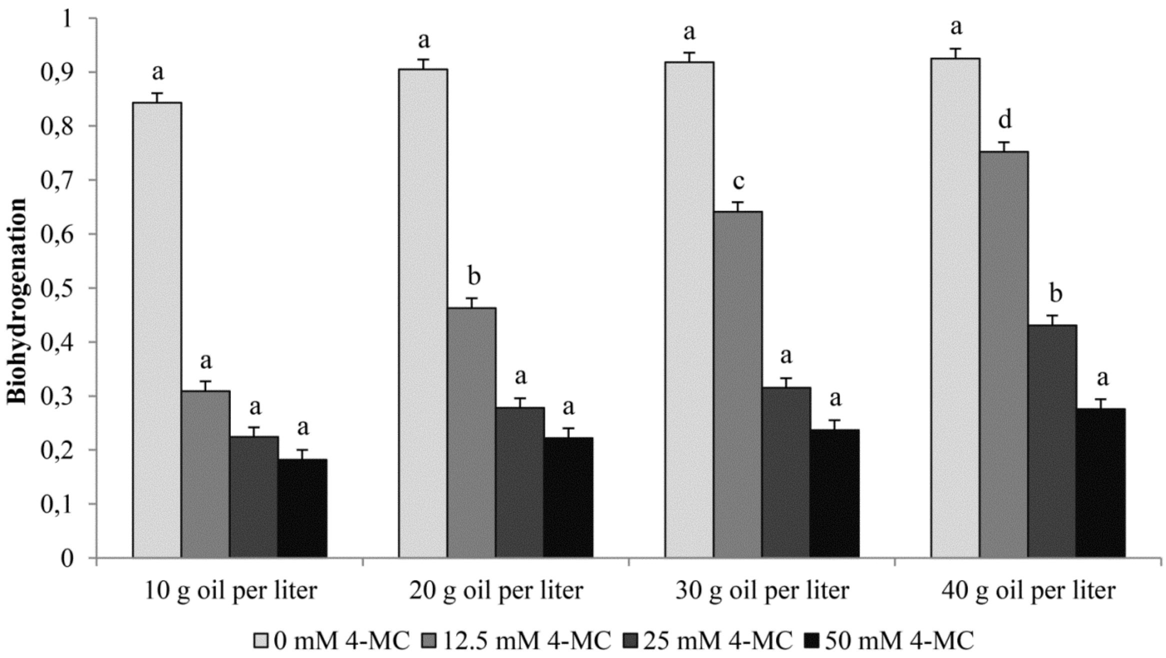

Figure 4.5 Biohydrogenation of C18:3*n-3* after batch *in vitro* incubation for 24h is influenced by oil and diphenol concentrations. Emulsions used in experiment 6 (prepared by passing 5 times through a microfluidizer at 25 MPa) contain 4 different oil concentrations (10, 20, 30 or 40 g oil per liter protein extract), different levels of extra casein (0, 1 or 2 g extra casein per liter protein extract) and different diphenol concentrations (0, 12.5, 25 or 50 mM 4-methylcatechol). [a,b,c,d] indicates differences in biohydrogenation between oil concentration within 4-methylcatechol concentration at $P \leq 0.05$. Error bars represent the standard error of the mean (3 original emulsions)

4-MC ($P<0.001$), but largest significant differences in BH were found for the intermediate concentrations of diphenol (12.5 and 25 mM). Protecting higher amounts of oil seemed more difficult, as greater amounts of 4-MC were required to obtain a decreased BH and concomitantly increased protection efficiency. Rather unexpectedly, differences in BH were also found for casein ($P<0.001$), independent of the oil concentration ($P=0.372$), but dependent of diphenol ($P=0.029$). Adding 1 g of casein per liter protein extract did not result in a change in BH ($P=0.947$), however, when 2 g of casein per liter protein extract was added, a small but significant increase in BH was observed as compared with no casein or 1 g casein per liter ($P<0.001$). More protein thus resulted in higher levels of BH, despite the reduction in emulsion droplet size (Table 4.1). Increasing oil concentrations, resulting in more extensive BH, were linked with an increase in emulsion droplet size and decrease in specific surface area. Highest protection efficiencies of 0.716 and 0.795 for C18:2*n-6* and C18:3*n-3*, respectively, were found for the lowest oil concentration (10 g oil per liter) and highest 4-MC

concentration (50 mM). In this experiment fresh emulsions were used for rumen *in vitro* incubations, instead of emulsions stored at -80°C in the experiments 1 and 2. Emulsions stored at -80°C did not show major destabilization or coalescence after freezing and thawing. However it is noteworthy that protection efficiencies reached in the current experiment were greater than those in experiment 1, despite the lower PPO activity expressed per mg of protein.

3.7 Experiment 7: Effect of removing the continuous phase

Next, the influence of the continuous phase of the emulsions after 4-MC addition on the level of protection was assessed. It was hypothesized protection occurred because of cross-linking of interfacial protein, so replacing the continuous phase after reaction with 4-MC by water would not have an effect on the level of protection. Therefore, after protection was created upon addition of 4-MC, the continuous phase was removed by centrifugation (30 min; 30000 *g*; 4°C) and the remaining oil droplets resolubilized in water (called 'washed emulsions'). The process of washing and redissolving was executed twice to ensure the removal of the majority of quinones and polymers of the continuous phase. Red clover extracts (n=2) contained 1.62 g protein per liter and had a specific PPO activity of 1052 ΔA/min/mg protein. Emulsions (n=2) were exposed to 4-MC to reach a final concentration of 0, 12.5, 25 or 50 mM. The centrifuged oil droplets showed a gradual increase in brown color upon higher levels of 4-MC added. The removed continuous phase had, irrespective of the amount of 4-MC applied, the same color (results not shown). No differences were observed in BH of C18:3*n-3* between the original and washed emulsions ($P=0.776$) (Figure 4.6). Similar results were found for C18:2*n-6* (results not shown). Further, addition of the lowest amount of 4-MC already resulted in a steep decrease in BH ($P<0.001$). Also, original and washed emulsions showed a similar droplet size distribution profile (Figure 4.7; left), meaning emulsions remained stable after resolubilization, and the original versus washed emulsions could be compared. Upon addition of higher concentrations of 4-MC (Figure 4.7; right), a shift towards larger mean emulsion droplet sizes was observed, but all emulsions remained

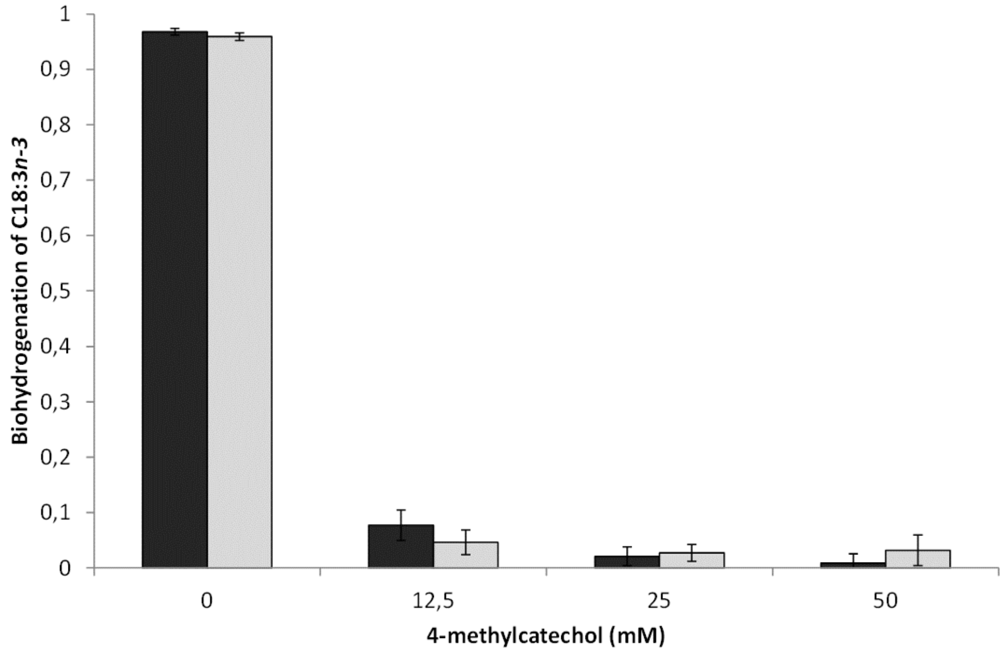

Figure 4.6 No differences were observed in biohydrogenation of C18:3*n-3* between original (dark bars) and washed (light bars) emulsions containing 20 mg linseed oil per ml red clover protein extract and increasing concentrations of 4-methylcatechol (experiment 7; error bars represent standard deviations of 6 analytical incubation replicates, i.e. 3 analytical replicates of 2 original emulsions)

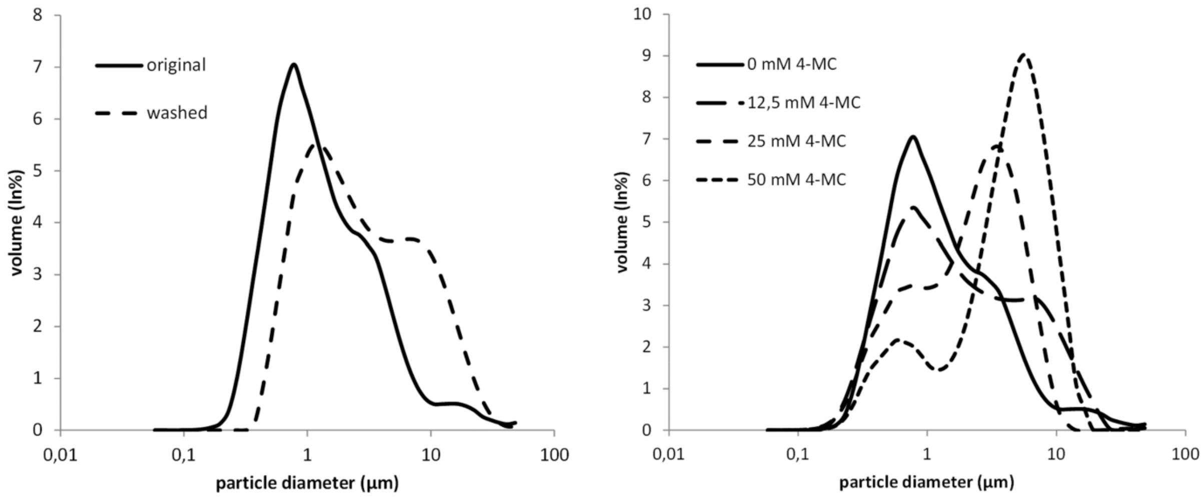

Figure 4.7 Droplet size distribution of original versus washed emulsions containing 20 mg linseed oil per ml red clover protein extract without 4-methylcatechol (4-MC) (left) and original emulsions with increasing concentrations of 4-MC (right) (experiment 7; 2 original emulsions)

stable. Washed emulsions showed a similar droplet distribution profile (results not shown). Overall, removing the continuous phase had no influence on the level of protection, which suggested the cross-linked interfacial protein barrier was responsible for protection.

3.8 Experiment 8: Effect of reaction upon addition of diphenol

Finally, experiment 8 was performed to assess the effect of reaction time after addition of 4-MC on the protection against BH and being stopped by adding 4-HR to inhibit PPO activity. The red clover extract (n=1) contained 2.07 g protein per liter and had a specific PPO activity of 2353 ΔA/min/mg protein. Fifteen g of linseed oil was emulsified per liter red clover protein extract and test emulsions (n=1) had a final concentration of 15 mM 4-MC. Results of fresh incubated emulsions, shown in Figure 4.8, clearly indicates that BH of C18:2*n-6* and C18:3*n-3* is reduced when time between addition of 4-MC and PPO inhibition increases. During the first two hours, no reduction of BH was found, while already after 4h a marked decrease in BH was noted. Even greater reductions were found after 8h and 24h, suggesting higher protection against BH is induced when the PPO catalyzed reaction is allowed to occur longer.

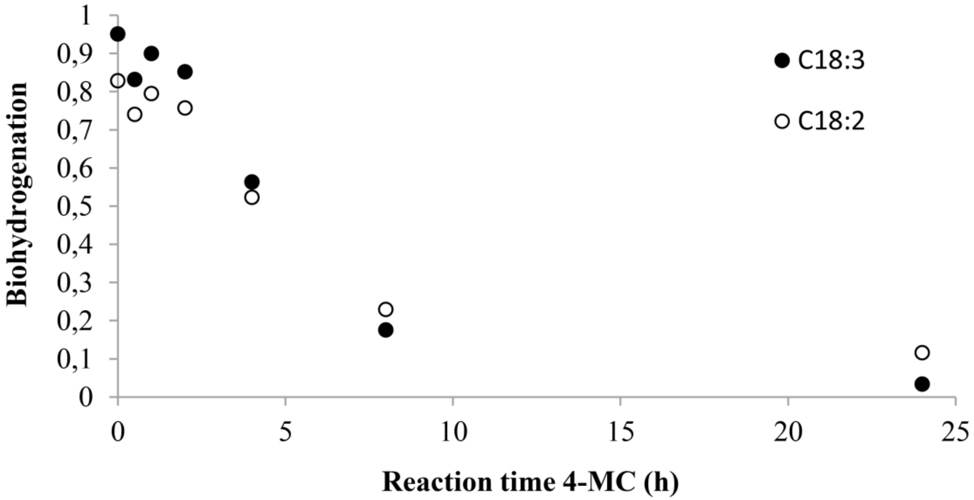

Figure 4.8 An increased time between the addition of 4-methylcatechol (4-MC) to red clover based linseed oil-in-extract emulsions (prepared by passing 5 times through a microfluidizer at 25 MPa) and PPO inactivation by 4-hexylresorcinol results in a decrease of biohydrogenation of C18:2*n-6* and C18:3*n-3* after 24h *in vitro* incubation (experiment 8; 1 original emulsion)

4 Discussion

The link between PPO from red clover and a reduction in proteolysis, lipolysis and BH in silo and in the rumen has been extensively discussed before (Van Ranst *et al.*, 2011; Lee *et al.*, 2004; Sullivan and Hatfield, 2006). However, to our knowledge, this is the first time a protein extract of red clover, as a source of PPO, is used to protect emulsified PUFA against ruminal BH. The current set of experiments demonstrates that BH is substantially reduced when a PUFA rich oil is first emulsified in a protein extract of red clover, showing PPO-activity, to which a diphenolic substrate was added to induce protection. Generally, in order to achieve a reduction in BH, emulsions should be stable and a sufficient amount of 4-MC should be combined with active PPO for at least a couple of hours. When no 4-MC was added, BH of PUFA was not reduced although PPO was present in the emulsions. Nevertheless, PPO does exhibit monophenolase activity (Yoruk and Marshall, 2003), meaning monophenols present in amino acids like tyrosyl residues in proteins are also susceptible to PPO-induced cross-linking. However, this activity in red clover is likely not big enough to create protection of the emulsion, as illustrated by the absence of a decrease in BH when no 4-MC was added. When a diphenol like 4-MC is added to the emulsions, cross-linking can occur due to diphenolase activity, probably because the diphenol acts as a mediator of cross-linking with proteins (Stanic *et al.*, 2010; Chung *et al.*, 2005). Accordingly, both PPO and a diphenolic substrate were required in the current set-up to obtain a reduction in BH. The hypothesis is made that protection is obtained because of an effective encapsulation of lipids in protein-phenol complexes, as suggested before by Van Ranst *et al.* (2011).

It is suggested that PPO has to be present near to the oil interface to induce cross-linking, based on following observations: first, a similar degree of protection between original and washed emulsions was found (experiment 7). The fact that removing the continuous phase, containing quinones and protein-phenol polymers, had no influence on the level of protection, could be considered as a proof of evidence that protection is indeed created at the emulsion

interface. Second, reduction in protection efficiency through addition of extra casein as emulsifier (experiment 6) may also be explained within this perspective. Indeed, this observation is in contrast to what was originally expected, as casein hydrolysate was added as extra emulsifier to reach smaller and hence more stable emulsions. Smaller emulsion droplet sizes were indeed obtained, which resulted in an increase of the oil-in-extract surface area. The latter obviously demands more protein to be withdrawn from the continuous phase to incorporate into the interface of the emulsion as an emulsifier. This phenomenon also had been observed in dairy emulsions with greater fat content and hence increased specific surface area, which resulted in an increased fraction of adsorbed protein (Tomas *et al.*, 1994). However, disordered 'soft' proteins like casein, tend to adsorb more easily to emulsion interfaces than globular 'hard' proteins, because these proteins unfold faster, leading to a faster reduction of the interfacial tension (Dickinson, 1999). It might be suggested that the peptides present in casein hydrolysate, having a molecular weight distribution between 0.1 and 1 kDa, according to the manufacturer, possibly act as a better emulsifier compared to the parental material (van der Ven *et al.*, 2001). Enzymes like PPO and other proteins in the red clover extract are typically globular proteins. When red clover globular proteins and proteins like casein or its peptides are present simultaneously, casein might be incorporated in the interface to a higher extent than does PPO, due to competitive adsorption (Dickinson, 1999). Hence, this negative effect of extra emulsifier on protection efficiency might indicate that PPO indeed has to be present at the oil interface to create protection. However, one might argue that the reduction in protection is the result of a simple dilution effect of the extra casein present in the emulsion. Indeed, it might still be possible that both types of emulsifiers, both the globular red clover proteins as well as the small peptides, adsorb to the interface with similar efficiencies and reflecting their relative abundance in the system. Therefore, studies elucidating the proteins which are effectively adsorbed at the emulsion interface are needed.

Furthermore, not only the presence of diphenol and PPO is important to protect against BH, also the reaction time and amount of diphenol plays a role, with greater amounts of diphenol and longer reaction periods resulting in greater ruminal protection. This may be due to the formation of a denser phenol cross-linked protein layer at the interface, resulting in improved protection efficiency. The amount of diphenol required probably depends on the total interfacial surface area in the emulsions, which is reflected in the specific surface area (Table 4.1). Indeed, it seems more difficult to protect higher amounts of oil against BH (experiment 4 and 6), probably because of a shortage of diphenols. Hence, in Figure 4.9, the amount of diphenolic substrate added to the emulsions per unit of emulsion interfacial area available (mmol 4-MC/m²) was related to the protection efficiencies for data of experiments 4 and 6. Experimental data points showed a logarithmic increase, reaching a plateau once more than 0.2 mmol 4-MC is available in the system per square meter surface area in the emulsion. This does not allow to assess the amount of 4-MC effectively present at the interface, as 4-MC or its derived quinone probably partitioned between the interface and the aqueous phase depending on its solubility, interfacial properties and its affinity for the interfacial proteins.

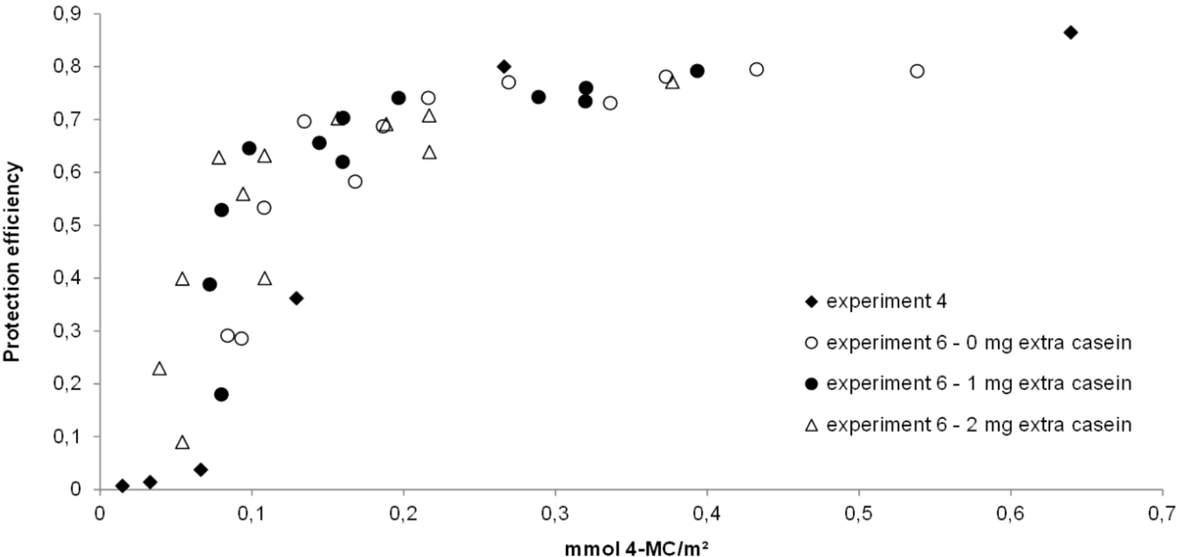

Figure 4.9 Protection efficiencies increased with increasing amounts of added 4-methylcatechol (4-MC) per unit of interfacial surface area of emulsified linseed oil droplets in oil-in-extract emulsions (experiment 4 and 6)

Nevertheless, the direct link between the amount of 4-MC present in the system relative to the total surface area available and the protection efficiency suggests 4-MC is indeed partitioning at the interface. Therefore, the suggestion is made that protection efficiency against BH might be caused by emulsion encapsulation. This re-enforces the hypothesis for the need of the PPO enzyme to be present near the droplet interface, to induce oxidation of 4-MC, in order to create quinones which can cross-link with the proteins at the emulsion interface.

Highest protection efficiencies reported in the current paper are comparable with those achieved by formaldehyde protection as described by Scott *et al.* (1971), who found 54 to 100 % protection of C18:2*n-6* against ruminal BH during *in vitro* incubations. There, dry particles were created with a 1:1 (w/w) protein to oil ratio, resulting in great amounts of oil entrapped in a protein matrix. In the current experiment, a much lower protein to oil ratio is needed to obtain protection, suggesting protein is only necessary as emulsifier to create oil-in-extract emulsions. Unfortunately, only low amounts of oil could be protected against BH, probably because of the large need of 4-MC per unit of emulsion interfacial area. More research is needed in this respect. Furthermore, it is yet unclear whether a decrease in ruminal BH with PPO-protected emulsions also leads to an increased level of PUFA in plasma or milk fat as observed for formaldehyde-treated PUFA oils in the study of Scott *et al.* (1971), because PUFA have to be liberated from protected emulsions during abomasal or intestinal passage.

5 Conclusions

Protection against ruminal BH of PUFA is possible after emulsification of PUFA in a red clover protein extract, containing PPO, when 4-MC is present in the continuous phase of the emulsion as diphenolic substrate. It is suggested that the reduction in BH is due to an effective encapsulation of PUFA, because of the PPO-induced cross-linking of proteins at the oil-water interface, mediated through the addition of a diphenolic substrate.

6 Acknowledgements

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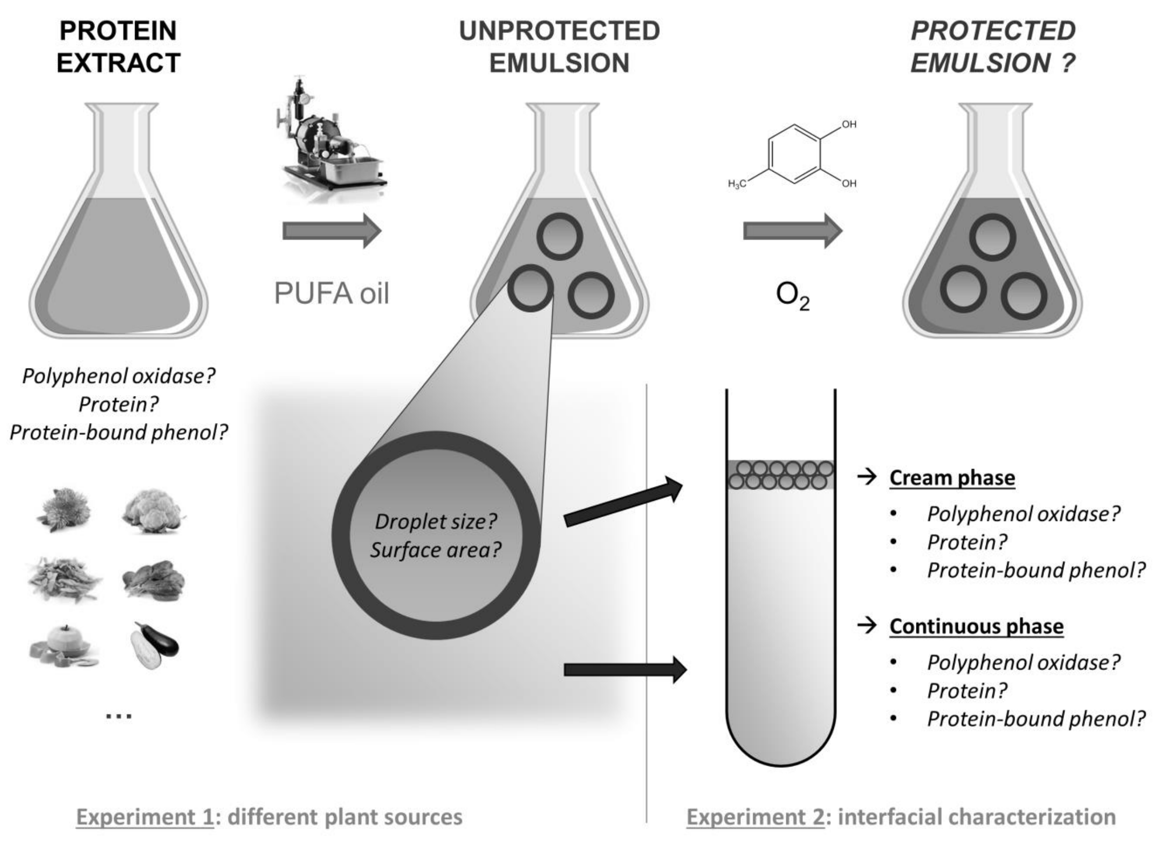
Chapter 5

Polyphenol oxidase containing sidestreams as emulsifiers of rumen bypass linseed oil emulsions: interfacial characterization and efficacy of protection against in vitro ruminal biohydrogenation



Redrafted after Gadeyne F., De Neve N., Vlaeminck B., Claeys E., Van der Meeren P. and Fievez V., 2016, Polyphenol oxidase containing sidestreams as emulsifiers of rumen by-pass linseed oil emulsions: interfacial characterization and efficacy of protection against *in vitro* ruminal biohydrogenation, Journal of Agricultural and Food Chemistry, 64, 3749-3759.

Chapter 5

Polyphenol oxidase containing sidestreams as emulsifiers of rumen bypass linseed oil emulsions: interfacial characterization and efficacy of protection against in vitro ruminal biohydrogenation

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Abstract. The low transfer in ruminants of dietary polyunsaturated fatty acids (PUFA) to the milk or peripheral tissues is largely due to ruminal biohydrogenation (BH). Lipids emulsified by a polyphenol oxidase (PPO) rich protein extract of red clover were shown before to be protected against this breakdown after cross-linking with 4-methylcatechol (4-MC). Protein extracts of thirteen other vegetal resources were tested. Surprisingly, the effectiveness to protect emulsified lipids against *in vitro* ruminal BH largely depended on the origin of the extract and its protein concentration but was not related to PPO activity. Moreover, PPO isoforms in vegetal sources, effectively protecting emulsified lipids, were diverse and their presence at the emulsion interface did not seem essential. Potato tuber peels were identified as the most interesting biological source of emulsifying proteins and PPO.

1 Hypothesis and objective

The seasonal availability and the low-fat content of red clover, which was used in Chapter 4, limits the potential applicability of the current technology. Nevertheless, PPO activity has been reported in almost any organism, both in plants, fungi, bacteria and animals (Mayer, 2006). Although only PPO extracted from red clover has been used up till now to create protection against ruminal BH, extrapolation to other resources could be hypothesized. In this perspective, industrial sidestreams of plant resources are widely available, some of which are now used in animal feeds or discarded, although they still might contain valuable molecules and characteristics. Therefore, the first aim of this chapter was to assess the

possibility to protect PUFA against ruminal BH using 4-MC as diphenolic cross-linking mediator after emulsification with protein extracts of different plant sources.

The second goal is to further characterize some of these protein extracts and emulsions using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), PPO zymography, conventional PPO activity assays and protein load tests to get further insight in factors affecting the protection efficiency (PE) in the rumen.

2 Materials and methods

2.1 Materials

Different vegetal sources were used for a screening test depending on their availability, previously reported PPO activity in literature (Bottino *et al.*, 2009; Gawlik-Dziki *et al.*, 2007; Dogan *et al.*, 2005; Constabel *et al.*, 1995; Rahman *et al.*, 2012; Wang *et al.*, 2015; Thada *et al.*, 2013; Plazas *et al.*, 2013; Kuijpers *et al.*, 2014; Zhang *et al.*, 2015; Van Ranst *et al.*, 2009c) or potential as upgradable industrial sidestream (Ravindran and Jaiswal, 2016; Duarte-Vazquez *et al.*, 2007; Niphadkar *et al.*, 2015): apple peels (*Malus domestica* Mill. cv. Jonagold), artichoke flower leaves (*Cynara scolymus* L.), broccoli stems (*Brassica oleracea* L. convar. *botrytis* var. *cymosa*), carrot peels (*Daucus carota* subsp *sativus* (Hoffm.) Schübl & G. Martens), cauliflower florets, stems and leaves (*Brassica oleracea* L. convar. *botrytis* var. *botrytis*), eggplant pulp (*Solanum melongena* L.), pineapple peels (*Ananas comosus* (L.) Merr.), potato tuber peels (*Solanum tuberosum* L.), red clover stems & leaves (*Trifolium pratense* L. cv. Lemmon), spinach leaves (*Spinacia olereacea* L.) and tomato stems & leaves (*Solanum lycopersicum* L.). Plant sources were either bought in a local grocery store or kindly provided by the Institute for Agricultural and Fisheries Research (ILVO, Belgium). Immediately upon arrival in the laboratory, plant material was cut into fine pieces, snap frozen in liquid nitrogen and stored at -80°C until further use.

Crude linseed oil (40, 194, 169 and 586 mg per g total FA of C18:0, C18:1*n-9*, C18:2*n-6* and C18:3*n-3*, respectively) was a gift from Dumoulin (Kortrijk, Belgium). 4-MC was purchased from Sigma-Aldrich (Bornem, Belgium). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (Bornem, Belgium), Merck (Darmstadt, Germany), Carl-Roth (Karlsruhe, Germany) or VWR (Heverlee, Belgium), unless stated otherwise. Any centrifugal step was performed using a refrigerated Beckman J2-HS centrifuge (Beckman Coulter, Brea, California, USA).

2.2 Preparation of protected PUFA emulsions and experimental set-up

Emulsions were prepared as described by Gadeyne *et al.* (2015) (see also section 2.2 of Chapter 4). In brief, a three-step process was performed: first, proteins were isolated from frozen plant material by extraction in 0.1 M sodium phosphate buffer (SPB) with ascorbic acid (pH=7), protein precipitation with acetone (4:1 volumetric acetone to SPB ratio) and recovery of soluble protein in 0.01 M SPB (pH=7); second, this protein extract was used to emulsify oil with a high speed Ultra-Turrax (T25 Basic, Ika Werke, Staufen, Germany) for pre-emulsification and passed 5 times at 25 MPa through a microfluidizer (M110S, Microfluidics Corporation, Newton, Massachusetts, USA); third, emulsions were treated with 4-MC and shaken for 24h to induce protein-phenol complexing. All emulsions were prepared using 20 mg linseed oil per ml of extract and stored at 4°C.

Two experiments were performed to assess the potential of different sources as emulsifier to protect against ruminal degradation. Emulsions in experiment 1 were created using protein extracts of the thirteen selected vegetal sources mentioned in the Materials sections. Emulsions (before addition of diphenol) were prepared in triplicate, starting from three different protein extracts. Afterwards, 4-MC was added to obtain a final concentration of 0, 10, 20 or 40 mM 4-MC. Next, in experiment 2 four vegetal sources were selected from the first experiment for further characterization of proteins in the continuous phase and being present at the interface: carrot peels, cauliflower florets, eggplant pulp and potato tuber

peels. Protein solutions were obtained as described before, but were diluted with 0.01 M SPB to obtain similar protein concentrations across treatments. Emulsions were prepared in triplicate starting from the same protein solution and contained a final concentration of 0 or 40 mM 4-MC.

2.3 Protein extract characterization

2.3.1 Spectrophotometric analysis

Protein solutions were characterized by measuring the PPO activity and the concentration of total protein and protein-bound phenol (PBP). The adaptations described by Winters and Minchin (2005) were used to perform a modified Lowry assay, in order to determine total protein and PBP concentration simultaneously. Protein content and PBP were expressed as mg of protein per ml extract and mg tyrosine-equivalents per mg protein, respectively, assuming 1 g of bovine serum albumin, used for the standard solution, contains 0.043 g tyrosine-equivalents. Further, PPO activity was determined by measuring the absorbance at 400 nm each 5 seconds for 1 min. For this, 0.75 ml 0.04 M 4-MC (containing 5 mM HCl to prevent autoxidation) was added to a solution of 2.2 ml 0.01 M SPB (pH=7) and 50 µl of protein extract. The increase in absorbance of the initial linear part of the curve was retained as a measure of activity. PPO activity was finally expressed in nkatal by recalculating the increase in absorbance per time unit to amounts of formed quinones using a standard series of 4-methylbenzoquinone, obtained by treating 3 ml of 4-MC with 3 ml 0.045 M sodium periodate and measuring the absorbance at 400 nm after 10 min. Specific PPO activity was expressed as µkatal per mg protein. All spectrophotometric analyzes were done in triplicate (analytical replicates).

2.3.2 Gel electrophoretic analysis

Next to spectrophotometric analyzes of protein and PPO activity, gel electrophoresis was performed to visualize different protein patterns in the extracts using a vertical slab gel

system (SE600, Hoefer Scientific Instruments, Holliston, Massachusetts, USA). Gels were prepared as described by Greaser *et al.* (1983). Briefly, separating gels contained 10 or 12 % of total acrylamide and N,N′-methylene-bis-acrylamide. In experiment 1, wells were loaded with the protein solution, to which SDS (2 %, w/v), approximately 15 % sucrose (w/v) and 0.3 mg/ml bromophenol blue were added. Twenty µl of this mixture was loaded per well, while volumes loaded in the second experiment varied from 10 to 50 µl per well, depending on the protein concentration of the samples, in order to load equal amounts of protein. Separation was carried out at constant current. After the bromophenol front reached the bottom of the gel, proteins were fixed in a 20 % methanol / 10 % acetic acid solution and visualized by staining with Coomassie brilliant blue R250 according to Claeys *et al.* (1995), followed by scanning with a Bio-Rad computing densitometer (model CDS-100; Bio-Rad, Temse, Belgium). Further, similar gels were used to visualize PPO activity, based on the method described by Rescigno *et al.* (1997). For this, 10 % gels were prepared similarly as for the protein visualization. The presence of SDS is of particular interest as it is known to activate PPO isoforms from its latent state, in contrast to many enzymes which are inactivated by SDS (Moore and Flurkey, 1990). After proteins were separated, gels were gently washed with demineralized water and soaked in 0.1 M SPB (pH=7) for 3 min. Next, the gels were transferred to a solution containing 80 mM 4-MC (dissolved in 0.1 M SPB, pH=7). After 5 min, gels were washed in the original SPB solution and immediately transferred to a 16 mM 4-amino-N,N-diethylaniline sulfate solution, which reacts with 4-methylbenzoquinone, resulting in the visualization of PPO isoforms as purple blue bands. Zymograms were scanned at least 10 minutes after addition of 4-amino-N,N-diethylaniline sulfate.

2.4 Emulsion characterization

The particle size distribution of emulsions was assessed immediately after preparation with a Mastersizer S (Malvern Instruments, Malvern, UK) as described before (see section 2.2 of Chapter 4). Droplet sizes were characterized in terms of volume-weighted mean diameter (D_{43}), surface-weighted mean diameter (D_{32}), median volume-weighted distribution value

$D_{[v,0.5]}$ or 90 % percentile of the volume-weighted distribution $D_{[v,0.9]}$. Specific surface areas (in m²/g oil) were calculated as SSA = 6 / [D_{32} × ρ], assuming a linseed oil density ρ of 930 kg/m³.

Further, the protein load of emulsions from experiment 2 was determined. To separate proteins present in the continuous phase from proteins at the interface after emulsification, 25 ml of emulsion was centrifuged for at least 30 min (30000 x *g*; 4° C). The protein fraction of the continuous phase was obtained by removing 5 ml of the subnatant with a fine needle and was filtered through a 0.20 µm cellulose syringe filter (Chromafil, Macherey-Nagel, Düren, Germany). These unadsorbed proteins were further characterized using the modified Lowry and gel electrophoretic procedures (vide supra). As demonstrated before (Berton *et al.*, 2011a), the aqueous phases obtained after filtration only contained negligible volumes of oil (data not shown). Accordingly, adsorbed protein in the continuous phase can be assumed negligible as well. Protein loads were expressed as mg protein per m² interfacial area or relatively to the initial protein concentration in the aqueous extract (g per 100 g protein). Next, to recover the interfacial proteins for gel electrophoresis, an extra 15 ml was removed from the centrifuged sample and the remaining fraction was washed twice by redispersing in fresh 0.01 M SPB (pH=7), thorough vortexing and centrifuging for at least 30 min (30000 x *g*; 4° C). Then, 1 % sodium dodecyl sulphate solution was added to the remaining fraction, vortexed and gently shaken for 1 h to allow interfacial proteins to be replaced by sodium dodecyl sulphate, before centrifuging for at least 30 min (30000 x *g*; 4° C). Finally, 5 ml of the subnatant was removed and passed through a 0.20 µm cellulose syringe filter to obtain the adsorbed protein fraction for gel electrophoretic analysis.

2.5 Assessment of protection against ruminal biohydrogenation

Batch *in vitro* incubations were performed to assess protection of PUFA in emulsions against ruminal BH as described before (see section 2.4 of Chapter 4). In brief, incubation flasks contained 1 ml emulsion, 250 mg hay and 24 ml buffer/rumen fluid solution in a 4:1 ratio,

which has been shown before (Fievez *et al.*, 2007) to be appropriate to estimate BH of C18:2*n-6* or C18:3*n-3*. Rumen contents were collected before the morning feeding from three rumen fistulated sheep, which were fed grass hay ad libitum and a grain based concentrate (200 g/day), combined and filtered before incubation. Fistulation was approved by the ethical commission (file number 114, 2009) of the Institute for Agricultural and Fisheries Research (ILVO, Belgium). Flasks were flushed with CO_2 and incubated under intermittent shaking at 39°C for 24h in a batch culture incubator (Edmund Bühler GmbH, Hechingen, Germany). All emulsions were incubated in duplicate (analytical replicates). To monitor the quality of the incubations, gas composition (Hassim *et al.*, 2010), pH (Hanna Instruments, Temse, Belgium) and volatile FA (Gadeyne *et al.*, 2016f) (see also section 2.4.1 of Chapter 2) was assessed. Gas composition was converted to absolute gas production rates based on the pressure accumulation in the flask (Infield 7C handheld read-out device equipped with a T1 Stitch-Tensiometer; UMS GmbH, München, Germany). Incubation characteristics are not reported as no major differences were observed between treatments, indicating changes in the extent of BH were not due to changes in microbial activity.

To calculate BH, 5 ml of incubation fluid was taken before and after incubation, freeze-dried and analyzed for long chain FA after direct transesterification using gas chromatograph (see section 2.5 of Chapter 4). Peaks were identified based on their retention times, compared to external standards (GLC463, Nu-Check Prep Inc., Elysian, Minnesota, USA). Quantification of FA methyl esters was based on the area of the internal standard (C21:0 for experiment 1 and C13:0 for experiments 2) and on the conversion of peak areas to the weight of FA by a theoretical response factor for each FA (Ackman and Sipos, 1964; Wolff *et al.*, 1995). Finally, BH, which is the disappearance of C18:3*n-3* after incubation, was calculated as [(proportion of C18:3*n-3* in total C18 FA)$_{0h}$ – (proportion of C18:3*n-3* in total C18 FA)$_{24h}$] / (proportion of C18:3*n-3* in total C18 FA)$_{0h}$. PE of C18:3*n-3* was calculated as [(BH of C18:3*n-3*)$_{\text{non-protected (0 mM)}}$ – (BH of C18:3*n-3*)$_{\text{protected (10, 20 or 40 mM)}}$] / (BH of C18:3*n-3*)$_{\text{non-protected (0 mM)}}$. The formation of C18:0, the end product of C18 FA BH, was calculated as [(proportion of C18:0 in total C18

FA)$_{24h}$ – (proportion of C18:0 in total C18 FA)$_{0h}$] / [(proportion of C18:3*n-3* and C18:2*n-6* in total C18 FA)$_{0h}$ – (proportion of C18:3*n-3* and C18:2*n-6* in total C18 FA)$_{24h}$].

2.6 Statistics

Results were analyzed with the MIXED procedure of SAS (SAS Enterprise Guide 7, SAS Institute Inc., Cary, North Carolina, USA). Analytical replicates were averaged prior to statistical analysis. Linear regression analyzes and two-sample T-tests were performed using the REG and TTEST procedure of SAS, respectively.

For experiment 1, the following model was used: $Y_{ij} = \mu + P_i + D_j + P_i \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, P_i the fixed effect of protein source (i = apple peel, artichoke flower leaf, broccoli stem, etc.), D_j the fixed effect of diphenol concentration (j = 0, 10, 20 or 40 mM 4-MC) and ε_{ij} the residual error. Hierarchical cluster analysis was performed on C18:3*n-3* BH using 10, 20 and 40 mM 4-MC to distinguish between protein source using the CLUSTER and TREE procedures of SAS with average linkage.

In experiment 2, the same model as for experiment 1 was used, with P_i the fixed effect of protein source (i = carrot peel, cauliflower floret, eggplant pulp or potato tuber peel for) and D_j the fixed effect of diphenol concentration (j = 0 or 40 mM 4-MC).

Principal component analysis was performed to determine components which account for most of the variation in PE. The PRINCOMP procedure of SAS was applied using data from the 40 mM 4-MC emulsions of experiment 1 and 2, whereby PE of C18:3*n-3*, protein concentration, PPO activity and D_{32} were included as variables.

Differences were assigned at the 0.05 significance level and differences among least square means evaluated using Tukey’s multiple comparison test.

3 Results

3.1 Experiment 1: screening of different plant sources

In the first experiment, a screening test was performed to assess whether PPO containing protein extracts of different sources could be used to protect PUFA against ruminal BH. Therefore, thirteen possible vegetal sources were selected, proteins extracted and used as emulsifiers.

Protein concentrations of the extracts largely differed (Table 5.1), ranging from 0.204 to 2.73 mg of protein per ml extract for apple peel and potato tuber peel, respectively. Negligible concentrations of PBP were observed in the extracts (Table 5.1), except for artichoke flower leaf (10.5 mg tyrosine equivalents per mg protein), which was also reflected in a dark discoloration during the extraction procedure prior to acetone precipitation. The protein extracts of the latter also contained the highest specific PPO activity (5.23 µkatal per mg protein). Substantial amounts of quinones were formed in most extracts after addition of 4-MC, ranging from 1.53 nkatal for pineapple peel to 116 nkatal for artichoke flower leaf (Table 5.1). Very low PPO activities were found for broccoli, carrot and cauliflower.

Gel electrophoretic analysis was performed to separate proteins according to their molecular weights. Large variation in molecular weight and intensity of protein bands within and between vegetal extracts was observed (Figure 5.1A). Also, reactivity towards 4-MC of these proteins, attributed to the activity of PPO (Figure 5.1B), varied to a large extent among the protein sources. Most sources showed one or more bands indicating PPO activity, except for carrot peel. Remarkably, protein sources were quite diverse in PPO isoforms as indicated by the lack of uniformity in PPO bands, showing proteins with varying molecular weight being responsible for PPO activity. No relation between the spectrophotometric PPO activity (Table 5.1) and intensity of bands after electrophoretic analysis was observed: for example, no (detectable) or very low PPO activity was measured spectrophotometrically for protein

Table 5.1 Protein extracts (n=3) of thirteen selected plant sources in experiment 1 varied in protein concentration, PBP concentration and (specific) PPO activity

protein source	protein *mg/ml extract*	PBP *mg Tyr-eq/mg protein*	PPO activity *nkatal*	specific PPO activity *µkatal/mg protein*
apple (peel)	0.204^{a}	0.409^{a}	1.58^{a}	0.197^{a}
artichoke (flower leaf)	0.517^{a}	10.5^{b}	116^{b}	5.23^{b}
broccoli (stem)	0.916^{a,c,f}	0.327^{a}	BDL	BDL
carrot (peel)	0.462^{a}	0.373^{a}	BDL	BDL
cauliflower (floret)	1.39^{b,c,d,e}	0.243^{a}	BDL	BDL
cauliflower (stem)	0.817^{a,c,d}	0.246^{a}	BDL	BDL
cauliflower (leaf)	0.601^{a}	0.397^{a}	BDL	BDL
eggplant (pulp)	0.637^{a}	1.20^{a}	48.5^{a,b}	1.55^{a}
pineapple (peel)	0.705^{a,d}	0.736^{a}	1.53^{a}	0.0434^{a}
potato tuber (peel)	2.73^{g}	0.229^{a}	74.4^{b,c}	0.537^{a}
red clover (stem & leaf)	1.45^{b,c}	0.551^{a}	62.1^{b}	0.891^{a}
spinach (leaf)	1.57^{b,f}	0.333^{a}	4.79^{a}	0.0593^{a}
tomato (stem & leaf)	0.788^{a,c,e}	0.301^{a}	40.2^{a,c}	1.18^{a}
SEM	0.140	1.858	13.19	0.51
P-value	<0.001	0.006	<0.001	<0.001

PBP, protein-bound phenol; Tyr-eq, tyrosine equivalents; PPO, polyphenol oxidase; BDL, below detection limit; SEM, standard error of the mean

$^{a-g}$ indicates differences within columns between protein sources at P≤0.05

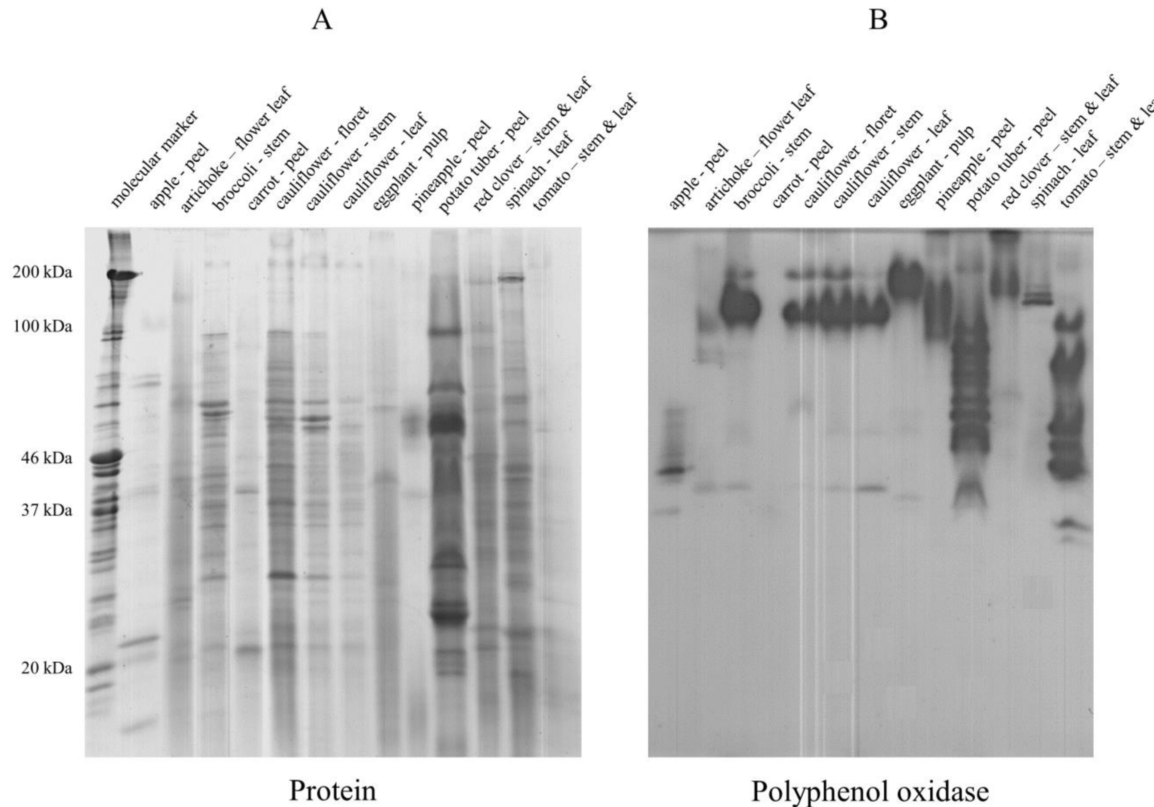

Figure 5.1 Gel electrophoretic analysis showed different patterns of proteins (A) and polyphenol oxidase (PPO) isoforms (B) for protein extracts of thirteen plant sources (experiment 1), revealing large variation in PPO activity

extracts of cauliflower, broccoli, spinach and pineapple, whereas intense bands were detected on gels. Furthermore, intensities of bands with high PPO activity (Figure 5.1B) were not related to intensities of protein as such (Figure 5.1A), meaning for some sources PPO represented only a minor proportion of the proteins.

Emulsion droplet sizes and specific surface areas (Table 5.2) illustrate the protein concentrations of the extracts were sufficient to obtain stable emulsions with a D_{32} ranging from 0.67 to 3.91 µm for cauliflower floret and carrot peel, respectively, associated with the highest (9.69 m²/g oil) and lowest (1.67 m²/g oil) specific surface area. Artichoke, broccoli, cauliflower and spinach protein extracts resulted in emulsions with the smallest droplet sizes, with 90 % of the droplet population showing a diameter below 10 µm. Regression analysis revealed no linear link between D_{32} of the emulsions and the protein concentration of the different extracts (P=0.927).

Table 5.2 Protein extracts (n=3) of thirteen selected plant sources in experiment 1 were used to emulsify 20 mg oil per ml extract, resulting in differences in emulsion droplet characteristics and protection against C18:3*n-3* biohydrogenation after 24h *in vitro* rumen incubation of emulsions with 40 mM 4-methylcatechol

protein source	D_{43} *µm*	D_{32} *µm*	$D_{(v,0.5)}$ *µm*	$D_{(v,0.9)}$ *µm*	SSA *m²/g oil*	PE C18:3*n-3* *-/-*
apple (peel)	8.90	$2.52^{a,b}$	$6.05^{a,b}$	21.0	$2.12^{a,b}$	$0.026^{a,c}$
artichoke (flower leaf)	3.87	$1.15^{a,c}$	2.97^{a}	8.50	$9.08^{a,c}$	$0.468^{a,b}$
broccoli (stem)	2.38	$1.49^{a,c,d}$	1.85^{a}	4.78	$4.59^{a,c,d}$	0.758^{b}
carrot (peel)	9.95	3.91^{b}	9.25^{b}	19.1	1.67^{b}	$0.075^{a,c}$
cauliflower (floret)	1.05	0.67^{c}	0.84^{a}	1.99	9.69^{c}	0.889^{b}
cauliflower (stem)	1.79	$1.26^{a,c}$	1.56^{a}	3.25	$5.33^{a,c}$	0.683^{b}
cauliflower (leaf)	4.65	$2.03^{a,c,e}$	2.56^{a}	8.97	$3.25^{a,c,e}$	0.657^{b}
eggplant (pulp)	5.06	$1.91^{a,c,f}$	3.11^{a}	12.3	$3.63^{a,c,f}$	$0.069^{a,c}$
pineapple (peel)	7.42	$3.37^{b,e,f}$	$5.88^{a,b}$	15.3	$1.93^{b,e,f}$	0.006^{a}
potato tuber (peel)	7.46	$1.61^{a,c,g}$	$4.04^{a,b}$	19.8	$3.99^{a,c,g}$	0.817^{b}
red clover (stem & leaf)	5.61	$1.15^{a,c}$	2.10^{a}	11.2	$6.36^{a,c}$	$0.545^{b,c}$
spinach (leaf)	1.79	$0.97^{a,c}$	1.26^{a}	3.16	$8.06^{a,c}$	0.868^{b}
tomato (stem & leaf)	6.32	$2.81^{b,d,e,f,g}$	$5.40^{a,b}$	12.9	$2.39^{b,d,e,f,g}$	$0.380^{a,b}$
SEM	1.78	0.29	1.07	3.89	1.18	0.104
P-value	0.033	<0.001	<0.001	0.022	<0.001	<0.001

D_{43}, volume-weighted mean diameter; D_{32}, surface-weighted mean diameter; $D_{(v,0.5)}$, 50 % median volume distribution diameter, meaning 50% of the population shows droplet sizes with a diameter below the value mentioned; $D_{(v,0.9)}$, 90 % volume distribution diameter, meaning 90% of the population shows droplet sizes with a diameter below the value mentioned; SSA, specific surface area; PE, protection efficiency; SEM, standard error of the mean

$^{a-g}$ indicates differences within columns between protein sources at P≤0.05

In order to create protection, 4-MC was added to the emulsions to obtain final concentrations of 0, 10, 20 or 40 mM. Results for BH of C18:3*n-3* are depicted in Figure 5.2A, while the associated PE for 40 mM 4-MC emulsions are presented in Table 5.2. Numeric averages and standard deviations for BH of C18:3*n-3* and C18:2*n-6* as well as the PE of C18:3*n-3* and the formation of C18:0 for all treatments are reported in Addendum B.1. On average across all protein sources, a proportion of 0.963 of C18:3*n-3* in emulsions without 4-MC was hydrogenated after 24h *in vitro* incubation. Upon addition of 4-MC, a decrease in BH of C18:3*n-3* was observed with the extent of the decrease depending on the source of protein used as emulsifier. C18:3*n-3* BH of emulsions containing 10 mM 4-MC, prepared using potato tuber peel, was significantly lower compared with other protein sources ($P<0.05$), except for red clover stem & leaf ($P=0.163$). Increasing the diphenol concentration to 20 mM 4-MC resulted in a further decrease of C18:3*n-3* BH, although only cauliflower floret ($P=0.015$) and potato tuber peel ($P<0.001$) emulsions were significantly different from their 0 mM counterparts. Emulsions with the highest 4-MC concentration resulted in more protein sources being different from the 0 mM 4-MC emulsions. Cluster analysis of emulsions with added 4-MC revealed three major groups (Figure 5.2B): first, a cluster of protein sources with C18:3*n-3* BH levels which did not differ from the 0 mM treatment (pineapple peel, apple peel, carrot peel and eggplant pulp); second, a cluster resulting in intermediary reductions in C18:3*n-3* BH as compared with their 0 mM control; and third, a cluster with large reductions in C18:3*n-3* BH from the lowest 4-MC concentration onwards (potato tuber peel). A further distinction could be made for the intermediary group, showing groups with large reduction in C18:3*n-3* BH from 20 mM onwards (cauliflower floret and spinach leaf) or only showing reduced C18:3*n-3* BH at the highest level of 4-MC (40 mM). All BH levels for protein sources in the second or third cluster using 40 mM 4-MC were significantly different from the emulsions without 4-MC ($P<0.012$), except for artichoke flower leaf ($P=0.132$) and tomato stem & leaf ($P=0.486$). Protein sources of the second cluster were associated with the highest standard deviations for C18:3*n-3* BH, which generally diminished at increased 4-MC concentrations (Addendum B.1). Overall, the lowest BH of C18:3*n-3* (0.106) and

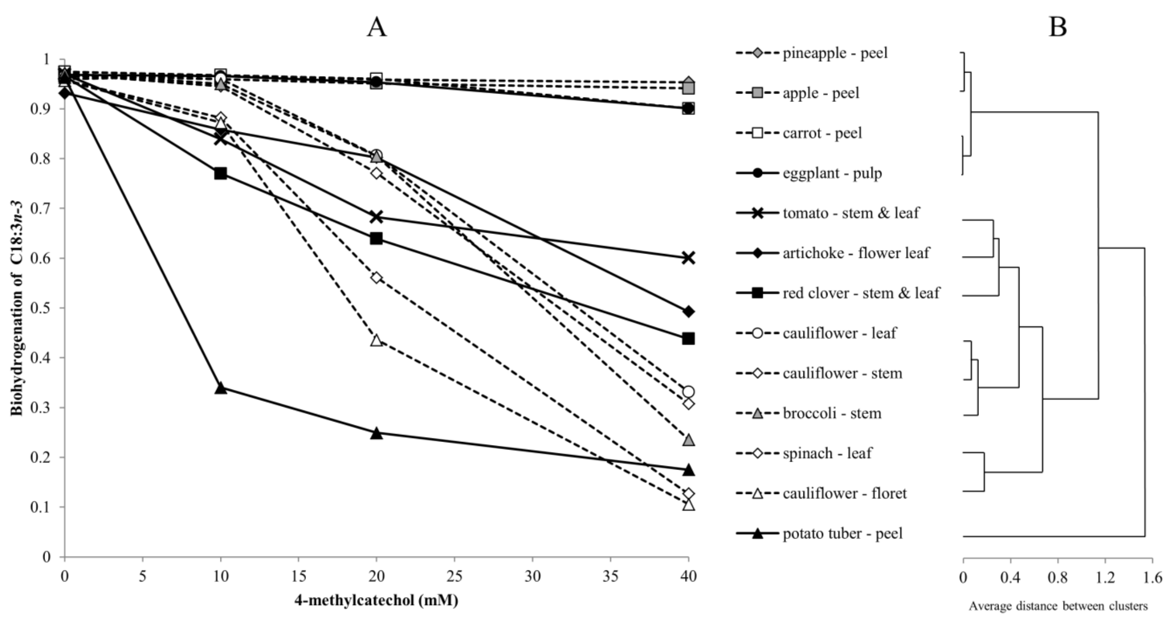

Figure 5.2 Biohydrogenation of C18:3*n-3* after 24h *in vitro* ruminal incubation (A) is influenced by diphenol concentration and plant source from which protein extracts were obtained for further emulsification (experiment 1). Emulsions contained 20 mg oil per ml protein extract and varied in diphenol concentration (0, 10, 20 or 40 mM 4-methylcatechol) (n=3). Full and dotted lines connecting data points within protein source represent high (>0.2 µkatal/mg protein) or low (<0.2 µkatal/mg protein) specific PPO activity, respectively. Cluster analysis (B) was performed for C18:3*n-3* BH of emulsions with 10, 20 and 40 mM 4-methylcatechol

concomitantly highest PE (0.889) was observed for extracts of cauliflower floret using 40 mM 4-MC. However, an effect of diphenol (P<0.001) and protein (P<0.001) on the formation of C18:0, expressed relative to the amount of hydrogenated C18:2*n-6* and C18:3*n-3* after 24h *in vitro* incubation was observed (Addendum B.1), indicating a stronger protection resulted in a more complete BH due to a slower and more gradual release of the PUFA. Finally, linear regression analysis revealed no link between the level of protection against C18:3*n-3* BH and PPO activity (P=0.909) or specific PPO activity (P=0.620) of the extract.

3.2 Experiment 2: selected plant sources with similar protein extract concentrations

As no link was found between the spectrophotometric PPO activity and protection against BH in experiment 1, protein partitioning between the continuous phase and the emulsion droplet interface was assessed for four sources with diverse characteristics. Selection of vegetal

sources (potato tuber peel, cauliflower floret, eggplant pulp and carrot peel) was based on PPO activity (low versus high) and rumen protection (low versus high) observed in experiment 1. Proteins were extracted as in experiment 1, but protein solutions were diluted with SPB to obtain similar protein concentrations of about 0.9 mg/ml across treatments. Dilutions were based on preliminary results of the Lowry assay in the presence of copper, representing the combined response of protein and phenolic groups.

True protein concentrations of the diluted extracts (Table 5.3) were somewhat lower than 0.9 mg/ml, due to later correction for PBP using the modified Lowry assay. Although we failed to have true protein concentrations exactly the same in all four extracts, the variation in protein concentration was considerably smaller as compared with experiment 1 and absolute concentrations were generally lower. Similarly as for the first experiment, high (specific) PPO activity was found for potato tuber peel and eggplant pulp extracts, in contrast to low values for cauliflower floret and carrot peel.

Table 5.3 Protein concentration, PBP and (specific) PPO activity in the extracts (n=1) of four selected plant sources and the resulting continuous phase after emulsifying 20 mg oil per ml extract (experiment 2). Protein concentration and (specific) PPO activity in the continuous phase of the emulsions was smaller than in the original extract, although PBP concentration increased

protein source	protein *mg/ml extract*		PBP *mg Tyr-eq/mg protein*		PPO activity *nkatal*		specific PPO activity *µkatal/mg protein*	
	Extract	CP	Extract	CP	Extract	CP	Extract	CP
potato tuber (peel)	0.870	0.741	0.058	0.074	14.0	3.47	0.321	0.0940
cauliflower (floret)	0.665	0.458	0.139	0.316	BDL	BDL	BDL	BDL
eggplant (pulp)	0.500	0.355	0.478	1.070	48.1	6.68	1.923	0.378
carrot (peel)	0.768	0.463	0.130	0.386	BDL	BDL	BDL	BDL
P-value	<0.001*		0.008*		0.028*		0.047*	

PBP, protein-bound phenol; Tyr-eq, tyrosine equivalents; PPO, polyphenol oxidase; CP, continuous phase; BDL, below detection limit;
*P-values are the result of a paired two-sample T-test, depicting differences between variables measured in the extract and the continuous phase of the emulsion, irrespective of protein source

Extracted proteins were used to emulsify linseed oil resulting in emulsions with varying characteristics (Table 5.4). D_{32} was similar for emulsions with cauliflower floret, eggplant pulp or carrot peel, but larger for potato tuber peels. Specific surface areas differed between the four sources, with the largest interfacial area measured for emulsions created with

Table 5.4 Protein extracts (n=1) of four selected plant sources in experiment 2, having relatively similar protein concentrations (Table 5.3), resulted in differences in emulsion (3 original emulsions created by using the same protein extract) droplet characteristics after emulsifying 20 mg oil per ml extract and protection against 24h *in vitro* rumen C18:3*n-3* biohydrogenation of emulsions with 40 mM 4-methylcatechol.

protein source	D_{32} *µm*	SSA *m²/g oil*	protein load *g/100 g adsorbed*	protein load *mg/m²*	PE C18:3*n-3* *-/-*
potato tuber (peel)	5.95^{a}	1.17^{a}	14.9^{a}	7.26	0.562^{a}
cauliflower (floret)	1.05^{b}	6.18^{b}	$31.1^{b,c}$	1.74	$0.657^{a,b}$
eggplant (pulp)	1.31^{b}	4.93^{c}	29.1^{b}	1.54	0.015^{c}
carrot (peel)	2.05^{b}	3.17^{d}	39.7^{c}	5.04	0.421^{d}
SEM	0.61	0.24	2.3	2.38	0.023
P-value	0.002	<0.001	<0.001	0.117	<0.001

D_{32}, surface-weighted mean diameter; SSA, specific surface area; PE, protection efficiency; SEM, standard error of the mean
$^{a\text{-}d}$ indicates differences within columns between protein sources at P≤0.05

cauliflower floret proteins. The proportion of proteins adsorbing to the interface was limited for potato tuber peel proteins (0.149 g/g), while 0.400 g/g adsorbed in the case of carrot peel. Protein loads varied from 1.54 mg of protein per m² interfacial area using eggplant pulp up to 7.26 mg/m² in the case of potato tuber peel, although these values were not different between treatments (P=0.117). Smaller concentrations of protein were found in the continuous phase as compared to the original extract (P<0.001) (Table 5.3). This was not observed for PBP, where higher (P=0.008) PBP concentrations were found in the continuous phase for all protein sources.

Next, proteins distributed at the interface or remaining in the continuous phase of the emulsions were loaded on gels. No major differences in protein bands (Figure 5.3A) were observed between those present in the whole protein extract and the ones residing in the continuous phase of the emulsion. However, protein bands recovered in the cream phase differed from those in the whole extract: for potato tuber peel and eggplant pulp only a few bands from the original extract were present at the interface, while a smear of proteins was observed for the cauliflower floret and carrot peel emulsion interface. The original extract showed multiple bands for PPO activity (Figure 5.3B) in the case of potato tuber peel and a single band for cauliflower floret. Multiple but closely eluting bands seem to be responsible

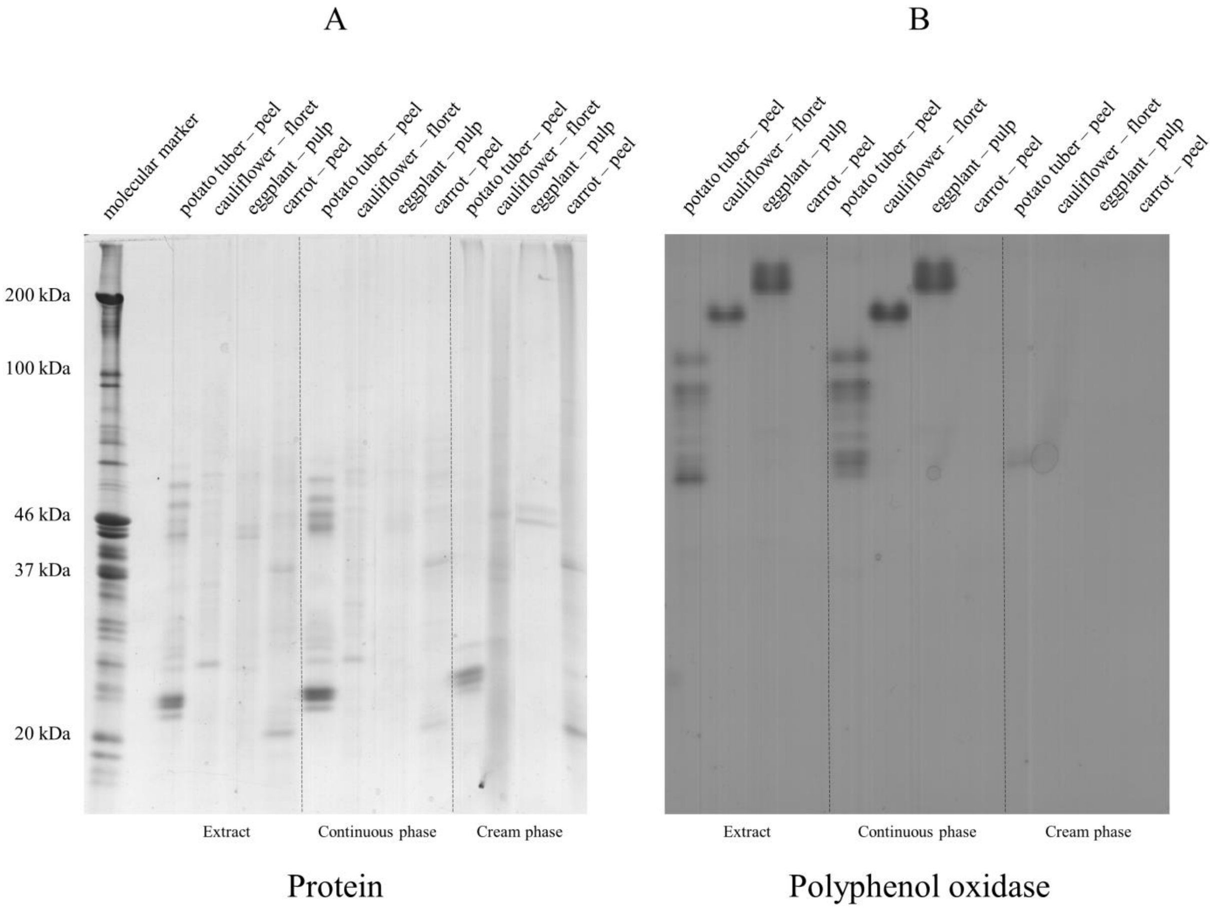

Figure 5.3 Gel electrophoretic analysis revealed different proteins (A) participating in the cream phase of emulsions containing 20 mg oil per ml protein extract of four selected plant sources with similar protein extract concentrations, compared with the continuous phase (experiment 2). Polyphenol oxidase (PPO) isoforms (B) were not detected in the cream phase. Loaded volumes per lane varied from 10 to 50 µl in order to load equal amounts of protein

for the large PPO activity in eggplant pulp. Fewer bands for those three vegetal sources were observed as compared with experiment 1, which might be related to the lower protein load on the gels of experiment 2. As in experiment 1, no PPO bands were observed in carrot peel extracts. Furthermore, proteins in the continuous phase of the emulsions showed a similar PPO activity profile as observed for whole protein extracts. However, proteins of the droplet interface did not show any PPO activity, except for a very weak and hazy band appearing in the potato tuber peel interface.

Finally, the highest protection against ruminal BH (using 40 mM 4-MC) was obtained when potato tuber peel or cauliflower floret was used, while negligible protection was found using

eggplant pulp (Table 5.4). In contrast to the first experiment, protection was found when using carrot peel proteins as emulsifier.

4 Discussion

4.1 Valorization potential of plant and sidestream recovered proteins

Previous research showed the possibility of red clover protein extracts, rich in PPO, to protect emulsified unsaturated FA against *in vitro* ruminal BH (Gadeyne *et al.*, 2015) (see also Chapter 4). Logically, it could be hypothesized that any protein extract showing PPO activity can be used as emulsifier to obtain rumen protected PUFA-rich emulsions. As a plethora of papers demonstrated PPO activity in many plants (Mayer, 2006; Yoruk and Marshall, 2003), a selection was made of thirteen vegetal sources to test their capacity to create rumen protected emulsions. The results of this study confirmed the feasibility to use protein solutions from other sources than red clover to obtain substantial rumen protection. However, the origin of the protein extract had a profound effect on the ruminal PE. This varied from very low (e.g. carrot peel in experiment 1 and eggplant pulp) to very high (e.g. potato tuber peels, floret of cauliflower, broccoli waste and spinach leaves).

Even though only few vegetal sources were tested in this study, much more vegetal protein sources could be hypothesized to result in ruminal protection. From the current set of protein sources, potato tuber peels could be regarded as the most valuable sidestream, especially since potato is the world’s fourth most important crop, after rice, wheat and corn (Mullins *et al.*, 2006).

4.2 Factors affecting ruminal protection efficiency

4.2.1 Polyphenol oxidase activity of the protein extract

Within the current concept, PPO is hypothesized to be the key component in order to create protection against ruminal BH (Gadeyne *et al.*, 2015; Gadeyne *et al.*, 2016f). Although

several protein sources in this study resulted in ruminal protection of emulsified PUFA, no direct link was found between PPO activity of the protein extract and PE.

The lack of correlation between PPO activity and PE might be related to the methodology of PPO measurement. Indeed, PPO activity was spectrophotometrically quantified by measuring the change in absorbance upon addition of 4-MC during one minute. During this short time frame, PPO activities of some extracts remained below the detection limit of 1.5 nkatal, as absorbances increased inadequately. Nevertheless, these extracts also resulted in dark discoloration upon addition of 4-MC after a sufficient amount of time (> 8 h; data not shown). Therefore, the lack of relation between PPO activity measured within a one minute time frame and PE might be related to the relatively long time (24h) emulsions were exposed to 4-MC to create rumen bypass emulsions, which might have resulted in adequate cross-linking, even with sources showing low PPO activity. Indeed, protection of emulsified lipids has been shown before to rise upon longer exposure to diphenol (Gadeyne *et al.*, 2015) (see also section 3.8 of Chapter 4; experiment 8). On the other hand, the detection of PPO activity in the protein extract does not automatically make the protein extract suitable to create protection against *in vitro* ruminal BH even when stable small-sized emulsions were formed (e.g. eggplant pulp shows high PPO activity and is useful to create stable emulsions with relatively small droplet sizes but results in low protection against BH). Therefore, it isn’t possible to simply deduce from a standard PPO activity test whether or not rumen protection can be obtained. This makes it also impossible to derive conclusions on PE from PPO activities reported in literature, in addition to the problem that reported PPO activities may largely differ due to measurement methodologies and procedures for extraction or purification.

On the other hand, gel electrophoretic analysis revealed high reactivity of some extracted proteins towards 4-MC, despite spectrophotometric measurements were below the detection limit. Conflicting results between a conventional spectrophotometric PPO assay and a PAGE-blot have been reported before and were explained by an endogenous PPO inhibitor

present in the extract (Cheng *et al.*, 2007). The presence of such inhibitors (or activators) in the protein extract most likely does not affect the activity observed by means of PAGE (Marri *et al.*, 2003). However, in case the action of an inhibitor would explain the discrepancy between the spectrophotometric PPO assay (below detection limit) and PAGE observations (obvious PPO band) for cauliflower extracts, the potential to create emulsions which are effectively protected against rumen BH through addition of 4-MC remains unexpected. Indeed, both PPO as well as the inhibitor would be expected to be present in the extract of cauliflower floret. Apart from that, gel electrophoretic analysis also revealed reactivity towards 4-MC was not exclusively linked to one single protein band, which might indicate the existence of various enzyme isoforms of PPO, as observed before in unpurified protein extracts of potato tubers (Cho and Ahn, 1999; Marri *et al.*, 2003) and other biological materials (Cheng *et al.*, 2015; Cheng *et al.*, 2007).

4.2.2 Interfacial involvement of polyphenol oxidase activity

In order to gain more insight on the role of PPO on PE, four protein sources from the first experiment were selected for further investigation in experiment 2. Here, proteins at the interface or those remaining in the continuous phase were visualized. Differences in PE were hypothesized to be linked to variation in the presence of PPO at the interface rather than PPO in the total extract. The aim of experiment 2 was to have lower protein concentrations compared with experiment 1, but which were similar across treatments. It was still ensured that interfacial protein loads were larger than 1 mg/m², to exclude that differences in PE between protein extracts would be related to varying amounts of protein as emulsifier.

Logically, protein concentrations in the continuous phase were lower compared to the original un-emulsified extract due to protein inclusions in the interface. PPO activity was also lower in the continuous phase. Such reduction in PPO activity could be due to the high shear during emulsification besides its incorporation in the interface. Spectrophotometric measurements also indicated PBP present in the extract prior to 4-MC addition particularly

participated in the continuous phase. The latter suggests PBP, formed during the extraction, to be less suitable emulsifiers than non-bound proteins. Unexpectedly, SDS-PAGE analysis of the cream phase of the emulsions revealed no reactivity towards 4-MC at the interfacial level. Loss of enzyme activity upon adsorption at an emulsion interface has been observed before (de Roos and Walstra, 1996). The present observation is, however, in contrast with Gadeyne *et al.* (2015) (see also section 4 of Chapter 4), who suggested active PPO to be part of the emulsion interface, but no electrophoretic confirmation was given in the latter study.

Much research has been done on cross-linking enzymes, such as transglutaminase, laccase or tyrosinase/PPO to improve the stability and functionality of food dispersions (Zeeb *et al.*, 2014). The involvement of higher molecular weight protein fractions in strengthening the interfacial layer seems plausible as their occurrence, for example induced by transglutaminase cross-linking (Partanen *et al.*, 2013), in the cream phase of emulsions has been observed before. However, only few papers reported on the use of PPO to cross-link interfacial protein membranes (Ercili-Cura *et al.*, 2012; Gadeyne *et al.*, 2015; Isaschar-Ovdat *et al.*, 2015). The co-occurrence of protein and phenol in emulsified systems, either cross-linked (Ali *et al.*, 2013; Reinkensmeier *et al.*, 2016) or not (Conde *et al.*, 2011) beforehand, has also been shown before to improve the stability of emulsified lipids. There, cross-linked interfacial layers were presumably obtained by a quinone reaction with nucleophilic residues of the emulsifying proteins. Such cross-linking due to PPO seems to be responsible for protection against microbial breakdown in the current study too, as emulsions without diphenol addition did not result in any protection in the present study.

4.2.3 Contribution of other factors to variation in ruminal protection efficiency

It was possible to obtain substantial protection against BH using several protein sources, but large variation in PE was observed between sources. To elucidate which factors explained most of the variation in PE besides PPO, principal component analysis was performed using

the 40 mM 4-MC treated emulsions of both experiments. Variation in PE seemed mainly explained by variation in protein concentration, while the variation seemed rather independent from PPO activity (Figure 5.4A). This might explain why PE of emulsions with carrot peels extracts in the second experiment are larger compared with the first. Indeed, a shift along the first principal component axis was found between experiments (Figure 5.4B) due to differences in protein concentrations, although in both cases stable emulsions were obtained. Nevertheless, it has been shown before that stable emulsions, active PPO and an appropriate diphenol source such as 4-MC are needed to obtain protection (Gadeyne *et al.*, 2015) (see also Chapter 4). Combination of current and previous observations could imply stable emulsions, with sufficiently small oil droplet size, in combination with 4-MC and some level of PPO activity are a prerequisite for protection, but once these conditions are met the protein concentration of the extract is the largest contributor to acquire protection against BH. This might be related to varying emulsifying properties of different proteins or a varying amino acid profile which might affect polymerization and protection.

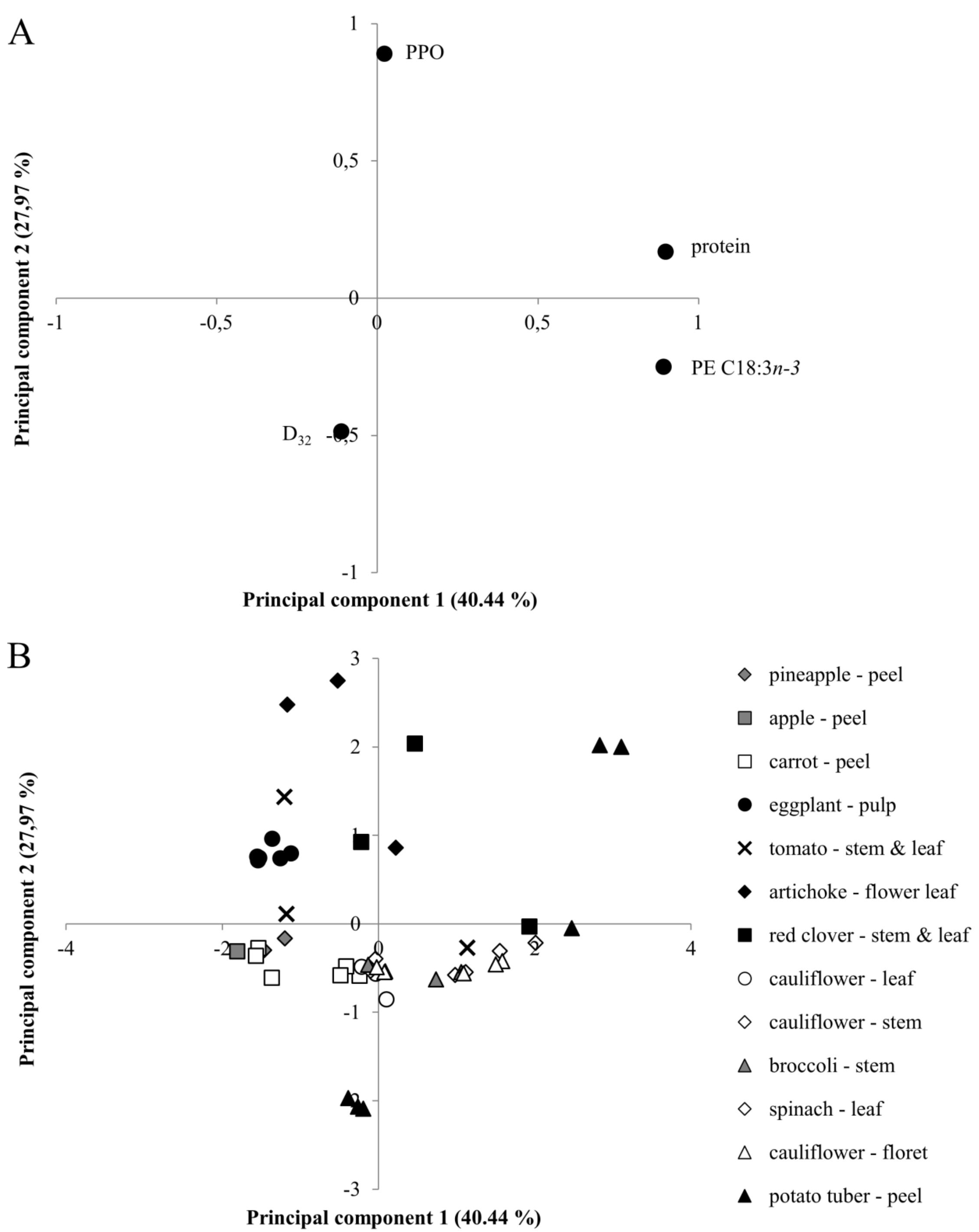

Figure 5.4 Principal component loadings (A) and scores (B) using data from the 40 mM 4-methylcatechol emulsions of experiment 1 and 2 showed variation in protection of C18:3*n-3* (PE) against ruminal biohydrogenation was mainly explained by variation in protein concentration of the extract, while variation in the surface-weighted mean diameter (D_{32}) of the emulsions was less determining and PE was fairly independent of polyphenol oxidase (PPO) activity of the extract

5 Conclusions

It is possible to protect PUFA against ruminal BH using 4-MC after emulsification in protein extracts of several plant resources, despite major differences in PPO isoforms and activity. However, PPO activity and degree of rumen bypass were not correlated. Furthermore, current results showed PPO isoforms do not need to be part of the emulsion interface. The origin and concentration of the protein extract, however, determined to a large extent the ruminal PE. Potato tuber peels showed to be an interesting source of PPO and protein to create ruminal PUFA protection.

6 Acknowledgements

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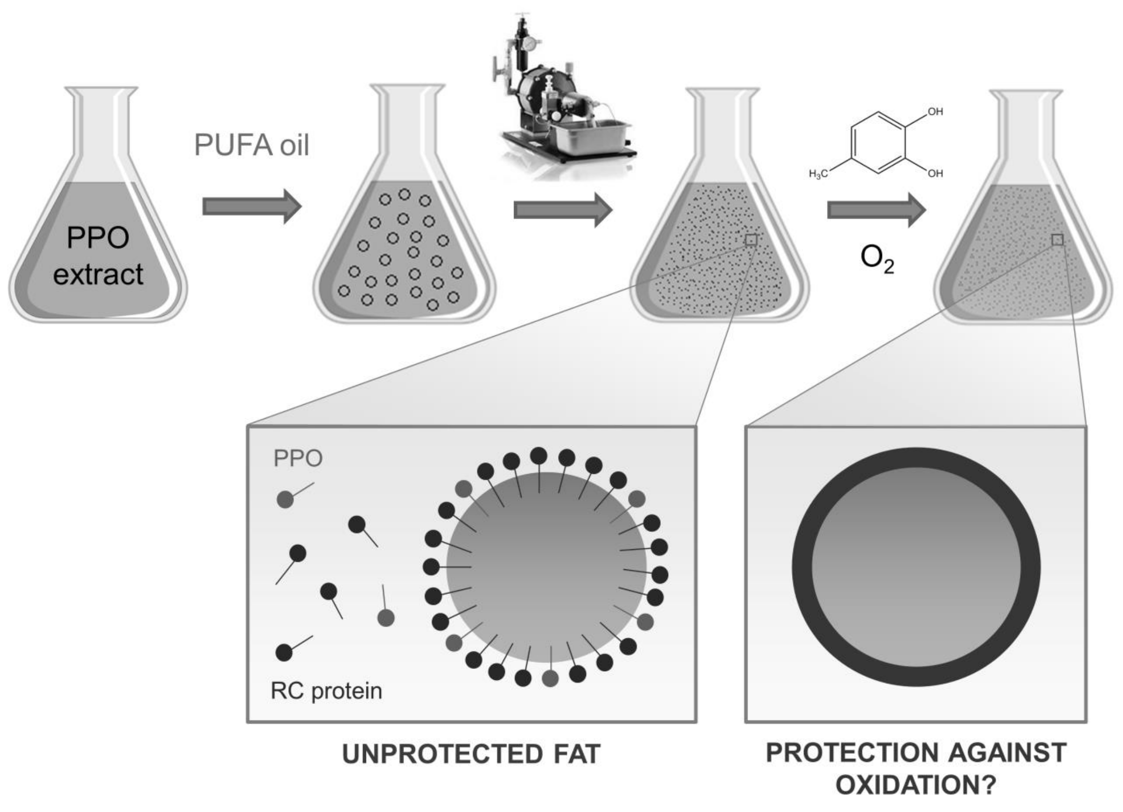
Chapter 6

Protection of polyunsaturated oils and vitamin E against oxidation using a polyphenol oxidase containing extract



Partially redrafted after Gadeyne F., Van Ranst G., Vlaeminck B., Vossen E., Van der Meeren P. and Fievez V., 2015, Protection of polyunsaturated oils against ruminal biohydrogenation and oxidation during storage using a polyphenol oxidase containing extract from red clover, Food Chemistry, 171, 241-250.

Partially redrafted after Fievez V., Gadeyne F. and Van Ranst G., 2015, Method to protect lipophilic nutrients against ruminal degradation, International Publication Number: WO 2015091840 A1

Chapter 6

Protection of polyunsaturated oils and vitamin E against oxidation using a polyphenol oxidase containing extract

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Abstract. Polyunsaturated fatty acids (PUFA) are to a large extent prone to biohydrogenation (BH) in a ruminal environment, resulting in the loss of its healthy features when being fed to ruminants. However, PUFA might also be prone to processes of oxidation during storage, making their healthy value is already lost before being fed to ruminants. It has been shown before that emulsions created using protein extracts rich in polyphenol oxidase (PPO), an enzyme responsible for browning of plant tissues, can be protected against *in vitro* ruminal BH upon addition of a synthetic diphenol, e.g. 4-methylcatechol (4-MC). Therefore, it was hypothesized that the same process might also protect the encapsulated content against oxidation during storage. Indeed, results confirmed the potential of the PPO technology to protect PUFA both against ruminal BH as well as oxidation during storage. It was also demonstrated that the pre-ingestive oxidation of other co-emulsified lipophilic compounds such as vitamin E is hampered upon addition of 4-MC. Overall, this further illustrates the benefits and potential of the PPO technology.

1 Hypothesis and objective

Results from Chapter 4 and Chapter 5 demonstrated the potential of protecting emulsified PUFA against rumen BH with PPO. In this chapter, it was hypothesized that the same process might also protect the encapsulated content against oxidation during storage. Therefore, the goal was to evaluate whether PUFA and other lipophilic compounds such as vitamin E might also be protected against oxidation during storage.

2 Materials and Methods

2.1 Experiment 1: Oxidation during storage of PUFA

2.1.1 Set-up

It was examined if the applied protection technology allowed to protect PUFA against oxidation during storage. Two emulsions (n=1) were prepared as described before (see section 2.1 and 2.2 of Chapter 4): an emulsion with red clover (*Trifolium pratense* L.) protein extract and 2 mg/ml casein on the one hand (RC+CAS) and on the other hand an emulsion containing 2 mg/ml casein only (CAS). Emulsions were prepared without or in the presence of 20 mM 4-MC. To stimulate oxidation, fresh emulsions containing 30 g of linseed oil per liter were shaken under aerobic conditions for 1, 2, 4 or 6 days at 50°C. The degree of oxidation was assessed both through the measurement of volatile components (SPME-GC/MS) and thiobarbituric acid reactive substances (TBARS). A summary of emulsion characteristics in experiment 1 is shown in Table 6.1.

Table 6.1 Emulsion characteristics for the experiment assessing protection of polyunsaturated fatty acids (experiment 1; n=1) or alpha-tocopherol (experiment 2; n=2) in oil-in-extract emulsions against oxidation during storage. Emulsions were either or not treated with 4-methylcatechol to create protein-bound phenol complexes to achieve rumen bypass products

	Emulsion treatment			D[4,3]	D[3,2]	D[v,0.5]	D[v,0.9]	SSA
	Protein	Oil[a]	CAS[b]	(µm)	(µm)	(µm)	(µm)	(m²/g oil)
Experiment 1	Red clover	30	2	0.46	0.39	0.42	0.76	16.5
	-	30	2[c]	0.47	0.39	0.42	0.72	16.5
Experiment 2	Potato peel	20	0	10.0	1.86	7.48	23.9	3.60

CAS, casein; D[4,3], volume-weighted mean diameter; D[3,2], surface-weighted mean diameter; D[v,0.5], 50 % median volume distribution diameter, meaning 50 % of the population shows droplet sizes with a diameter below the value mentioned; D[v,0.9], 90 % volume distribution diameter, meaning 90 % of the population shows droplet sizes with a diameter below the value mentioned; SSA, specific surface area (m²/g oil)

[a] amount of oil emulsified in g per liter protein extract; linseed oil was used in experiment 1, while the oil phase in experiment 2 contained 90 wt % Tonalin and 10 wt % vitamin E

[b] amount of casein in g per liter protein extract, added to the continuous phase of the emulsion before emulsification

[c] only casein was used to emulsify oil, so no red clover proteins were present in the emulsion

2.1.2 SPME-GC/MS analysis of volatile compounds

With SPME-GC/MS (Solid Phase Micro-Extraction – Gas Chromatography/Mass Spectroscopy) volatile components, formed during oxidation of unsaturated fatty acids, are measured. The analysis is based on the method as described by Jelen *et al.* (2007). Volatiles were extracted from the headspace of the emulsions with a carboxen-polydimethylsiloxane (CAR/PDMS) fiber (85 µm thickness) (Supelco, Bellefonte, Pennsylvania, USA). For this, 3 g of emulsion, put in a 10 ml vial, were incubated in a heating block for 45 min at 35°C. Extracted volatiles were analyzed with a gas chromatograph (Agilent model 6890N) coupled to a mass-selective detector (Agilent model 5973, Agilent Technologies, Diegem, Belgium). Compounds were separated on a HP-5 column (30 m x 250 µm x 1 µm, 5 % phenyl methyl siloxane, Agilent Technologies, Diegem, Belgium), at an inlet temperature of 280°C. Hydrogen flow was 1.1 ml/min and the temperature program was as follows: 40°C for 3 min; increased at 8°C/min to 280°C. N-alkanes were run under the same conditions to calculate the Kovats index (KI) values for the compounds. Compounds were identified by comparing their mass spectra with those contained in the NIST05 mass spectral library and by comparison of KI with those reported by Jelen *et al.* (2007). Samples were analyzed in duplicate and results for the major volatile oxidation product of interest were provided in arbitrary area units (AAUx10^6).

2.1.3 Thiobarbituric acid reactive substances

TBARS were measured to assess the general oxidative state of the emulsions. The reaction medium was measured colorimetrically and expressed as malondialdehyde-equivalents, oxidation products of unsaturated fatty acids. Extraction and sample preparation were based on the method described by Grotto *et al.* (2007). In short, 125 µl of emulsion and 250 µl of distilled water were brought in an extraction tube, together with 250 µl of 1.5M NaOH and vortexed with a Multi-Tube Vortex (VX-2500, VWR International, Leuven, Belgium). For standard solutions, 375 µl of 1,1,3,3-tetramethoxypropane was used. Samples were put in a

water bath at 60°C for 30 min, before adding 625 µl of 6 % (w/v) H_3PO_4 and 625 µl of 2-thiobarbituric acid (0.8 g dissolved in 100 ml of distilled water). Next, samples were put in a water bath at 90°C for 45 min. After cooling down for 10 min, 250 µl of 10 % (w/v) sodium dodecyl sulphate (10 g dissolved in 100 ml of distilled water) and 2.5 ml of n-butanol was added to the extraction tubes. Samples were vortexed and immediately centrifuged for 10 min at 1500×g. The upper layer was carefully taken with a Pasteur pipette and brought into a cuvette, to measure the absorbance at 532 nm. The analysis was done in duplicate.

2.2 Experiment 2: Pre-ingestive oxidation of vitamin E

In this second experiment, it was tested whether emulsions containing alpha-tocopherol (vitamin E) could withstand pre-ingestive oxidation during storage. Therefore, emulsions (n=2) containing 18 mg oil, with high concentrations of trans-10, cis-12 conjugated linoleic acid as triacylglyceride (Tonalin® TG80, BASF-AG, Ludwigshafen, Germany), per ml potato (*Solanum tuberosum* L.) tuber peel protein extract (n=2) and 2 mg vitamin E (T3251, (±)-α-Tocopherol, Sigma-Aldrich, Bornem, Belgium) per ml protein extract were made as described before in section 2.2 of Chapter 4. A summary of emulsion characteristics in experiment 2 are also shown in Table 6.1. Emulsions were prepared with a final 4-MC concentration of 0 and 20 mM. To induce oxidation, emulsions were put on a shaker in a ventilated oven at 50°C for about 16h before vitamin E analysis (Pastsart *et al.*, 2013). Results are expressed as µg of vitamin E per ml emulsion.

2.3 Statistics

Results of experiment 1 were analyzed by the MIXED procedure of SAS (SAS Enterprise Guide 5.1, SAS Institute Inc., Cary, North Carolina, USA). Prior to statistical analysis, technical replicates were averaged. Hence, the design didn't allow assessment of all interactions, but did allow assessment of all (n-1)-way interactions (with n the number of factors tested). The following model was used: $Y_{ij} = \mu + P_i + D_j + S_k + P_i \times D_j + S_k \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, P_i the effect of protein (i = red clover extract with extra casein

(RC+CAS) or casein only (CAS)), D_j the effect of diphenol concentration in the emulsion (j = 0 or 20 mM 4-MC), S_k the effect of storage time (k = 1, 2, 4 or 6 days) and ε_{ij} the residual error. The interaction effect $P_i \times S_k$ was left out of the model, as this effect turned out to be non-significant in each case. All mentioned differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

3 Results

3.1 Experiment 1: Oxidation during storage of PUFA

In this experiment, emulsions with or without red clover, and with or without 4-MC, were treated to induce oxidation. Results from SPME-GC/MS of the emulsions after 1, 2, 4 and 6 days of exposure to oxidation, together with the mean values over all storage times, showing a detailed image of major end products of fat oxidation, are given in Table 6.2. Generally, all volatile oxidation products in RC+CAS emulsions were decreased upon addition of 4-MC. Values for hexanal were considerably higher in emulsions with the RC extract as compared with emulsions containing casein only. Significant differences between CAS emulsions and CAS emulsions with 4-MC for 2-pentenal and 3,5-octadien-2-one were observed, which might suggest an anti-oxidative action of 4-MC. Remarkably, only in the latter case, without red clover extract, 3-methylphenol was detected.

TBARS results, giving a more general image of lipid oxidation compared to SPME-GC/MS, are also presented in Table 6.2. Lower TBARS values were observed for RC+CAS emulsions to which 4-MC was added compared with the ones not containing 4-MC. This indicates that oxidation took place in the red clover emulsions, containing PPO, but that oxidation was strongly inhibited when 4-MC was added. When only casein was used as emulsifier (CAS), TBARS were significantly lower than in RC+CAS emulsions. This might indicate induction of oxidation for the RC+CAS emulsions, through oxidizing enzymes,

Table 6.2 Major volatile compounds in PUFA rich emulsions (expressed in arbitrary area units × 10^6), measured by SPME-GC/MS, and thiobarbituric acid reactive substances (expressed as µg malondialdehyde-equivalents per ml emulsion) generally increase after 1, 2, 4 or 6 days at 50°C, but are lower when 4-methylcatechol is added. Emulsions are made with red clover protein extract and 2 g/l casein (RC+CAS) or 2 g/l casein only (CAS), with or without 20 mM 4-MC and 30 g/l linseed oil after passing 5 times through a microfluidizer at 25 MPa (1 original emulsion)

protein	4-MC addition	storage time (days)	Hexanal	2-pentenal	2,4-hexadienal	3.5-octadien-2-one	3-methyl-phenol	TBARS
RC+CAS	no	1	22.9	1.89	1.61	9.89	0.000	6.75
		2	77.3	3.59	4.23	21.7	0.000	11.4
		4	125	5.96	7.74	35.9	0.000	18.6
		6	176	15.0	24.7	75.2	0.000	24.0
		mean	*100*^{a}	*6.61*^{a}	*9.58*	*35.7*^{a}	*0.000*^{a}	*15.2*^{a}
	yes	1	7.51	4.31	1.26	3.08	0.000	6.21
		2	9.78	3.15	1.09	3.56	0.000	6.13
		4	11.1	2.29	1.20	4.58	0.000	6.16
		6	11.8	2.01	1.35	6.70	0.000	6.20
		mean	*10.1*^{b}	*2.94*^{b}	*1.23*	*4.48*^{b,c}	*0.000*^{a}	*6.18*^{b}
CAS	no	1	2.42	2.26	0.265	4.11	0.000	0.727
		2	11.7	2.05	0.500	5.44	0.000	1.15
		4	35.5	4.78	1.16	14.7	0.000	2.10
		6	143	15.9	4.77	27.6	0.000	3.60
		mean	*48.3*^{b}	*6.26*^{a}	*1.68*	*13.0*^{a,c}	*0.000*^{a}	*1.90*^{b}
	yes	1	4.04	0.925	0.000	0.000	21.5	5.78
		2	6.23	2.72	0.148	0.000	29.0	5.94
		4	7.36	1.99	0.186	0.000	27.7	5.56
		6	10.7	3.76	0.230	0.000	28.4	5.26
		mean	*7.09*^{b}	*2.35*^{b}	*0.143*	*0.000*^{b}	*26.6*^{b}	*5.64*^{b}
SEM			7.87	0.607	2.46	6.53	0.872	1.86
P-value								
protein			0.013	NS	NS	0.067	<0.001	0.005
diphenol			<0.001	0.001	0.076	0.008	<0.001	NS
storage time			0.002	0.001	NS	NS	NS	NS
protein × diphenol			0.021	NS	NS	NS	<0.001	0.008
diphenol × storage time*			0.003	0.001	NS	NS	NS	NS

PUFA, polyunsaturated fatty acids; 4-MC, 4-methylcatechol; TBARS, thiobarbituric acid reactive substances; RC, red clover; CAS, casein; SEM, standard error of the mean; NS, non-significant at p≥0.10

^{a,b,c,d} indicates differences within columns between treatment means at P≤0.05

* if the two-way factor diphenol × storage time was not significant, it was omitted from the model

present in the red clover extract. Remarkably, both CAS as well as RC+CAS treatments to which 4-MC was added, showed considerable amounts of TBARS. This might suggest interference between TBARS measurements in case of 4-MC addition.

3.2 Experiment 2: Pre-ingestive oxidation of vitamin E

Results for the second test are presented in Table 6.3. A decrease in vitamin E is observed after incubation both in the presence or not of 4-MC. Nevertheless, this decrease is substantially lower if 4-MC is present in the emulsion. Simultaneously, fatty acids in these

Table 6.3 Oxidation of α-tocopherol is observed after 16h of induced oxidation at 50°C for emulsion containing 18 mg Tonalin oil and 2 mg α-tocopherol per ml potato tuber peel protein extract and 0 mM 4-methylcatechol, but is reduced when 20 mM 4-methylcatechol is present in the emulsion (n=2)

4-methylcatechol (mM)	Temperature induced oxidation?	Vitamin E (µg/ml)	SD (µg/ml)	% decrease (compared to control)
0	No	49.47	20.28	
	Yes	20.82	5.05	58.0
20	No	34.74	1.63	
	yes	25.42	0.69	26.8

SD, standard deviation

emulsions are also protected against *in vitro* ruminal BH (0.775 protection efficiency). Also, vitamin E in emulsions without 4-MC could easily be separated using a mild hexane extraction, while this wasn't the case when 4-MC was present, indicating vitamin E is indeed present within the emulsions. In conclusion, this demonstrates that vitamin E in emulsions created using potato tuber peels, rich in PPO, and treated with 4-MC are protected against pre-ingestive damage during storage.

4 Discussion

Results from this chapter illustrate the potential to protect PUFA against oxidation during storage, in addition to the protection against ruminal BH. Emulsions with red clover extract and 4-MC seem to limit oxidation of the emulsions during storage. Indeed, both values for volatile compounds and TBARS in experiment 1 were lower in red clover based emulsions with 4-MC as compared to the red clover emulsions without 4-MC. Also, the pre-ingestive oxidation of other co-emulsified lipophilic compounds such as vitamin E was hampered upon addition of 4-MC (experiment 2). However, a few remarks have to be made concerning these results. First, higher amounts of TBARS and other volatile oxidation products as determined by SPME-GC/MS in the RC+CAS treatment, compared to the CAS emulsions were detected. This high rate of oxidation when PPO is present, is probably due to the high abundance of enzymes in the red clover extract: the red clover extract probably contains lipoxygenases, which are widely spread in nature and are responsible for the catalysis of the dioxygenation of PUFA (Porta and Rocha-Sosa, 2002). Second, the fact that non-negligible values for TBARS were found in the RC+CAS and CAS treatments when 4-MC was added. Most

probably, this is due to an interfering effect of 4-MC on the TBARS measurement. Indeed, it has been shown before that many substances including phenolic compounds can interfere with the measurement, leading to an overestimation of TBARS (Fernandez *et al.*, 1997; Ganhao *et al.*, 2011; Hodges *et al.*, 1999). Third, current data only give an indirect indication of lipid oxidation as only secondary products of lipid oxidation are presented, as SPME-GC/MS and TBARS measurements did not allow to measure the primary oxidation products such as hydroperoxides (Fereidoon Shahidi and Ying Zhong, 2014). Indeed, lower amounts of secondary products of lipid oxidation theoretically also could be the result of a reduced breakdown of the primary products of oxidation, rather than an effective decrease in lipid oxidation. Nevertheless, laboratory technicians and researchers qualitatively noticed a strong rancid smell in emulsions without 4-MC after 6 days of storage, which was completely absent in emulsions with 4-MC. Accordingly, 4-MC supplementation most likely reduces susceptibility to oxidation. However, it is not yet possible to unambiguously draw the conclusion that this protection against oxidation during storage is because of the action of PPO, as limited oxidation can be caused either by an anti-oxidative effect of the diphenol 4-MC or because of encapsulation in a protein-phenol complex. Indeed, it has been stated earlier that (poly)phenols have anti-oxidative properties (Rice-Evans *et al.*, 1997), so diphenols like 4-MC could act as an anti-oxidant, protecting PUFA without prior oil encapsulation. However, it also has been suggested that lipid oxidation of emulsions could be retarded by strengthening the interfacial protein layer between the oil and the continuous phase which might impair the transfer of oxygen and oxidation products (Ma *et al.*, 2012). This strengthening could possibly be generated here by protein-phenol complexation of the interface. Nevertheless, others failed to demonstrate the protective effect against oxygen transfer by, either or not reinforced, single-layered protein stabilized interfaces (Berton *et al.*, 2011b; Tikekar *et al.*, 2011).

In an attempt to elucidate the importance of both working mechanisms, attention can be drawn to the detection of 3-methylphenol in experiment 1. Unexpectedly, this compound was

not found in the RC+CAS emulsion containing 4-MC, but only in the CAS emulsion containing 4-MC. This may indicate that 4-MC disappeared in the red clover emulsions, most probably due to the formation of a protein-phenol complex, induced by PPO. The fact that 4-MC is not present anymore in a free form, might be a proof of evidence that 4-MC is bound to other molecules like proteins, resulting in reduced oxidation. On the other hand, in the absence of red clover, and hence PPO, 4-MC itself may be responsible for the anti-oxidative action. In this context, it has been suggested (Sullivan and Foster, 2013) that post-harvest proteolysis might be reduced either by PPO induced oxidation of phenolic substrates, or in a PPO-independent manner, due to the proteolytic inhibition by the phenolic substrates itself.

5 Conclusions

The release of volatile compounds during storage due to oxidation is reduced when emulsified PUFA oil is treated with 4-MC. It was also demonstrated that the pre-ingestive oxidation of other co-emulsified lipophilic compounds such as vitamin E is hampered upon addition of 4-MC. Therefore, the hypothesis that the PPO protection technology might protect the encapsulated content both against ruminal BH as well as (pre-ingestive) oxidation during storage seems plausible. This further illustrates the wide encapsulative potential of the PPO-based technology.

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(PaInT) of Ghent University is thanked for the use of the Microfluidizer and Mastersizer. We also highly appreciated technical assistance of the staff of LANUPRO, in particular Charlotte Melis and Erik Claeys.

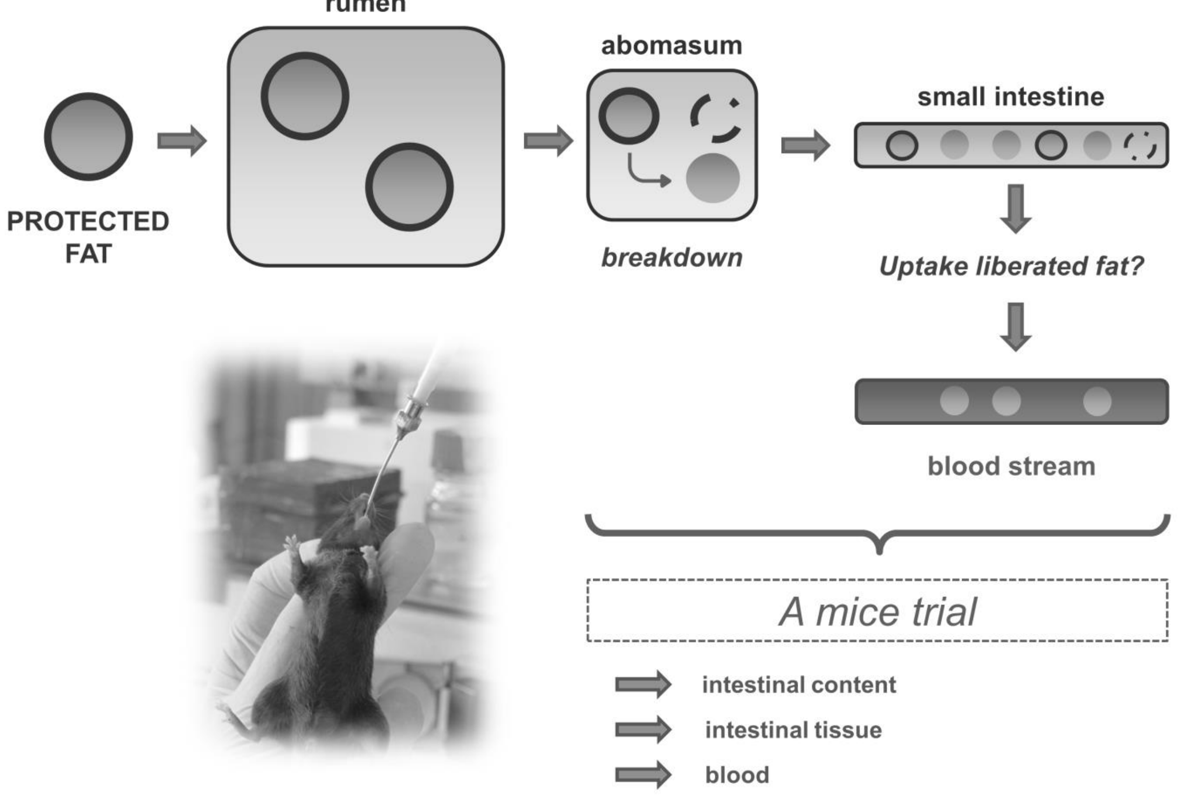
PART II B

PROTECTION OF EMULSIFIED FATTY ACIDS:

POST-RUMINAL AVAILABILITY

Chapter 7

Assessing post-ruminal digestion of rumen bypass emulsions created through red clover polyphenol oxidase: a mice trial



Redrafted after Gadeyne F., Druart C., De Neve N., Vlaeminck B., Van der Meeren P., Delzenne N. and Fievez V., 2014, Assessing post ruminal digestion of rumen bypass emulsions created through red clover polyphenol oxidase: a mice trial, 39th Animal Nutrition Research Forum, Utrecht, The Netherlands, p.5-6.

Chapter 7

Assessing post-ruminal digestion of rumen bypass emulsions created through red clover polyphenol oxidase: a mice trial

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Abstract. The protection of polyunsaturated fatty acids (PUFA) by emulsification in a polyphenol oxidase (PPO) rich protein extract and cross-linking of interfacial protein upon diphenol addition has been shown as a promising technology to bypass the process of rumen biohydrogenation (BH). Of course, PUFA have to be liberated from its protective shell post-ruminally, otherwise the added value of protection is meaningless. Therefore, the aim of this chapter was to assess the bioavailability of PUFA-rich linseed oil-in-extract emulsions, whether or not protected by PPO-induced cross-linking. An *in vivo* trial was conducted in which mice were force-fed with PUFA-rich emulsions. C18:3*n-3* levels in intestinal digesta and tissue were similar when un- or protected emulsions were fed, suggesting an equal bioavailability at intestinal level. However, a significantly lower increase in plasma C18:3*n-3* was found upon feeding of protected emulsions compared with their unprotected counterparts, suggesting a substantial level of overprotection. Accordingly, as PUFA originating from the protected emulsions did not seem to be fully recovered in the sampled tissues, it was not possible to definitely conclude whether protected PUFA could be released and absorbed post-ruminally using the current methodology.

1 Hypothesis and objective

Results from the previous chapters have demonstrated that protecting PUFA-rich emulsions using PPO is a promising new approach to protect oils against ruminal BH. However, post-ruminal availability of PPO-protected emulsions has not been tested yet. Indeed, it is necessary to protect against ruminal BH in order to achieve an adequate transfer to the

duodenum without conversion, but protected PUFA have to be liberated during passage in the gastro-intestinal tract, otherwise the added value of protection is meaningless. Literature shows milk PUFA levels are higher when red clover silages are fed to cows, which has been attributed to the action of PPO (Van Ranst *et al.*, 2011). This suggests PUFA were at least partially protected against ruminal BH by PPO and were at least partially released post-ruminally, as they appear in higher concentrations in milk.

Hence, the aim of this experiment was to assess the bioavailability of PUFA-rich emulsions, whether or not protected by the action of PPO. Due to some problems with preliminary *in vitro* experiments, it was decided to first assess the bioavailability using a mice trial. The hypothesis was that ruminally protected PUFA can be released from its protective shell during gastro-intestinal passage and absorbed at intestinal level, resulting in the recovery of PUFA in the intestinal tissue or plasma of mice.

2 Materials and Methods

2.1 Treatments, emulsion preparation and characterization

Three treatments were considered: 1) a control treatment which consisted of a PPO-rich red clover (*Trifolium pratense* L.) protein extract, 2) a 25 % (w/v) linseed oil-in-extract emulsion and 3) a 25 % (w/v) linseed oil-in-extract emulsion treated with 20 mM 4-methylcatechol (4-MC; Sigma-Aldrich, Bornem, Belgium) in order to create emulsions which are protected against ruminal BH, assessed through *in vitro* incubation. Extracts and 2 % (w/v) emulsions were created in duplicate and characterized as described before (see section 2.2 of Chapter 4), emulsions were concentrated to 25 % (w/v) oil by centrifugation and resolubilizing, as described before in section 3.7 of Chapter 4 (Experiment 7), and assessed for ruminal BH as described in section 2.4 and 2.5 of Chapter 4.

2.2 Mice trial: procedures, sampling and analyzes

In order to gain insight into the post-ruminal bioavailability of protected emulsions, a mice trial was conducted, similar to the experiment conducted by Druart *et al.* (2014). Therefore, two batches of nine mice were allocated to the three treatments with the two batches being performed on two consecutive days. Mice were force-fed using a pipet with 200 µl of either one of the treatments, 30 minutes after retro-orbital injection of tyloxapol (0.5 mg/g body weight; Sigma-Aldrich, Bornem, Belgium). Tyloxapol is a lipoprotein lipase inhibitor, impairing the uptake of fatty acids (FA) in peripheral tissues so lipids accumulate in blood, which allows assessment of the FA profile of circulating lipids in relation to the rate of absorption (Druart *et al.*, 2014). Mice experiments were approved by and performed in accordance with the guidelines of the ethical committee for animal care of the Health Sector of the Université catholique de Louvain. Housing conditions were as specified by the Belgian Law of May 29, 2013 on the protection of laboratory animals (LA1230314).

Blood from the tail vein was taken before force-feeding (0 h) and one hour after force-feeding (1 h). Three hours after force-feeding, mice were anesthetized and blood samples were taken from the portal (3 h portal vein) and cava vein (3 h cava vein). Samples of digesta content and tissues of different parts of the gastro-intestinal tract (jejenum, ileum, caecum and colon) were taken after sacrifice and treated as previously described (Druart *et al.*, 2014). FA in emulsion, plasma, intestinal tissue and content were transesterified and quantified as described before (see section 2.5 of Chapter 4) and expressed as g FA per 100 g of total identified FA (C16:0, C18:0, *cis-9* C18:1, *cis-11* C18:1, C18:2*n-6*, C18:3*n-3*, C20:4*n-6* and C22:6*n-3*).

2.3 Statistics

Results were analyzed using the MIXED procedure of SAS (SAS Enterprise Guide 5.1, SAS Institute Inc., Cary, North Carolina, USA) using the following model: $Y_{ijk} = \mu + A_i + B_j + A_i \times B_j + C_k + \varepsilon_{ijk}$, with Y_{ij} the variable of interest, A_i the fixed effect of treatment, B_j the fixed effect of

sampling time (plasma) or place (intestinal part), C_k the random effect of day when the experiment was performed and ε_{ij} the residual error. All mentioned differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

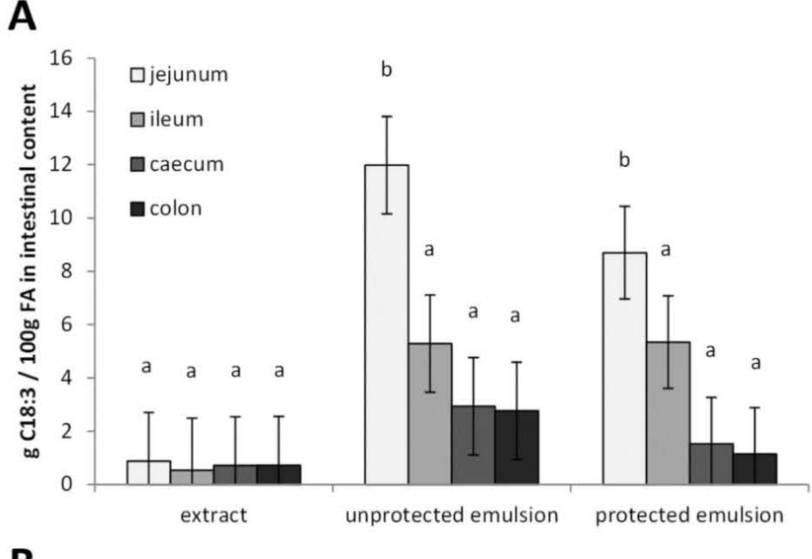
3 Results and Discussion

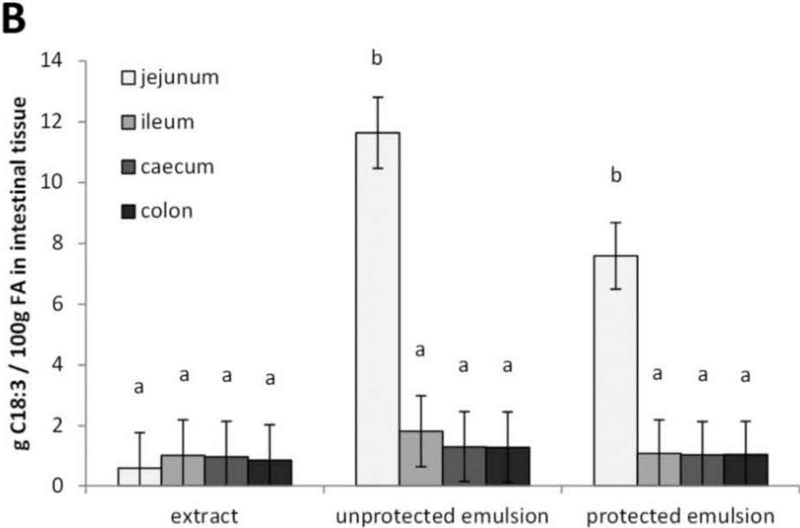
3.1 Emulsion characterization

Prior to the mice trial, the characteristics of the different extracts and emulsions were assessed. The red clover extracts (n=2) contained on average 1.86 g protein per liter and had a specific PPO activity of 1759 ΔA/min/mg protein, resulting in 2 % (w/v) emulsions (n=2) with a surface-weighted mean diameter D_{32} of 0.96 µm and a specific surface area (SSA) of 7.10 m² per g oil. BH of C18:3*n-3* of unprotected emulsions was 0.965 ± 0.006, whereas incubation of protected 2 % (w/v) and 25 % (w/v) emulsions resulted in BH levels of 0.370 ± 0.096 and 0.426 ± 0.080, respectively. Consequently, protected emulsions offered to mice reached a protection efficiency of 0.558. Unfortunately, this level of protection efficiency was much lower than observed in previous experiments, although small emulsion droplets with a high SSA were made, whereby the used extracts contained similar or even larger protein concentrations and levels of PPO activity as applied before. However, according to us, this rather intermediate level of protection efficiency cannot explain differences between un- and protected emulsions administered to the mice, but makes the interpretation of the *in vivo* results more difficult, as probably a major part of the administered PUFA was anyhow unprotected.

3.2 *In vivo* results

Concentrations of C18:3*n-3* found in the intestinal contents and tissues of the different parts of the mice gut are shown in Figure 7.1A and Figure 7.1B, respectively. Only results for C18:3*n-3* are shown as this was the only FA resulting in substantial differences, which could





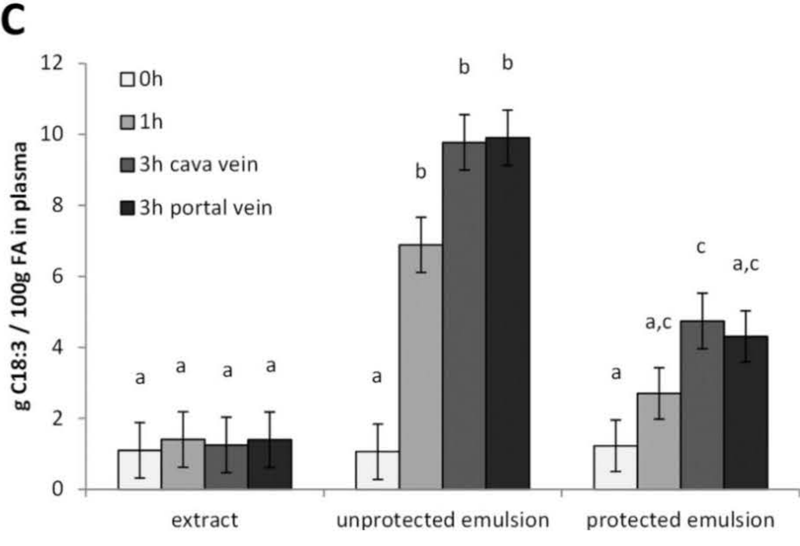


Figure 7.1 Levels of C18:3*n-3* sampled in different parts of the intestinal content (A), intestinal tissue (B) or at different times in plasma (C) differed after force-feeding mice similar volumes of either a red clover extract, an unprotected 25 % oil-in-extract emulsion (0 mM 4-methylcatechol) or a protected 25 % oil-in-extract emulsion (20 mM 4-methylcatechol). [a,b,c] indicates differences between treatments within intestinal part or plasma sampling time at P≤0.05. Error bars represent the standard error of the mean (n=6)

be expected as linseed oil used in this experiment contained about 51 g of C18:3*n-3* per 100 g of total identified FA. As expected, low levels of C18:3*n-3* were observed when only the extract was given, reflecting the basal C18:3*n-3* levels. When emulsions were administered, a steep increase in jejunal C18:3*n-3* was observed, showing FA are mainly taken up in the proximal parts of the gastro-intestinal tract. In the study of Druart *et al.* (2014), which investigated the production and absorption of gut microbiota-derived PUFA metabolites with a similar set-up, the total amount of FA in the jejunal content of linseed oil force-fed mice was also very high, but decreased dramatically in the following parts of the gastro-intestinal tract. However, results of Druart *et al.* (2014) indicated linseed oil force-feeding increased the content of C18:3*n-3* at a similar extent in the different parts of the gut, which is in contrast to the findings of the current study. Most interestingly in this experiment, no differences between un- and protected emulsions could be observed in digesta contents or tissues. This suggests that removal of C18:3*n-3* from the small intestine is similar for both types of emulsions. This would imply rumen un- and -protected PUFA are equally bio-available at intestinal level, suggesting no overprotection of the 4-MC treated emulsions.

Figure 7.1C gives an overview of the levels of C18:3*n-3* sampled at different time points in <u>plasma</u> of force-fed mice. Similar to the results of Druart *et al.* (2014), an increase in C18:3*n-3* per 100 g of total identified FA in circulating lipids was observed compared with the situation before force-feeding. Indeed, tyloxapol injection prior to force-feeding impaired the uptake of PUFA in peripheral tissues, allowing to assess the transfer from the intestinal content and tissue to the circulating system. However, a significantly lower increase in plasma C18:3*n-3* was found upon feeding of protected emulsions compared with the unprotected counterparts. This would suggest only the unprotected C18:3*n-3* was taken up and accumulated in the blood stream, suggesting a substantial level of overprotection of the 4-MC treated emulsions.

The discrepancy between observations at intestinal and plasma level is remarkable. It is also noteworthy C18:3*n-3* levels were numerically higher for unprotected emulsions than

protected emulsions in both the intestinal content, intestinal tissue and plasma. Figure 7.2 illustrates the potential routes which C18:3*n*-3 might have followed after being force-fed to the mice. The discrepancy could not have been related to storage of lipids in adipose tissues due to the tyloxapol injection. Arguably, its inhibiting action might have been less good for emulsions treated with 4-MC. Next, absorption from the lumen to the epithelial cells and further on to the circulatory system or outflow to the distal parts of the gastrointestinal tract might have differed, but seems unlikely based on the results of tissue versus blood and the levels in caecum and colon. Maybe, more C18:3*n-3* was unintentionally administered to the mice who received the unprotected emulsion than the ones who received the protected emulsion. The reason for this remains unclear. However, it is not possible to express C18:3*n-3* levels in intestinal tissue or plasma relatively to C18:3*n-3* in intestinal content, because no steady state conditions were reached with the current experimental force-feeding set-up.

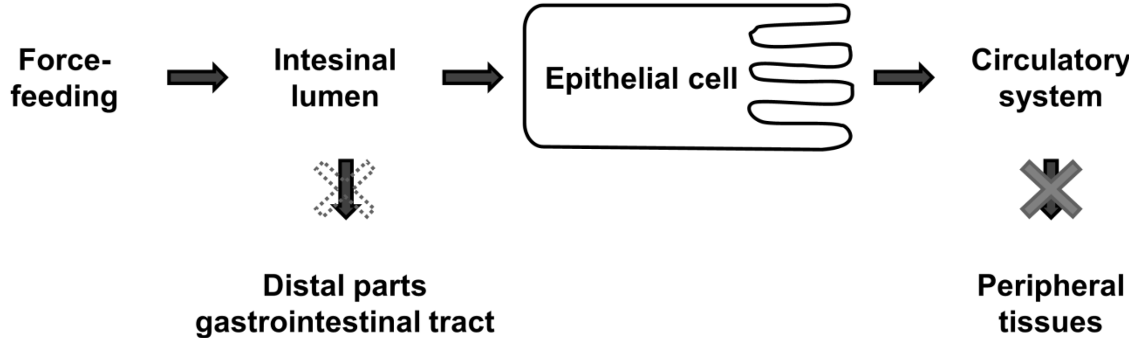


Figure 7.2 Potential routes for the fatty acids after being force-fed to the mice

4 Conclusions

C18:3*n-3* originating from the protected emulsions do not seem to be fully recovered in the sampled tissues, which impairs conclusions on intestinal absorption of PUFA from un- or protected emulsions. Accordingly, it was not possible to unequivocally conclude whether protected PUFA could be released and absorbed during gastro-intestinal passage with the current set-up.

5 Acknowledgements

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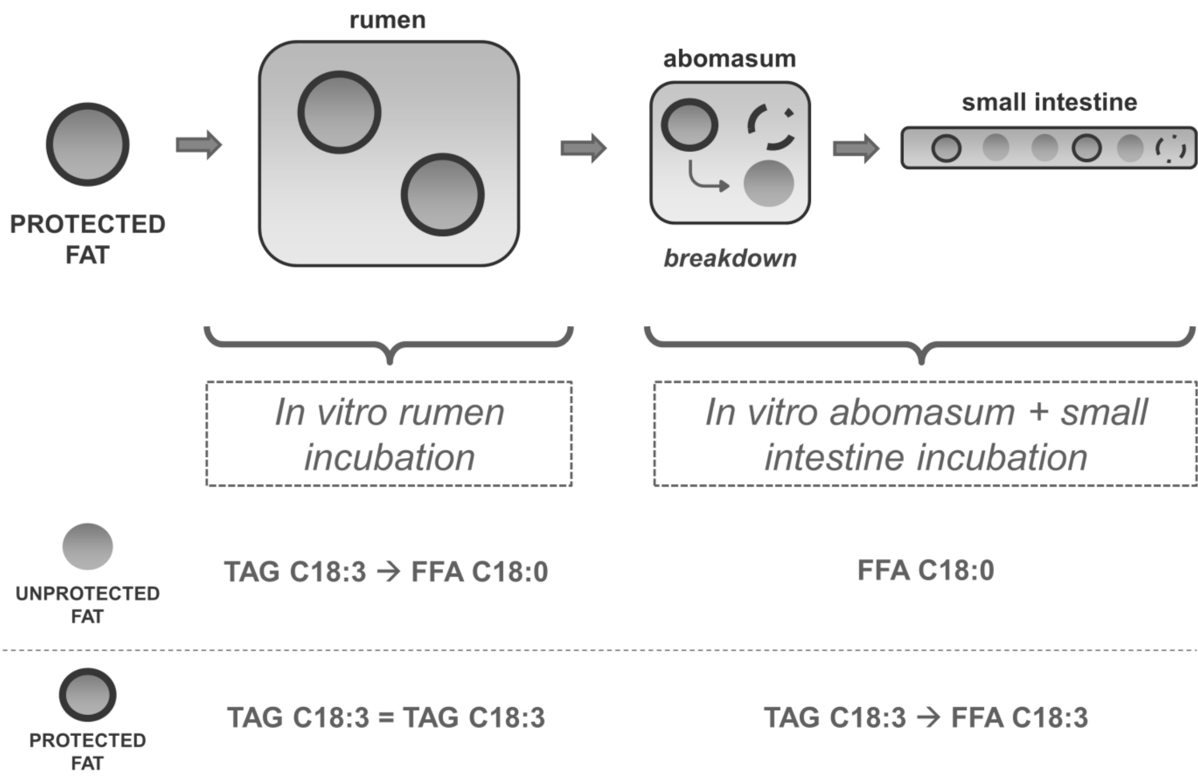
Chapter 8

In vitro post-ruminal digestion of rumen bypass emulsions created by polyphenol oxidase rich protein extracts of potato tuber peels



Redrafted after Gadeyne F., De Neve N., Vlaeminck B., Van der Meeren P. and Fievez V., 2017, *In vitro* post-ruminal digestion of rumen bypass emulsions encapsulated by interfacial crosslinking using polyphenol oxidase from potato tuber peels, 2017 American Dairy Science Association Annual Meeting, Pittsburgh, Pennsylvania, United States of America, *[submitted]*.

Chapter 8

In vitro post-ruminal digestion of rumen bypass emulsions created by polyphenol oxidase rich protein extracts of potato tuber peels

Abstract. An *in vitro* experiment was designed to assess the post-ruminal availability of rumen bypass emulsions created using polyphenol oxidase (PPO) rich protein extracts and the diphenol 4-methylcatechol (4-MC). Therefore, ruminal, abomasal and a time series of intestinal incubations were performed using linseed oil, which is rich in polyunsaturated fatty acids (PUFA) as triacylglyceride (TAG). Results confirmed PUFA in protected emulsions were bio-available for intestinal uptake, as C18:3*n-3* survived the process of biohydrogenation (BH) during ruminal incubation and was detected in the free fatty acid (FFA) fraction after subsequent abomasal and intestinal incubations, while C18:3*n-3* almost completely disappeared for unprotected emulsions. However, post-ruminal incubations indicated emulsions showing the highest level of rumen protection (0.907) were associated with some degree of overprotection.

1 Hypothesis and objective

Results from the previous chapter were inconclusive regarding absorption from the intestine of PUFA from protected emulsions. Therefore, an *in vitro* experiment was designed to assess the post-ruminal availability of rumen bypass emulsions created using PPO-rich protein extracts. As PUFA in linseed oil are mainly present in the TAG fraction and TAG have to be hydrolyzed to free fatty acids (FFA) before the process of ruminal BH can occur, it was hypothesized that protected PUFA during ruminal incubation would remain in the TAG fraction during ruminal incubation. Subsequently, protected PUFA have to be released from their protective shell in order to be bio-available at post-ruminal level. As the *in vitro* system

does not allow to directly assess absorption, it was assumed that PUFA in the FFA fraction would be available for intestinal absorption. Hence, in case of overprotection of emulsions, PUFA would still be present in the TAG fraction after intestinal incubation.

In a first preliminary experiment, the distribution (TAG or FFA) of PUFA from linseed oil in a 4-MC treated PPO-rich emulsion before and after ruminal incubation is determined in order to verify the potential of this protocol. Indeed, the set-up and hypothesis of this experiment to test post-ruminal bioavailability would not be valid if protected PUFA do not remain in the TAG fraction after ruminal incubation. Second, the potential post-ruminal overprotection of PUFA in 4-MC treated PPO-rich emulsions was assessed by ruminal, abomasal and a time series of intestinal incubations.

2 Materials and methods

2.1 Experiment 1: preliminary test

Fatty acids (FA) in linseed oil are mainly present as TAG. It is known that TAG have to be hydrolyzed before hydrogenation can occur (Buccioni *et al.*, 2012). However, lipases might occur in the protein extract (Van Ranst *et al.*, 2009c), which was used as emulsifier of the linseed oil. Therefore, in a first preliminary experiment it was tested whether PUFA in emulsions were esterified or not. Next, these emulsions were *in vitro* ruminally incubated and the esterification fate of the contained lipids determined, in order to check the potential of the proposed post-ruminal bioavailability test.

Emulsions (n=1) were made with a protein extract of potato (*Solanum tuberosum* L.) tuber peels (n=1), which contained 20 mg linseed oil per ml protein extract and had a final 4-MC concentration of 0 or 15 mM. Methods and measurements of emulsions and protein extracts are as described before in section 2.2 of Chapter 4. *In vitro* batch incubations were performed in triplicate to evaluate the protection of PUFA in the various emulsions against ruminal BH (see section 2.4 of Chapter 4). Emulsions and samples after incubation were

acidified with formic acid and lipids were extracted using chloroform, methanol and water in a 8/4/3 ratio (Folch et al., 1957) and separated by solid phase extraction (Van Ranst et al., 2010) into two lipid fractions: neutral lipids (mainly composed of TAG - and further designated as such - next to minor amounts of diacylglycerides and monoacylglycerides) and FFA. TAG-C13:0 (0.5 mg/ml chloroform; Nu-Check Prep Inc., Elysian, Minnesota, USA) and C21:0 (0.5 mg/ml chloroform; Sigma-Aldrich, Bornem, Belgium) were added before extraction and used as internal standard for the TAG and FFA fractions, respectively. The polar lipid fraction was not considered as there were no FA in this fraction in the emulsified oil (results not shown). Fractions were methylated and FA quantified using gas chromatography as described in section 2.4.2 of Chapter 2.

2.2 Experiment 2: *in vitro* post-ruminal digestion

The *in vitro* post-ruminal overprotection of PUFA in 4-MC treated PPO-rich emulsions was assessed in experiment 2. The objectives of this experiment were twofold. First, it was assessed whether there was an effect of variation in 4-MC concentration in the emulsions on the rate of lipolysis of PUFA from the TAG fraction in the linseed oil emulsions to the FFA fraction under abomasal and intestinal conditions. Varying amounts of 4-MC were used as these have been associated with variation in rumen protection efficiency (section 3.5 of Chapter 4, experiment 5). Second, consecutive rumen, abomasal and intestinal incubations were performed on PUFA-rich linseed oil emulsions with varying levels of rumen protection to assess the bioavailability after ruminal and gastro-intestinal passage.

2.2.1 Experiment 2a: time series of intestinal digestion of emulsions

In experiment 2a, emulsions with varying 4-MC concentrations were subjected to a sequential simulation of the abomasum and small intestine. Therefore, emulsions (n=3) were made with a protein extract of potato tuber peels (n=3), contained 20 mg linseed oil per ml protein extract and had a final 4-MC concentration of 0, 5, 10 or 20 mM. The emulsion preparation and measurements of emulsions and protein extracts are as described in section

2.2 of Chapter 4 and section 2.3.1 of Chapter 5, respectively. Unfortunately, a mistake happened in the addition of 4-MC to the emulsions, resulting in the following real statistical repetitions: n=3, n=3, n=4 and n=2 for 0, 5, 10 and 20 mM 4-MC, respectively. To have enough replicates, it was decided to keep all samples for statistical analysis. Further, the protection of PUFA in the various emulsions against *in vitro* ruminal BH was evaluated in duplicate as described before (see section 2.4 and 2.5 of Chapter 4).

In vitro incubations to assess the PUFA release in the small intestine after abomasal passage were performed according to the standardized test conditions of *in vitro* digestion models for emulsified products as described by McClements and Li (2010). Therefore, a two-step batch protocol was performed. First, 15 ml of emulsion (resulting in about 300 mg of fat per flask) was incubated for 2h at 39°C in a rubber-stoppered 125-ml incubation flask with 4 ml pepsin (10 g/L; >250 units/mg according to the manufacturer, Sigma-Aldrich, Bornem, Belgium) solution in 0.75 M HCl to simulate abomasal conditions. Next, the pH was adapted to 8.00 using 1 M NaOH and 4 ml porcine bile solution (6.25 g per 100 ml 0.2 M preheated sodium phosphate buffer; Sigma-Aldrich, Bornem, Belgium), 1 ml $CaCl_2.2H_2O$ (5.6 g per 100 ml 0.2 M preheated sodium phosphate buffer; J.T. Baker, Deventer, Netherlands) and 24 ml 0.2 M preheated sodium phosphate buffer were added. Before any further steps, the pH was adjusted to 9.00 using 1 M NaOH, if necessary. To assess the lipid bioavailability after small intestinal incubation, 2 ml porcine pancreatin (6 g per 100 ml 0.2 M sodium phosphate buffer, 8xUSP, Sigma-Aldrich, Bornem, Belgium) was added and incubated for 5, 10, 20, 60 or 120 min at 39°C. Further, samples for FA analysis were taken after the two-step incubation (0.4 ml incubation fluid, resulting in about 2.4 mg of fat per sample) and acidified using formic acid. Lipids were extracted, separated in lipid fractions, methylated and analyzed using gas chromatography as described before (section 2.1 of Chapter 8). Finally, only C18:3*n-3* was considered, as this is the most abundant FA in linseed oil, and results were expressed as [mg of C18:3*n-3* in the TAG or FFA fraction] / [mg of C18:3*n-3* in both fractions].

2.2.2 Experiment 2b: Subsequent ruminal, abomasal and intestinal digestion of emulsions

In contrast to experiment 2a, abomasal and intestinal digestion were preceded by a rumen incubation. The same emulsions as in experiment 2a were used. *In vitro* batch incubations were performed in duplicate as described in section 2.4 of Chapter 4. Duplicate rumen incubations were pooled prior to the abomasal and intestinal incubation of emulsions. Fifteen ml of pooled rumen fluid (resulting in about 12 mg of fat per reaction flask) was used for the subsequent post-ruminal simulations. Conditions of abomasal (2h) and small intestinal (2h) incubation were as described earlier (section 2.2.1 of Chapter 8). Samples for FA analysis of the three-step incubation were taken after rumen (3 ml incubation fluid, resulting in about 2.4 mg of fat per sample) and intestinal incubation (10 ml incubation fluid, resulting in about 2.4 mg of fat per sample) and acidified using formic acid. Lipids were extracted, separated in lipid fractions, methylated and analyzed using gas chromatography as described before (section 2.1 of Chapter 8). Again, only C18:3*n-3* was considered, but results were expressed as [mg of C18:3*n-3* in the TAG or FFA fraction] / [mg of all FA across both fractions]. Finally, the *in vitro* bioavailability of lipid, i.e. including both the process of ruminal hydrogenation and release upon gastro-intestinal passage, was expressed as $[(\text{mg of C18:3}n\text{-}3 \text{ in the FFA fraction}) / (\text{mg of all FA across both fractions})]_{\text{after rumen, abomasum and intestinal digestion}} / [(\text{mg of C18:3}n\text{-}3 \text{ in both fractions}) / (\text{mg of all FA across both fractions})]_{\text{original emulsion}}$.

2.3 Statistics

Results were analyzed with the MIXED procedure of SAS (SAS Enterprise Guide 7, SAS Institute Inc., Cary, North Carolina, USA). Analytical replicates were averaged prior to statistical analysis.

The following model was used for experiment 2a: $Y_{ij} = \mu + T_i + D_j + T_i \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, T_i the fixed effect of time (i = 0, 5, 10, 20, 60 or 120

min of intestinal digestion), D_j the fixed effect of diphenol concentration (j = 0, 5, 10 or 20 mM 4-MC) and ε_{ij} the residual error.

The following model was used for experiment 2b: $Y_{ij} = \mu + S_i + D_j + S_i \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, S_i the fixed effect of sample type (i = emulsion, after rumen incubation or after rumen plus abomasum plus small intestine incubation), D_j the fixed effect of diphenol concentration (j = 0, 5, 10 or 20 mM 4-MC) and ε_{ij} the residual error.

Differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

3 Results

3.1 Experiment 1: preliminary test

A potato tuber peel extract (n=1) was used and contained 3.46 g protein per liter and had a specific PPO activity of 251 ΔA/min/mg protein, resulting in emulsions (n=1) with a surface-weighted mean diameter D_{32} of 2.51 µm and a specific surface area SSA of 2.58 m² per g oil. Results presented in Figure 8.1 are the averages of unprotected (0 mM 4-C) and protected (15 mM 4-MC) emulsions, before ruminal incubation. As expected, the FA profile of the emulsions reflected the composition of the original oil: about 50 g/100 g of FA were C18:3*n-3*, followed by approximately 15 g/100 g of C18:2*n-6* and 20 g/100 g *cis*-9 C18:1. Most FA (85 g/100 g) were present as TAG, meaning plant lipases induced only minimal lipolysis.

When unprotected emulsions (0 mM 4-MC) were incubated *in vitro* for 24h to simulate ruminal circumstances, most of the FA present in the original emulsion were hydrogenated (Figure 8.2): C18:2 intermediates, C18:1 intermediates as well as the final hydrogenation end-product stearic acid (C18:0) were formed. Hydrogenated products were mainly in the FFA form, as expected. The small amounts of remaining C18:2*n-6* and C18:3*n-3* were mainly in the TAG-form.

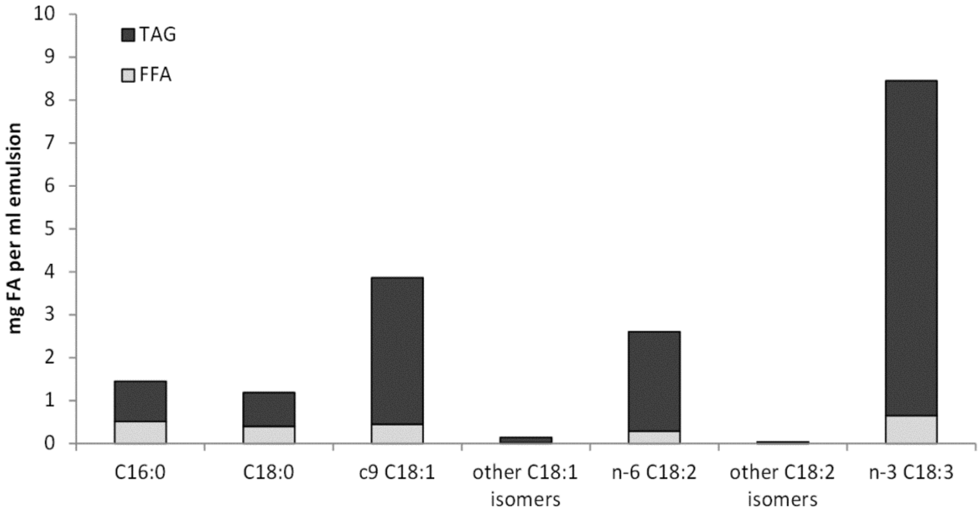


Figure 8.1 The average fatty acid composition of emulsions (prepared by emulsifying 20 mg triacylglycerides (TAG) of linseed oil per ml potato tuber peel protein extract and exposed over 24h to either 0 or 15 mM 4-methylcatechol (4-MC)) reflects the fatty acid and lipid classes composition of original linseed oil, whereby most fatty acids are present as TAG and not as free fatty acids (FFA) (1 original emulsion was prepared of which half was exposed to 15 mM 4-MC and the other half was not treated with 4-MC; experiment 1)

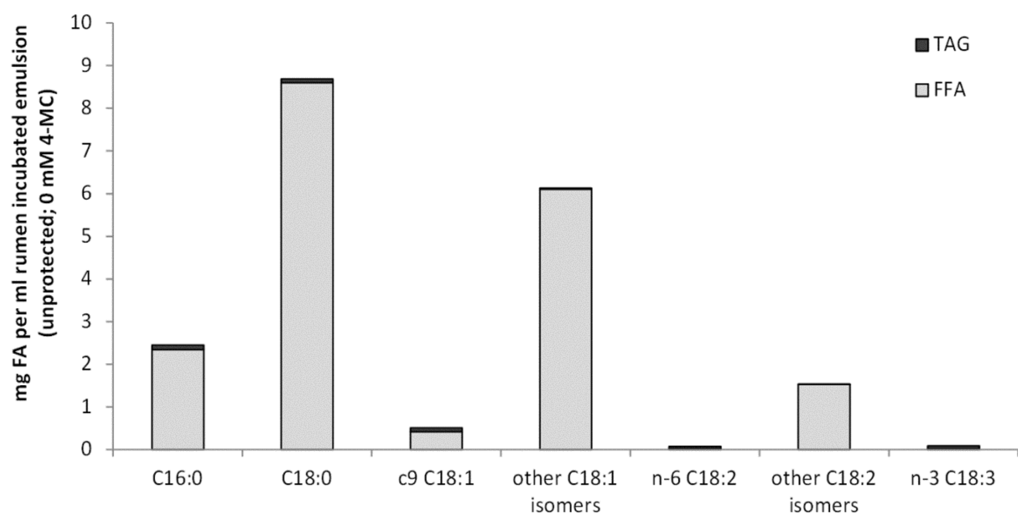


Figure 8.2 The fatty acids in unprotected emulsions (prepared by emulsifying 20 mg triacylglycerides (TAG) of linseed oil per ml potato tuber peel protein extract and containing 0 mM 4-methylcatechol) are lipolyzed (FFA) and hydrogenated after 24h *in vitro* rumen incubation (1 original emulsion; experiment 1)

On the other hand, when emulsions were protected by addition of 4-MC, 24h *in vitro* rumen incubation resulted in a FA profile similar to the original emulsion (Figure 8.3): hardly any C18:1 or C18:2 intermediates were formed, whereas most of the original *cis-9* C18:1,

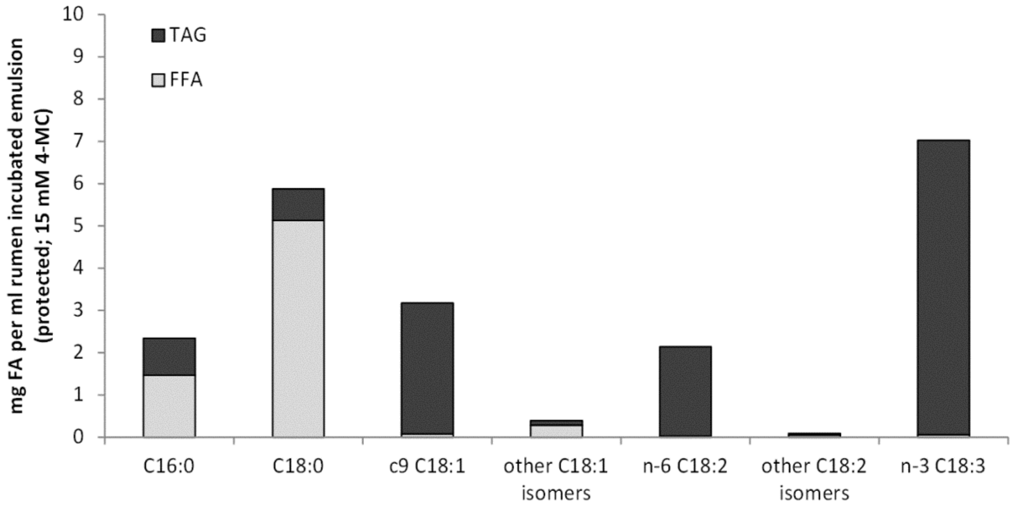


Figure 8.3 The fatty acids in protected emulsions (prepared by emulsifying 20 mg triacylglycerides (TAG) of linseed oil per ml potato tuber peel protein extract and containing 15 mM 4-methylcatechol) are rather unaffected after 24h *in vitro* rumen incubation and not lipolyzed to free fatty acids (FFA) (1 original emulsion; experiment 1)

C18:2*n-6* and C18:3*n-3* were recovered, still in the original TAG-form. Any intermediates or stearic acid which were formed were mainly present as FFA. Protection of C18:3*n-3* against BH was calculated to be 0.850.

As a conclusion, TAG in unprotected fat was lipolyzed and finally hydrogenated to C18:0 or other C18 intermediates. However, TAG in protected oil remained as TAG, so no lipolysis occurred. Also, as no FFA were formed, no hydrogenation occurred and FA were protected. Obviously, only minimal action of plant lipases in the extract was observed on TAG. The fact that most protected PUFA remained in the TAG fraction after ruminal incubation illustrates that the proposed post-ruminal bioavailability protocol, in which bioavailability is assessed through evaluation of the increase in the FFA fraction, is suitable and will be used in the next experiment.

3.2 Experiment 2: *in vitro* post-ruminal digestion

In both experiment 2a and experiment 2b, extracts of potato tuber peels (n=3) were used and contained 3.49 g protein per liter and had a specific PPO activity of 263 nkatal per mg protein, resulting in emulsions (n=3) with a surface-weighted mean diameter D_{32} of 3.54 µm

and a specific surface area SSA of 1.92 m² per g oil. Emulsions containing 0, 5, 10 or 20 mM 4-MC showed BH levels for C18:3*n-3* of 0.946 ± 0.017, 0.429 ± 0.266, 0.291 ± 0.204 and 0.088 ± 0.059, respectively, after *in vitro* ruminal incubation, resulting in a concomitant ruminal protection of 0.546, 0.693 and 0.907 for 5, 10 or 20 mM 4-MC, respectively.

Results for C18:3*n-3* in the FFA fraction after *in vitro* abomasal and intestinal incubation in experiment 2a are presented in Figure 8.4, which clearly illustrates an effect of time (P<0.001). Indeed, upon prolonged intestinal incubation, an increase in C18:3*n-3* as part of the FFA fraction, and a concomitant decrease as part of TAG fraction was observed, due to the lipolytic action of pancreatin (0 min < 5 min ≤ 10 min ≤ 20 min < 60 min = 120 min). Also an effect of 4-MC (P<0.001) was observed: no difference in behavior between emulsions without, with 5 mM or 10 mM of 4-MC was found (P>0.270), but C18:3*n-3* was found less as part of the FFA fraction in case of the 20 mM 4-MC emulsions (P<0.002). No interaction between time and 4-MC occurred (P=0.996).

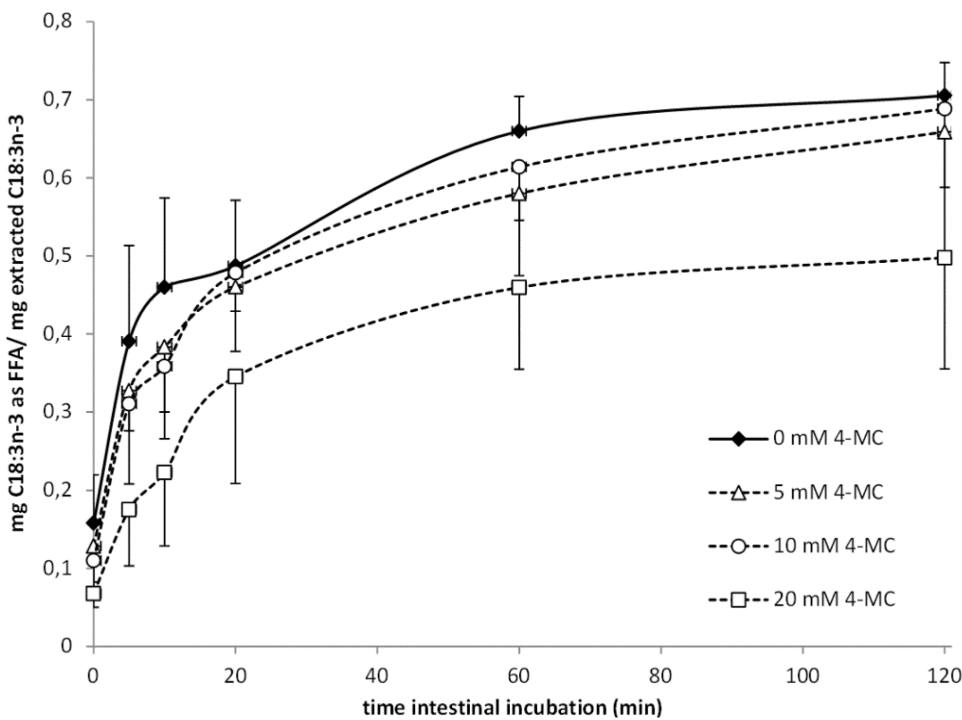


Figure 8.4 C18:3*n-3* in emulsions prepared by emulsifying 20 mg linseed oil per ml potato tuber peel protein extract and containing 0, 5, 10 or 20 mM 4-methylcatechol (4-MC) are increasingly lipolyzed to free fatty acids (FFA) during 120 min *in vitro* abomasal and 0, 5, 10, 20, 60 or 120 min intestinal incubation (experiment 2a)

The same emulsions as in experiment 2a were used for a consecutive *in vitro* rumen, abomasum and small intestine incubation in experiment 2b. Results for C18:3*n-3* in the TAG and FFA fraction after ruminal, abomasal and intestinal simulation are presented in Figure 8.5 and Figure 8.6, respectively. C18:3*n-3* disappeared from the TAG fraction, but this was dependent of the 4-MC dose (P<0.001), sample type (P<0.001) as well as their interaction effect (P=0.005) (Figure 8.5). When no 4-MC was added to the emulsions, almost all C18:3*n-3* which was present in the original emulsions disappeared from the TAG fraction after rumen incubation (P<0.001). Less C18:3*n-3* disappeared in case of 5 (P=0.006) or 10 (P=0.024) mM 4-MC and with 20 mM 4-MC, the emulsions after rumen incubation did not differ from the original emulsions (P=0.879). However, after rumen incubation TAG levels of 0 mM 4-MC emulsions were lower than for the other 4-MC concentrations (P≤0.026). Compared with C18:3*n-3* in the original emulsions, no C18:3*n-3* was remaining as TAG after abomasal and intestinal incubation (P<0.001). Similarly, C18:3*n-3* in the FFA fraction was affected by both 4-MC addition (P<0.001), sample type (P=0.008) and their interaction (P=0.004) (Figure 8.6). Levels of C18:3*n-3* in the FFA fraction were similar and very low irrespective of the site of digestion (rumen or a combination of rumen, abomasum and intestinal incubation) for the unprotected 0 mM 4-MC emulsions (P≥0.188) and no differences between levels in the original emulsions or after rumen incubation were observed (P≥0.191). However, compared with the levels after ruminal incubation, increasing levels of C18:3*n-3* as bio-available FFA were observed when 4-MC was added to the original emulsion after abomasal and intestinal incubation (P<0.004), but no differences between 5, 10 or 20 mM 4-MC within this post-ruminal stage were found (P>0.555). Finally, the *in vitro* bioavailability of C18:3*n-3* as FFA, i.e. including both the process of ruminal BH and release upon gastro-intestinal passage and expressed as a fraction of C18:3*n*-3 in the original emulsion (prior to digestive simulation), was calculated as 0.018, 0.223, 0.237 and 0.303 for emulsions which contained 0, 5, 10 or 20 mM 4-MC, respectively.

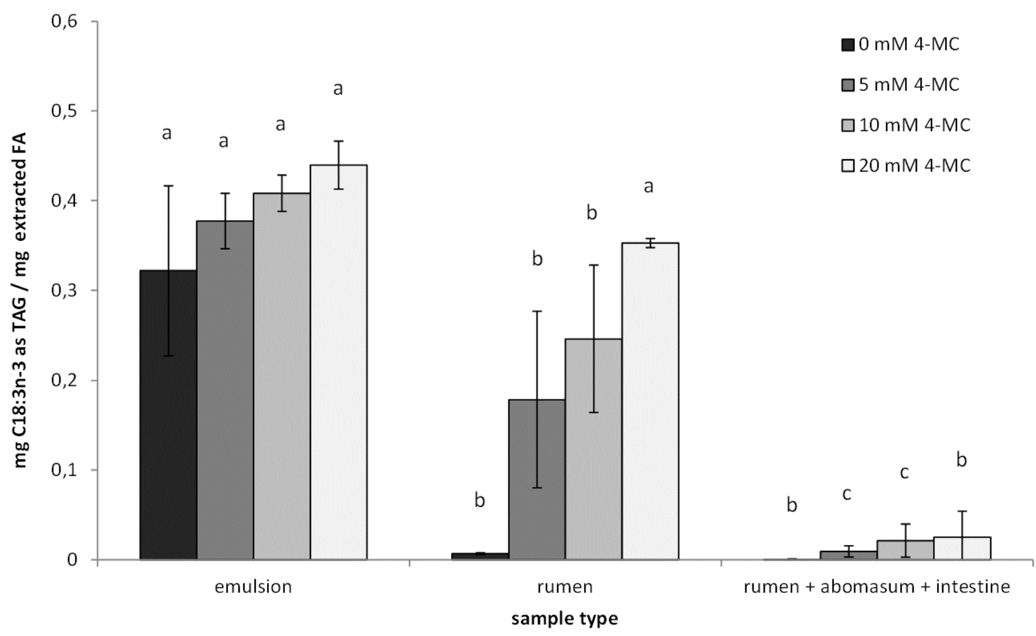


Figure 8.5 C18:3*n-3* in emulsions prepared by emulsifying 20 mg linseed oil per ml potato tuber peel protein extract and containing 0, 5, 10 or 20 mM 4-methylcatechol (4-MC) disappeared less from the triacylglycerides (TAG) fraction after 24h *in vitro* ruminal incubation with higher levels of 4-MC, whereas no or only traces of C18:3*n-3* remained in the TAG fraction after 2h *in vitro* abomasal and 2h *in vitro* intestinal incubation. [a,b,c] indicates differences in levels of C18:3*n-3* as TAG between sample type within 4-MC concentration at P≤0.05 (experiment 2b)

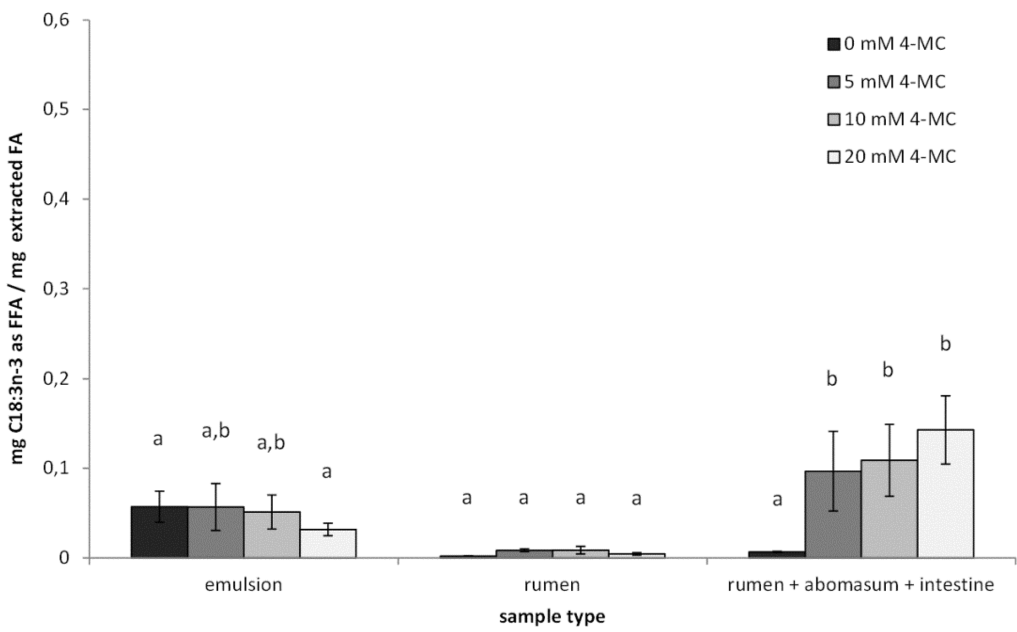


Figure 8.6 C18:3*n-3* in emulsions prepared by emulsifying 20 mg linseed oil per ml potato tuber peel protein extract and containing 0, 5, 10 or 20 mM 4-methylcatechol (4-MC) disappeared from the free fatty acid (FFA) fraction after 24h *in vitro* ruminal incubation, but increasing levels of C18:3*n-3* were observed in the FFA fraction with higher levels of 4-MC after 2h *in vitro* abomasal and 2h *in vitro* intestinal incubation. [a,b,c] indicates differences in levels of C18:3*n-3* as FFA between sample type within 4-MC concentration at P≤0.05 (experiment 2b)

4 Discussion

Results from this experiment confirmed PUFA such as C18:3*n-3* in emulsions created using a PPO-rich protein extract of potato tuber peels in combination with 4-MC was lipolyzed and present in the FFA fraction after ruminal and post-ruminal simulations. As all free C18:3*n-3* disappeared during rumen incubation, any C18:3*n-3* found as FFA after abomasal and intestinal simulation must have been hydrolyzed from the TAG fraction during this second step of the consecutive incubation set-up. This suggests that lipids in emulsions which were protected against ruminal BH by adding increasing levels of 4-MC escaped from microbial metabolism in the rumen and entered the post-ruminal stage of the gastro-intestinal tract in an esterified form which could then be hydrolyzed by the lipolytic action of pancreatin. However, results of experiment 2a indicated some level of post-ruminal overprotection with the highest concentration of 4-MC and the concomitant highest protection against ruminal BH, as they show the lowest release of PUFA into the bio-available FFA fraction. This is in contrast to results of previous experiments, whereby FFA release from encapsulated lipid droplets was irrespective of the presence of an interfacial barrier, either obtained by transglutaminase-induced cross-linking of hydrogel microspheres (Matalanis and McClements, 2012), heat-induced cross-linking of adsorbed proteins (Sandra *et al.*, 2008) or laccase-induced cross-linking of gelatin-pectin multilayered emulsions (Zeeb *et al.*, 2015a). Despite the risk of overprotection at post-ruminal level at the highest levels of rumen protection (20 mM 4-MC), the overall balance of C18:3*n-3* in the bio-available FFA fraction after a combination of rumen and post-ruminal digestion in experiment 2b was still positive. Nevertheless, levels of C18:3*n-3* in the bio-available FFA fraction were similar for all 4-MC doses (5, 10 or 20 mM 4-MC). Obviously, these results are only a proxy for the potential intestinal uptake, as the presence of PUFA in the FFA fraction was measured after *in vitro* incubation, whereas absorption was not assessed directly. This means no quantitative extrapolation should be made to the *in vivo* uptake of encapsulated PUFA into the bloodstream and transport to the target cells.

The mechanism for releasing PUFA from its protective shell is currently unknown. With the applied protocol no distinction can be made between release of the oil by proteolytic cleavage of the protein-phenol interface during exposure to abomasal and intestinal conditions or whether cross-linked barriers were unaffected but allowed access of lipases to perform their enzymatic action. Both mechanisms are theoretically possible. Indeed, the reaction of phenolic compounds with proteins may induce cross-linking of the proteins, which renders them hydrophilic in nature (Ozdal et al., 2013). It has been shown before that lipases can adsorb at both hydrophobic and hydrophilic interfaces (Reis et al., 2006). In the study of Maldonado-Valderrama et al. (2013), hydrolysis of proteins adsorbed at the oil-water interface by pepsinolytic enzymes differed depending on the type of interfacial protein, but similar lipolytic behavior by pancreatic lipase was detected, which indicated that proteins did not comprise a barrier to lipolysis. Nevertheless, the rigidity of the interface due to the cross-linked proteins in the current experiment is unknown, but could have impacted the adsorption of lipases. However, in this case, it seems unlikely that conversion of TAG to FFA post-ruminally is caused by lipases migrating through the cross-linked protein interface, as it seems illogic that pancreatic lipases but not (rumen) microbial lipases or hydrogenases would have access to the oil in the protected emulsions. This favors the hypothesis that FA are protected from ruminal lipolysis and BH with the described bypass methodology, but the protective shell is (partially) destroyed post-ruminally to make the content available again for further enzymatic modifications.

5 Conclusions

Results confirmed PUFA in emulsions, created using a PPO-rich protein extract of potato tuber peels, are protected against ruminal BH upon addition of 4-MC and become bio-available in the small intestine. C18:3*n-3* was found in the FFA fraction after consecutive ruminal, abomasal and intestinal incubations when emulsions were treated with 4-MC, while

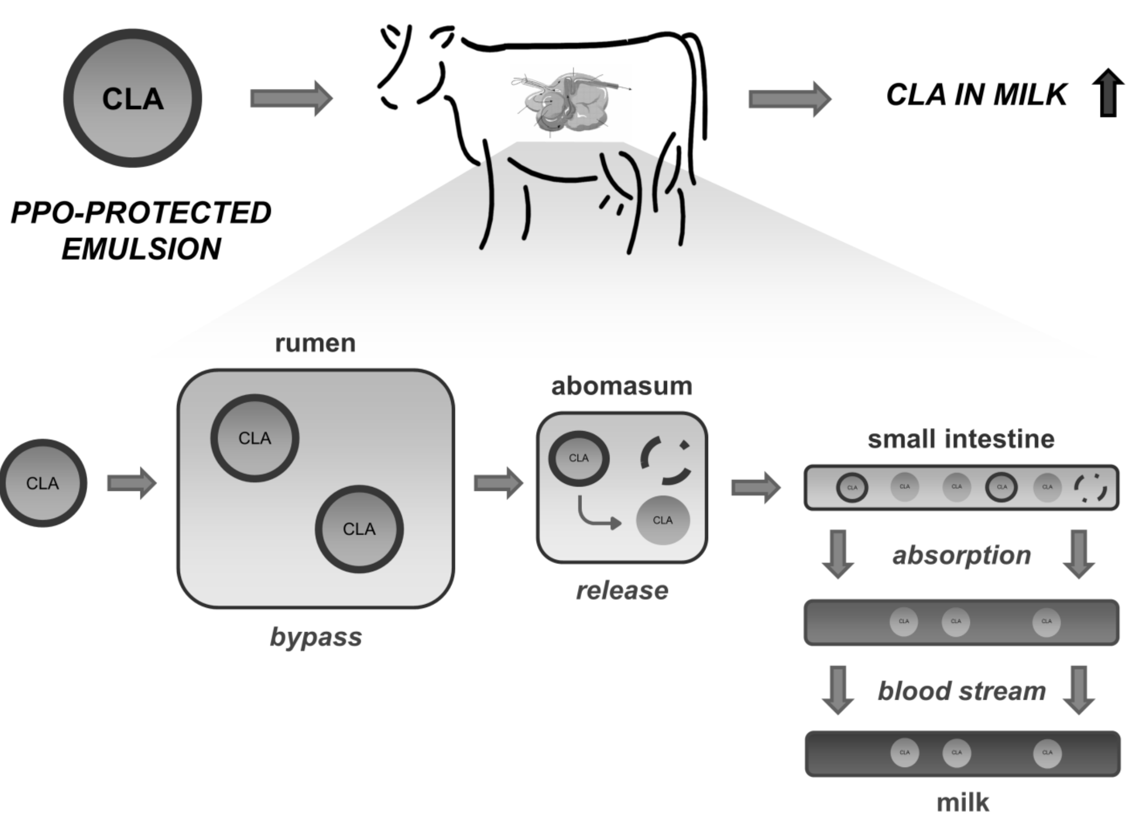
this wasn't the case for unprotected emulsions. However, post-ruminal incubations indicated some risk for overprotection at the highest levels of rumen protection.

6 Acknowledgements

Silas Claes is thanked for the extensive analyzes he performed during his laboratory internship at LANUPRO. The Particle and Interfacial Technology Group (PaInT) of Ghent University is thanked for the use of the Microfluidizer and Mastersizer. We also highly appreciated technical assistance of the staff of LANUPRO, in particular Charlotte Melis.

Chapter 9

In vivo bioavailability of conjugated linoleic acid in dairy cows supplemented with rumen bypass emulsions created by polyphenol oxidase rich protein extracts of potato tuber peels



Redrafted after Fievez V., Gadeyne F. and Van Ranst G., 2015, Method to protect lipophilic nutrients against ruminal degradation, International Publication Number: WO 2015091840 A1

Chapter 9

In vivo bioavailability of conjugated linoleic acid in dairy cows supplemented with rumen bypass emulsions created by polyphenol oxidase rich protein extracts of potato tuber peels

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Abstract. Rumen bypass technologies aim at protecting nutrients against microbial degradation, e.g. biohydrogenation (BH) of polyunsaturated fatty acids (PUFA), to ensure an unaffected passage through the rumen and enhanced intestinal absorption. Recently, a new encapsulation technology has been proposed based on the properties of the naturally occurring enzyme polyphenol oxidase (PPO). However, up till now no *in vivo* proof of concept for the PPO-based bypass technology nor direct evidence of post-ruminal absorption was available. Proteins and PPO in this study were extracted from potato tuber peels and used to prepare an oil-in-extract emulsion of triacylglycerides of a mixture of conjugated linoleic acid (CLA) isomers in water. Encapsulation was created by cross-linking the interfacial emulsifying proteins upon addition of 4-methylcatechol (4-MC). Rumen bypass CLA emulsions were used to dose 7 g *trans*-10, *cis*-12 C18:2 per day to four dairy cows during five consecutive days. Moreover, four other cows were given a commercially available protected CLA product. Milk fat content and fatty acid composition was monitored to assess the transfer to the milk of dietary CLA. Evidence for the *in vivo* protection against ruminal degradation, post-ruminal uptake and transfer to the milk was given as increased proportions of *trans*-10, *cis*-12 C18:2 were found in milk fat of cows compared with the periods before and after supplementation in case of both additives. Extra proof for the transfer to the milk was given because both supplements reduced milk fat content, since *trans*-10, *cis*-12 C18:2 is known as an inhibitor of milk fat synthesis. At the end of the supplementation period, transfer efficiencies of *trans*-10, *cis*-12 C18:2 from the diet to the milk for the rumen bypass

CLA emulsions were comparable with the commercially available protected CLA product, but showed lower faecal recoveries. In conclusion, the PPO-based lipid protection technology could provide a worthy alternative for current rumen bypass products as the encapsulated CLA was effectively transferred to the milk.

1 Hypothesis and objective

The aim of the *in vivo* experiment described in this chapter was to evaluate the transfer to the milk of PUFA when dairy cows were fed an emulsion, protected by the PPO-based technology using potato tuber peels. So far, protection against ruminal BH and post-ruminal bioavailability has been assessed *in vitro*, while no *in vivo* proof of increased transfer to the milk has been demonstrated yet. An oil source rich in *trans*-10, *cis*-12 C18:2 was chosen for this experiment, as it is a potent inhibitor of milk fat synthesis by impairing the production of several enzymes essential for fat synthesis in the mammary gland (Jenkins and Harvatine, 2014), even when administered at limited doses, but only when it is transferred to the mammary gland. Accordingly, monitoring of a simple characteristic, i.e. milk fat content, could be used as an immediate proxy for the protection against ruminal BH of the test product, release from its protective shell during abomasal and intestinal passage and (partial) transfer to the milk (de Veth *et al.*, 2005). Moreover, comparison was made with the case of feeding a commercially available rumen bypass product.

The hypothesis was that dietary CLA in an oil emulsified with a PPO rich protein extract of potato tuber peels and treated with 4-MC could be protected against ruminal breakdown, would be released from its protected shell during gastro-intestinal passage, absorbed post-ruminally and transferred to the mammary gland. *In vivo* proof of concept for the bioavailability of CLA would be given as increasing proportions of *trans*-10, *cis*-12 C18:2 were observed in milk fat of cows, which concomitantly would induce milk fat depression (MFD). As prototype production was not possible yet, it was chosen to keep production circumstances exactly the same as for the previous in vitro experiments.

2 Materials and methods

2.1 Materials

A triacylglyceride mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Tonalin® TG80, BASF-AG, Ludwigshafen, Germany) was used as oil source throughout the whole experiment. Tonalin contained 4.47, 28.6, 152, 1.78, 393 and 391 mg C16:0, C18:0, *cis*-9 C18:1, *cis*-9, *cis*-12 C18:2, *cis*-9, *trans*-11 C18:2 and *trans*-10, *cis*-12 C18:2 per g of total FA, respectively. For comparative reasons, Lutrell Combi® (LUT; BASF-AG, Ludwigshafen, Germany) was also included in the trial as a commercially available rumen-protected product which contains a methyl ester mixture of the same FA. This product contained 315 g/kg crude fat according to the manufacturer and was comprised of 92.6, 447, 151, 32.9, 124 and 124 mg C16:0, C18:0, *cis*-9 18:1, *cis*-9, *cis*-12 C18:2, *cis*-9, *trans*-11 C18:2 and *trans*-10, *cis*-12 C18:2 per g of total FA, respectively. Lutalin® (BASF-AG, Ludwigshafen, Germany), a methyl ester mixture of the same FA in a liquid form, was assessed as unprotected counterpart of LUT and contained 52.4, 42.6, 280, 8.02, 280 and 277 mg C16:0, C18:0, *cis*-9 C18:1, *cis*-9, *cis*-12 C18:2, *cis*-9, *trans*-11 C18:2 and *trans*-10, *cis*-12 C18:2 per g of total FA, respectively. Potato (*Solanum tuberosum* L.) tuber peels originated from different Belgian household kitchen wastes, were immediately frozen after peeling and stored at -18°C until extraction. Chemicals were of analytical grade and were purchased from Sigma-Aldrich (Bornem, Belgium), Merck (Darmstadt, Germany), Carl-Roth (Karlsruhe, Germany) or VWR (Heverlee, Belgium), unless stated otherwise.

2.2 Preparation and Characterization of CLA Emulsions

2.2.1 Preparation of CLA emulsions

CLA-rich emulsions were prepared as described before by Gadeyne *et al.* (2015) (see also section 2.2 of Chapter 4). Briefly, the protection process consisted of three steps: first, a protein extract of potato tuber peels, containing PPO, was made; second, oil was emulsified

in this protein extract using a microfluidizer (M110S, Microfluidics Corporation, Newton, Massachusetts, USA); and third, emulsions were treated with 4-MC (Sigma-Aldrich, Bornem, Belgium) to create protection. All emulsions contained 20 mg Tonalin per ml of protein extract and 20 mM 4-MC and were stored in a refrigerator at 4°C until further usage. For the *in vivo* experiment, 10 batches of 1.8 liter were produced in a period of 14 days, providing sufficient amounts to the animals while maintaining the current laboratory scale production procedures. Products were stored at 4°C in liquid form. Storage up to two weeks using this procedure did not cause deterioration of the emulsions (results not shown).

2.2.2 Extract Characterization

Batches of protein extracts were thoroughly characterized. Protein, protein-bound phenol and specific PPO activity was assessed spectrophotometrically as described before (Gadeyne *et al.*, 2016a) (see section 2.3.1 of Chapter 5) and expressed as mg of protein per ml extract, µg tyrosine-equivalents per mg protein and µkatal per mg protein, respectively. Spectrophotometric analyzes were done in triplicate. Gel electrophoretic detection of PPO isoforms being present in the extract batches was also performed (Gadeyne *et al.*, 2016b) (see also section 2.3.2 of Chapter 5).

2.2.3 Emulsion Characterization

Particle size distributions of the emulsions, immediately after preparation and prior to 4-MC addition, were assessed with a Mastersizer S (Malvern Instruments, Malvern, UK) as described by Gadeyne *et al.* (2015) (see also section 2.2 of Chapter 4). Droplet size distributions were characterized in terms of surface-weighted mean diameter (D_{32}) and specific surface areas (in m²/g oil) were calculated as $SSA = 6 / [D_{32} \times \rho]$, assuming an oil density ρ of 930 kg/m³.

2.2.4 In vitro Ruminal Biohydrogenation of CLA Emulsions

Incubations were done to assess the protection of CLA in the 10 batches of emulsion against *in vitro* ruminal BH. The CO_2-flushed incubation flasks containing 250 mg hay, 24 ml buffer/rumen fluid solution (4:1 ratio, with the buffer containing 3.58 g $Na_2HPO_4.12H_20$, 1.55 g KH_2PO_4, 0.124 g $MgCl_2.6H_20$, 8.74 g $NaHCO_3$ and 1 g NH_4HCO_3 per liter of demineralized water) and 1 ml of emulsion, either or not treated with 4-MC, were intermittently shaken for 24h at 39°C in a batch culture incubator (Edmund Bühler GmbH, Hechingen, Germany). Rumen contents were collected before the morning feeding from three rumen fistulated sheep, which were fed grass hay *ad libitum* and a grain based concentrate (200 g/day) twice daily to meet their maintenance requirements and had free access to drinking water. Fistulation was approved by the ethical commission (file number 114, 2009) of the Institute for Agricultural and Fisheries Research (ILVO, Belgium). Rumen contents of the three sheep were combined and filtered (sieve with a pore size of 1 mm) before incubation. Emulsions were incubated in triplicate, using three different sources of hay (analytical replicates). To monitor the quality of the incubations, gas (Hassim *et al.*, 2010), pH (Hanna Instruments, Temse, Belgium) and volatile FA were assessed (Gadeyne *et al.*, 2016f) (see also section 2.4.1 of Chapter 2). Gas composition was converted to absolute gas production rates based on the pressure accumulation in the flask (Infield 7C handheld read-out device equipped with a T1 Stitch-Tensiometer; UMS GmbH, München, Germany).

To calculate BH, 5 ml of incubation fluid was taken before and after incubation, freeze-dried and FA analyzed after direct transesterification, according to Gadeyne *et al.* (2015) (see also section 2.5 of Chapter 4), using gas chromatography. Fatty acid methyl esters (FAME) were quantified with a Hewlett-Packard 6890 gas chromatograph (Hewlett-Packard, Brussels, Belgium) equipped with a Solgel-wax column (30 m x 0.25 mm x 0.25 µm; SGE Analytical Science, Ringwood, Victoria, Australia) and a flame ionization detector. The temperature program was as follows: 150°C for 2 min; increased at 3°C/min until 250°C. The injector and detector temperature were 250°C and 280°C, respectively. Two µl was injected with a split

ratio of 50:1 with H_2 as carrier gas at a flow rate of 1 ml/min. FA peaks were identified based on their retention times, compared to external standards (GLC463, Nu-Check Prep Inc., Elysian, Minnesota, USA). Quantification of FAME was based on the area of the internal standard (C13:0, 0.2 mg/ml toluene; Sigma-Aldrich, Bornem, Belgium) and on the conversion of peak areas to the weight of FA by a theoretical response factor for each FA (Ackman and Sipos, 1964; Wolff *et al.*, 1995). BH was then calculated as [(proportion of *trans*-10, *cis*-12 C18:2 in total C_{18} FA)$_{0h}$ – (proportion of *trans*-10, *cis*-12 C18:2 in total C_{18} FA)$_{24h}$] / (proportion of *trans*-10, *cis*-12 C18:2 in total C_{18} FA)$_{0h}$. Protection efficiency of *trans*-10, *cis*-12 C18:2 in CLA emulsions was calculated as [(BH of *trans*-10, *cis*-12 C18:2)$_{\text{non-protected (0 mM 4-MC)}}$ – (BH of *trans*-10, *cis*-12 C18:2)$_{\text{protected (20 mM 4-MC)}}$] / (BH of *trans*-10, *cis*-12 C18:2)$_{\text{non-protected (0 mM 4-MC)}}$. Similarly, the protection efficiency of LUT was calculated by comparison with unprotected Lutalin oil, a methyl ester mixture of the same FA in a liquid form.

2.3 Animals, diets, experimental set-up and sampling

Eight dairy cows in mid-lactation (100 to 220 days in milk, 562 to 712 kg live weight, minimum daily production of 20 to 25 kg milk) were used in a sequential set-up to test the transfer from the diet to the milk. Cows were divided into two groups of four animals. Groups were organized to ensure a homogenous distribution based on parity, weight, milk production, milk composition (protein and fat) and days in milk. Animals were kept in a tie barn and had free access to drinking water. The trial was approved by the ethical commission (file number 231bis, 2014) of the Institute for Agricultural and Fisheries Research (ILVO, Belgium)

Two supplements were tested: CLA emulsion, prepared as described before (vide supra) using Tonalin (PPO; n=4), and a commercially available rumen-protected CLA product (LUT; n=4). Cows received one of these two supplements twice a day, which were administered in such amounts to deliver 7 g of *trans*-10, *cis*-12 C18:2 per day per cow. Supplements were mixed with 0.5 kg of soybean meal which was offered separately to ensure total supplement

intake. The experimental period consisted of six weeks (Table 9.1). The first 21 days (period 1) were used as adaptation period, in which cows were adapted to a basal diet consisting of roughage (60/40 w/w maize silage/grass silage), soybean meal, Mervobest (rumen-protected protein; Nuscience, Ghent, Belgium), two types of concentrate (protein corrector and concentrate balanced for net energy and protein digestible in the small intestine for milk production) and urea to meet individual requirements, which was fed throughout the whole experimental period. Roughage intake was *ad libitum* during the first three adaptation weeks (period 1), but fixed during the next three weeks of the experiment (period 2, 3 and 4) based on individual intakes during the adaptation period. Diet formulation was based on the chemical composition and nutritional values of the ingredients (Table 9.2). *Trans*-10, *cis*-12 C18:2 containing supplements were administered in period 3 for five consecutive days, immediately followed by five post-treatment days in period 4.

Table 9.1 Overview of the experimental periods of the *in vivo* experiment with 8 dairy cows (n=4)

	Period 1 (21 days)	Period 2 (7 days)	Period 3 (5 days)	Period 4 (5 days)
	Adaptation	Pre-treatment	Treatment	Post-treatment
Milk sampling	-	Day[1,2] - 4 / - 3	Day 1 / **2** / **3** / **4** / **5**	Day 6 / 7 / 8 / 9 / 10
Feces sampling	-	Day - 3 / - 2	Day 4 / 5	Day 9 / 10
Supplement	-	-	PPO / LUT	-

PPO, CLA emulsion containing 20 mg Tonalin oil per ml potato tuber peel extract and 20 mM 4-methylcatechol; LUT, a commercially available protected product (Lutrell Combi)
[1] sampling days are relative to the first day of supplementation
[2] full underlined sampling days are used for hypothesis 1, days in bold case represent the ones used in hypothesis 2 (as described in section 2.5 of Chapter 9)

Table 9.2 Chemical composition of the feed ingredients

	DM (g/kg)	NE_L[1] (MJ/kg DM)	DVE[2] (g/kg DM)	OEB[3] (g/kg DM)
Maize silage	348	6.68	59	-47
Corn silage	380	6.01	68	3
Soybean meal	870	8.10	272	197
Mervobest	870	8.21	482	-8
Concentrate 1	870	7.92	122	18
Concentrate 2	870	7.62	84	-14

[1] NE_L = net energy of lactation, calculated with the Dutch net energy evaluation (VEM) system (Van Es, 1975)
[2] DVE = intestinal digestible protein (Tamminga *et al.*, 1994)
[3] OEB = degraded protein balance (Tamminga *et al.*, 1994)

Milk was sampled 4 and 3 days before the first supplementation day (period 2, pre-treatment), on day 1, 2, 3, 4 and 5 relative to the first day of supplementation (period 3, treatment) and on day 6, 7, 8 and 9 and 10 after first supplementation (period 4, post-treatment) (Table 9.1). Cows were milked twice a day and milk yield was recorded. Evening and morning milk samples were pooled and stored at -18°C prior to further analysis. Fecal spot samples of about 100 g were taken rectally 3 and 2 days before the first supplementation (period 2, pre-treatment), on day 4 and 5 (period 3, treatment) and on day 9 and 10 (period 4, post-treatment) after the first supplementation. Feces was immediately stored at -18°C, freeze-dried and ground with a mortar before analysis.

2.4 Analyzes

2.4.1 Milk Components

Fat, protein, lactose and urea content in pooled samples (evening and morning milk) was determined by Fourier Transform Infrared analysis (FTIR Delta Instuments, Drachten, The Netherlands) and presented as g per kg of milk produced per day for fat, protein and lactose content and as mg per liter sample per day for urea content.

2.4.2 Milk Fatty Acids

The FA profile of the pooled milk samples was analyzed using gas chromatography. Briefly, milk fat was extracted (mini Röse-Gottlieb method, adapted from Chouinard *et al.* (1997)), FA methylated (Stefanov *et al.*, 2010) and FAME analyzed after injection in an Agilent Technologies 7890A or 6890N gas chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with a Supelco column (75 m × 180 µm × 0.14 µm, SP-2560; Sigma-Aldrich, Bornem, Belgium) and a flame ionization detector. The temperature program was as follows: 70°C for 2 min; increased at 15°C/min to 150°C followed by an increase of 1°C/min to 165°C, then 165°C for 12 min, followed by an increase of 2°C/min to 170°C and held for 5 min, next an increase of 5°C/min to 215°C and held for 10 min, to end up with an

increase of 5°C/min to 225°C and held for 2 min. The injector and detector temperature were 250°C and 255°C, respectively. One µl was injected with a split ratio of 50:1 with H_2 as carrier gas at a flow rate of 1 ml/min. FA peaks were identified based on their retention times, compared to external standards (GLC463, Nu-Check Prep Inc., Elysian, Minnesota, USA; CLA *cis*-9, *trans*-11 C18:2 and *trans*-10, *cis*-12 C18:2, Larodan Fine Chemicals AB, Malmö, Sweden). Also, aliquots of methylated samples were pooled and subsequently fractionated by Ag^+-Solid Phase Extraction as described by Kramer *et al.* (2008a). FA commercially not available were identified by order of elution based on Shingfield *et al.* (2006) and Kramer *et al.* (2008b). Triacylglyceride-C13:0 (1 mg/ml chloroform; Nu-Chek Prep Inc., Elysia, Minnesota, USA) was used as internal standard. Peak areas were corrected by a theoretical relative response factor for each FA (Ackman and Sipos, 1964; Wolff *et al.*, 1995) and FA expressed as g per 100 g of detected FA. Also, analysis of milk *trans*-10, *cis*-12 C18:2 secretion allowed the calculation of the transfer efficiency of this FA (g/100 g) from the diet (7 g/day) to the milk.

2.4.3 Fecal Fatty Acids

Freeze-dried and grounded fecal grab samples were analyzed for their FA profile after direct transesterification, according to Gadeyne *et al.* (2015) (see section 2.5 of Chapter 4). Gas chromatographic conditions, identification and quantification of FAME was as described before for the BH determination (vide supra). Fecal FA were expressed as µg FA per g freeze-dried feces.

2.5 Statistics

All results were analyzed using the MIXED procedure of SAS (SAS Enterprise Guide 7, SAS Institute Inc., Cary, North Carolina, USA). Analytical replicates were averaged prior to statistical analysis. Differences were significant at $P<0.05$ and differences among least squares means evaluated using Tukey's multiple comparison test.

Two main questions were considered for milk data of the *in vivo* trial: first, did administration of the supplements induce changes in the milk FA composition or fat content compared to untreated periods, and second, did both supplements induce similar shifts. For the first question, the following model was used for both treatments: $Y_{ij} = \mu + C_i + P_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, C_i the random effect of cow, P_j the fixed effect of period (j = pre-treatment or treatment) and ε_{ij} the residual error. Samples taken at day 3 days prior to first supplementation (period 2) and day 5 after first supplementation (period 3) were used for the pre-treatment and treatment period, respectively (Table 9.1). For the second question, the model was: $Y_{ijk} = \mu + C_i + S_j + T_k + S_j \times T_k + \varepsilon_{ij}$, with Y_{ijk} the variable of interest, μ the overall mean, C_i the random effect of cow, S_j the fixed effect of supplement (j = CLA emulsions or LUT), T_k the fixed effect of time (k = 2, 3, 4 or 5 days after the start of the supplementation) as repeated measure and ε_{ijk} the residual error. Further, to study the daily evolution of the milk composition, comparison between daily measurements (day 1 to 10) and measurements during period 2 (day -4 and -3) was made for both supplements using a CONTRAST statement with a similar model as for hypothesis 1: $Y_{ij} = \mu + C_i + T_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, C_i the random effect of cow, T_j the fixed effect of time (j = day -4, -3, 1, 2, 3, 4 ,5, 6, 7, 8, 9 and 10) and ε_{ij} the residual error.

Finally, fecal FA were compared using the following model: $Y_{ijk} = \mu + C_i + S_j + P_k + S_j \times P_k + \varepsilon_{ij}$, with Y_{ijk} the variable of interest, μ the overall mean, C_i the random effect of cow, S_j the fixed effect of supplement (j = CLA emulsion or LUT), P_k the fixed effect of period (k = pre-treatment, treatment or post-treatment period) and ε_{ijk} the residual error.

3 Results

3.1 Properties of CLA Emulsions

In order to establish *in vivo* proof of concept for transfer to the milk with the described technology, 10 batches of 4-MC treated CLA emulsion were prepared using potato tuber peel

proteins and Tonalin oil. This was done to obtain sufficient amounts in order to dose the cows 7 g of *trans*-10, *cis*-12 C18:2 daily. Characteristics of extracts and the resulting emulsions of all ten batches used in the *in vivo* trial were assessed. Extracts of potato tuber peels contained on average (n=10) 2.11 ± 0.23 mg of protein per ml extract, 63.8 ± 12.7 μg tyrosine-equivalents per mg protein as a measure of protein-bound phenols and showed a specific PPO activity of 1.60 ± 0.27 μkatal per mg protein, resulting in emulsions with a surface-weighted mean diameter D_{32} of 3.20 ± 0.51 μm and a specific surface area SSA of 2.67 ± 0.39 m^2 per g oil. An average protection efficiency of 0.660 ± 0.051 and 0.672 ± 0.048 for *trans*-10, *cis*-12 C18:2 and *cis*-9, *trans*-11 C18:2, respectively, was measured by *in vitro* incubation. No major differences in incubation characteristics (i.e. pH, gas composition and volatile FA) were observed between treatments and batches (results not shown). However, two out of ten batches were found to have a lower protection efficiency compared with the other batches. More specifically, they were given in the afternoon of day 1 (batch 2) and the morning of day 2 (batch 3). *In vitro* protection efficiencies of CLA emulsions, administered to the cows during the five supplementation days, are shown in Figure 9.1A. Gel electrophoretic analysis revealed differing patterns of PPO isoforms in the potato tuber peel extracts which were used to create the emulsions (Figure 9.1B). Particularly, batches 2 and 3, resulting in lower *in vitro* protection efficiencies, showed larger absorbance areas as compared with the other batches. On the other hand, *in vitro* assessment of ruminal BH for LUT revealed the protection efficiency was lower (P=0.005) than for the CLA emulsions, more specifically 0.433 ± 0.032 for *trans*-10, *cis*-12 C18:2.

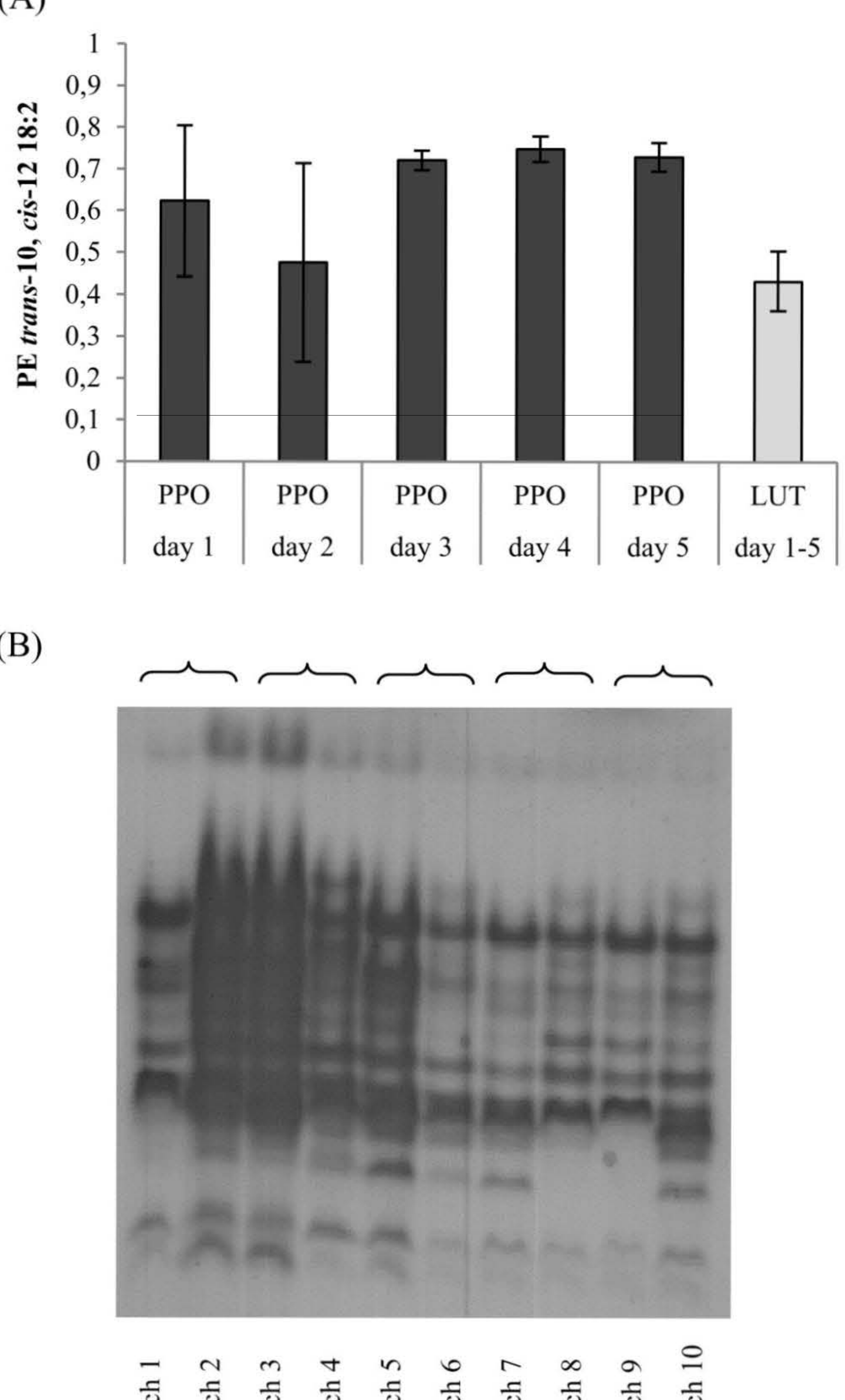

Figure 9.1 Protection efficiencies against *in vitro* ruminal biohydrogenation (PE) of *trans*-10, *cis*-12 C18:2 in CLA emulsions of the five daily doses made with polyphenol oxidase (PPO) rich extracts of potato tuber peels and treated with 20 mM 4-methylcatechol (A) differed and seemed to be related to different patterns of PPO isoforms visualized through gel electrophoretic analysis (B) of the ten batches of potato tuber peel extracts, which were used to emulsify CLA oil, but was lower for a commercially available protected product (LUT)

3.2 Milk Components and Fatty Acids

3.2.1 Is it possible to induce changes in milk composition by supplement feeding?

Milk was analyzed for its major components (fat, protein, lactose and urea) and fatty acid profile, in order to evaluate whether changes in the milk fat content could be related to an increased level of *trans*-10, *cis*-12 C18:2 upon supplementation. Milk yield and the major constituents in milk of cows administered with CLA emulsions (Table 9.3) were not significantly different between periods of the *in vivo* trial with or without supplement. Milk fat of cows during the pre-treatment period (day -3) contained negligible levels of *trans*-10, *cis*-12 C18:2, but a large increase (P=0.002) was observed when cows were given CLA emulsions (day 5). Similarly, no increase in milk yield was observed (P=0.880) when LUT (Table 9.4) was given, but milk fat decreased (P=0.025) from 43.7 ± 4.2 g/kg during the pre-treatment period to 34.7 ± 3.3 g/kg during treatment. Milk lactose content also decreased, but protein and urea levels in milk were unaltered. Similarly, *trans*-10, *cis*-12 C18:2 increased (P<0.001) from 0.001 ± 0.001 to 0.043 ± 0.005 g per 100 g FA when cows were fed LUT. Here, higher proportions of *cis*-9, *trans*-11 C18:2 were also found upon treatment. More FA were affected upon treatment when LUT (Table 9.4) was fed, compared to the CLA emulsions (Table 9.3), but proportions of most FA in both cases were generally only slightly changed upon administration. The major saturated FA C14:0, C16:0 and C18:0 showed a similar behavior in both cases. Most monounsaturated C_{18} FA only increased significantly upon administration of LUT. Finally, given the fact that 7 g *trans*-10, *cis*-12 C18:2 (and 7 g *cis*-9, *trans*-11 C18:2) was administered per cow per day, an average transfer efficiency of 4.04 ± 0.35 g/100 g and 4.81 ± 0.63 g/100 g of dietary *trans*-10, *cis*-12 C18:2 to the milk was calculated during the supplementation period for the CLA emulsions and LUT, respectively.

Table 9.3 Milk yield, components and FA composition for dairy cows supplemented with a conjugated linoleic acid emulsion containing 20 mg Tonalin oil per ml potato tuber peel extract and 20 mM 4-methylcatechol (n=4)

	Pre-treatment[1]	Treatment[2]	SEM	P-value
Milk Yield (kg/day)	28.5	27.9	1.98	0.769
Milk Components				
Fat (g/kg)	46.3	39.9	2.06	0.114
Protein (g/kg)	34.2	34.7	0.44	0.427
Lactose (g/kg)	47.1	47.1	0.50	0.922
Urea (mg/L)	279	279	14.9	0.994
Milk FA composition (g/100g)				
C4:0	3.83	3.46	0.108	0.096
C5:0	0.03	0.03	0.003	0.056
C6:0	2.46	2.13	0.074	0.048
C7:0	0.03	0.02	0.003	0.074
C8:0	1.45	1.25	0.052	0.045
C9:0	0.03	0.02	0.005	0.052
C10:0	3.07	2.79	0.152	0.049
C10:1 *cis*-9	0.41	0.34	0.012	0.026
C11:0	0.05	0.05	0.009	0.607
C12:0	3.52	3.39	0.207	0.435
C12:1 *cis*-9	0.11	0.11	0.007	0.349
C14:0	11.9	12.0	0.38	0.552
iso-C14:0	0.12	0.10	0.012	0.373
C14:1 *cis*-9	1.44	1.43	0.106	0.912
C15:0	1.07	1.01	0.033	0.226
iso-C15:0	0.23	0.22	0.010	0.083
anteiso-C15:0	0.41	0.40	0.015	0.291
C16:0	36.4	35.9	1.01	0.403
iso-C16:0	0.29	0.27	0.025	0.551
C16:1 *cis*-9	2.04	2.02	0.158	0.817
C17:0	0.49	0.58	0.013	0.001
C18:0	7.29	7.42	0.508	0.798
C18:1 *trans*-6-8	0.18	0.22	0.020	0.070
C18:1 *trans*-9	0.14	0.16	0.013	0.194
C18:1 *trans*-10	0.21	0.27	0.028	0.105
C18:1 *trans*-11	0.64	0.82	0.114	0.206
C18:1 *cis*-9	14.9	15.7	0.65	0.130
C18:1 *cis*-11	0.43	0.47	0.030	0.188
C18:1 *cis*-12	0.18	0.21	0.015	0.063
C18:1 *cis*-13	0.04	0.04	0.003	0.371
C18:2 *cis*-9, *cis*-12	1.03	1.09	0.074	0.258
C18:2 *cis*-9, *trans*-11	0.36	0.49	0.053	0.113
C18:2 *trans*-10, *cis*-12	0.001	0.029	0.002	0.002
C18:3 *cis*-9, *cis*-12, *cis*-15	0.27	0.29	0.007	0.088
C20:0	0.13	0.13	0.008	0.770
C20:1 *cis*-9	0.12	0.12	0.006	0.499
Other FA	4.69	4.99	0.146	0.040
Sum FA by source (g/100g)				
< C16	30.2	28.7	0.52	0.058
> C16	26.4	28.1	1.30	0.084
Saturated FA	71.8	70.2	0.96	0.108
Monounsaturated FA	20.8	21.9	0.76	0.149
Polyunsaturated FA	1.66	1.91	0.085	0.058
Odd and branched-chain FA	2.75	2.71	0.077	0.681
TE to the milk (g/100g)				
C18:2 *trans*-10, *cis*-12	0	4.04	0.247	0.001

SEM, standard error of the mean; FA, fatty acid; TE, transfer efficiency
[1] values represent an average of samples obtained on day -3
[2] values represent an average of samples obtained on day 5

Table 9.4 Milk yield, components and FA composition for dairy cows supplemented with a commercially available protected product (Lutrell Combi) (n=4)

	Pre-treatment[1]	Treatment[2]	SEM	P-value
Milk Yield (kg/day)	25.8	26.0	2.62	0.880
Milk Components				
Fat (g/kg)	43.7	34.7	3.80	0.025
Protein (g/kg)	33.7	33.7	1.60	0.981
Lactose (g/kg)	47.8	46.6	0.81	<0.001
Urea (mg/L)	234	239	26.2	0.710
Milk FA composition (g/100g)				
C4:0	3.64	3.53	0.105	0.250
C5:0	0.03	0.03	0.003	0.014
C6:0	2.40	2.10	0.074	0.016
C7:0	0.02	0.02	0.002	0.625
C8:0	1.43	1.23	0.062	0.028
C9:0	0.03	0.02	0.002	0.080
C10:0	3.15	2.73	0.189	0.031
C10:1 *cis*-9	0.37	0.31	0.044	0.183
C11:0	0.04	0.03	0.002	0.079
C12:0	3.55	3.26	0.186	0.091
C12:1 *cis*-9	0.10	0.10	0.014	0.451
C14:0	12.4	12.5	0.44	0.833
iso-C14:0	0.08	0.09	0.003	0.012
C14:1 *cis*-9	1.33	1.39	0.228	0.607
C15:0	1.00	0.97	0.033	0.527
iso-C15:0	0.23	0.23	0.009	0.546
anteiso-C15:0	0.41	0.42	0.009	0.528
C16:0	35.3	34.3	1.77	0.215
iso-C16:0	0.22	0.24	0.017	0.102
C16:1 *cis*-9	1.90	1.90	0.221	0.981
C17:0	0.52	0.59	0.027	0.078
C18:0	7.84	8.00	1.140	0.693
C18:1 *trans*-6-8	0.19	0.24	0.009	0.032
C18:1 *trans*-9	0.14	0.17	0.007	0.008
C18:1 *trans*-10	0.24	0.28	0.015	0.105
C18:1 *trans*-11	0.68	0.84	0.089	0.034
C18:1 *cis*-9	15.3	16.5	0.50	0.010
C18:1 *cis*-11	0.47	0.50	0.041	0.183
C18:1 *cis*-12	0.19	0.23	0.011	0.110
C18:1 *cis*-13	0.05	0.05	0.003	0.138
C18:2 *cis*-9, *cis*-12	0.96	1.04	0.065	0.081
C18:2 *cis*-9, *trans*-11	0.37	0.52	0.038	0.012
C18:2 *trans*-10, *cis*-12	0.001	0.043	0.004	0.004
C18:3 *cis*-9, *cis*-12, *cis*-15	0.29	0.31	0.019	0.316
C20:0	0.13	0.13	0.017	0.671
C20:1 *cis*-9	0.11	0.12	0.058	0.322
Other FA	4.82	5.10	0.218	0.013
Sum FA by source (g/100g)				
< C16	30.2	28.9	0.90	0.160
> C16	27.5	29.5	1.80	0.005
Saturated FA	71.5	69.4	0.80	0.014
Monounsaturated FA	21.1	22.6	0.50	0.029
Polyunsaturated FA	1.62	1.91	0.108	0.021
Odd and branched-chain FA	2.58	2.63	0.031	0.258
TE to the milk (g/100g)				
C18:2 *trans*-10, *cis*-12	0	4.81	0.445	0.005

SEM, standard error of the mean; FA, fatty acid; TE, transfer efficiency
[1] values represent an average of samples obtained on day -3
[2] values represent an average of samples obtained on day 5

3.2.2 Do both supplements induce similar shifts?

A time effect (P=0.001) was observed for milk fat contents (Table 9.5), indicating milk fat levels decreased over time within the treatment period. Milk yield and other milk constituents such as protein, lactose and urea content were unaffected by time or treatment. Similarly as for the milk fat content, an increase in *trans*-10, *cis*-12 C18:2 over time (P<0.001) was observed. Generally, proportions of FA synthesized de novo by the mammary gland (< C16) and saturated FA decreased over time upon supplementation, whereas FA derived from mammary uptake from circulation (> C16) and unsaturated FA increased. Further, no supplementation effect (P=0.200) on milk fat content was observed, meaning milk fat levels during administration of both supplements were similar, although higher concentrations for LUT compared with the CLA emulsions were found for *trans*-10, *cis*-12 C18:2 (P<0.001). A tendency for interaction between supplement and time was only found for milk fat content (P=0.062) and *trans*-10, *cis*-12 C18:2 (P=0.093). Finally, a time effect on the transfer efficiency of dietary *trans*-10, *cis*-12 C18:2 to the milk was also observed (P<0.001). This was mainly related to the fact that transfer efficiencies kept on increasing over time upon supplementation of the CLA emulsions (interaction effect: P<0.001; day 2 = day 3 ≤ day 4 ≤ day 5), while this wasn’t the case for LUT (day 2 = day 3 = day 4 = day 5). Therefore, at the end of the supplementation period transfer efficiencies of both supplements were similar (P=0.233 and P=0.912 on day 4 and 5, respectively).

Table 9.5 Milk yield, components and FA composition for dairy cows supplemented with a conjugated linoleic acid emulsion or LUT (n=4)

	Day 2		Day 3		Day 4		Day 5			P-value		
	PPO	LUT	PPO	LUT	PPO	LUT	PPO	LUT	SEM	S	T	S × T
Milk Yield (kg/day)	26.8	26.0	27.5	27.3	27.9	25.9	27.9	26.0	2.23	0.696	0.553	0.559
Milk Components												
Fat (g/kg)	45.3	42.9	45.1	41.1	45.2	34.6	39.9	34.7	3.20	0.200	0.001	0.062
Protein (g/kg)	34.2	34.4	34.2	34.5	34.0	34.5	34.7	33.7	1.23	0.986	0.978	0.104
Lactose (g/kg)	47.0	47.6	47.0	47.5	46.8	47.3	46.6	47.1	0.85	0.812	0.593	0.357
Urea (mg/L)	243	236	286	235	271	238	279	239	20.9	0.242	0.196	0.223
Milk FA composition (g/100g)												
C4:0	3.83	3.86	3.74	3.86	3.75	3.82	3.46	3.52	0.114	0.638	<0.001	0.844
C5:0	0.03	0.03	0.03	0.02	0.03	0.02	0.03	0.03	0.003	0.178	0.822	0.180
C6:0	2.43	2.49	2.38	2.44	2.37	2.38	2.13	2.10	0.089	0.840	<0.001	0.617
C7:0	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.003	0.157	0.094	0.374
C8:0	1.42	1.47	1.41	1.44	1.41	1.44	1.25	1.23	0.068	0.822	<0.001	0.552
C9:0	0.03	0.02	0.03	0.02	0.03	0.02	0.02	0.02	0.004	0.213	0.025	0.415
C10:0	3.02	3.17	3.00	3.11	3.07	3.14	2.79	2.73	0.179	0.785	<0.001	0.227
C10:1 *cis*-9	0.40	0.35	0.40	0.35	0.39	0.34	0.34	0.31	0.026	0.260	<0.001	0.762
C11:0	0.05	0.04	0.05	0.04	0.05	0.04	0.05	0.03	0.007	0.257	0.386	0.457
C12:0	3.47	3.53	3.48	3.49	3.58	3.55	3.39	3.26	0.200	0.924	0.004	0.368
C12:1 *cis*-9	0.11	0.10	0.11	0.10	0.11	0.10	0.11	0.09	0.009	0.314	0.189	0.960
C14:0	11.8	12.4	11.9	12.6	12.0	12.8	12.0	12.5	0.39	0.249	0.064	0.495
iso-C14:0	0.11	0.08	0.11	0.08	0.11	0.09	0.10	0.09	0.009	0.039	0.979	0.330
C14:1 *cis*-9	1.44	1.35	1.48	1.39	1.43	1.37	1.43	1.39	0.168	0.766	0.702	0.893
C15:0	1.04	0.94	1.03	0.95	1.02	0.97	1.01	0.97	0.023	0.032	0.960	0.159
iso-C15:0	0.22	0.23	0.22	0.23	0.22	0.23	0.22	0.23	0.009	0.575	0.866	0.968
anteiso-C15:0	0.39	0.41	0.40	0.42	0.39	0.43	0.40	0.42	0.013	0.190	0.410	0.270
C16:0	36.6	34.1	36.0	33.8	36.2	33.3	35.9	34.3	1.67	0.341	0.463	0.405
iso-C16:0	0.27	0.23	0.28	0.23	0.28	0.22	0.27	0.24	0.019	0.093	0.982	0.514
C16:1 *cis*-9	2.08	1.89	2.08	1.90	2.01	1.82	2.02	1.90	0.184	0.518	0.068	0.581
C17:0	0.48	0.49	0.48	0.48	0.49	0.50	0.58	0.59	0.016	0.799	<0.001	0.730
C18:0	6.97	8.19	7.04	8.15	7.12	8.20	7.42	8.00	0.856	0.415	0.820	0.177
C18:1 *trans*-6-8	0.19	0.21	0.20	0.22	0.19	0.22	0.22	0.24	0.019	0.348	0.005	0.930
C18:1 *trans*-9	0.16	0.15	0.16	0.15	0.16	0.16	0.16	0.17	0.013	0.842	0.180	0.679
C18:1 *trans*-10	0.26	0.27	0.25	0.28	0.25	0.28	0.27	0.28	0.028	0.538	0.720	0.620
C18:1 *trans*-11	0.82	0.82	0.77	0.81	0.80	0.81	0.82	0.84	0.115	0.915	0.831	0.979
C18:1 *cis*-9	15.1	15.6	15.5	15.8	15.1	16.0	15.7	16.5	0.62	0.474	0.003	0.432
C18:1 *cis*-11	0.44	0.49	0.44	0.49	0.44	0.49	0.47	0.50	0.040	0.431	0.013	0.335
C18:1 *cis*-12	0.19	0.21	0.19	0.22	0.19	0.22	0.21	0.23	0.016	0.224	0.017	0.360
C18:1 *cis*-13	0.04	0.05	0.05	0.05	0.05	0.05	0.04	0.05	0.005	0.421	0.148	0.621
C18:2 *cis*-9, *cis*-12	1.00	0.99	1.04	1.02	1.05	1.02	1.09	1.04	0.073	0.791	0.005	0.571
C18:2 *cis*-9, *trans*-11	0.45	0.44	0.45	0.46	0.45	0.47	0.49	0.52	0.053	0.860	0.093	0.873
C18:2 *trans*-10, *cis*-12	0.02	0.04	0.02	0.04	0.02	0.04	0.03	0.04	0.003	<0.001	<0.001	0.093
C18:3 *cis*-9, *cis*-12, *cis*-15	0.27	0.30	0.28	0.31	0.28	0.31	0.29	0.31	0.014	0.154	0.072	0.230
C20:0	0.12	0.13	0.11	0.13	0.12	0.13	0.13	0.13	0.013	0.534	0.017	0.176
C20:1 *cis*-9	0.12	0.11	0.12	0.11	0.12	0.12	0.12	0.12	0.005	0.516	0.045	0.113
Other FA	4.61	4.77	4.77	4.67	4.65	4.81	4.99	5.10	0.157	0.720	<0.001	0.069
Sum FA by source (g/100g)												
< C16	29.9	30.5	29.8	30.6	30.0	30.8	28.7	28.9	0.77	0.570	<0.001	0.713
> C16	26.6	28.5	27.1	28.8	26.8	29.0	28.1	29.5	1.64	0.442	0.003	0.625
Saturated FA	71.3	70.9	70.7	70.6	71.3	70.4	70.2	69.4	0.92	0.680	<0.001	0.375
Monounsaturated FA	21.3	21.6	21.7	21.9	21.3	22.0	21.9	22.6	0.69	0.634	0.005	0.428
Polyunsaturated FA	1.74	1.77	1.78	1.83	1.79	1.85	1.91	1.91	0.105	0.813	0.006	0.917
Odd and branched-chain FA	2.65	2.48	2.65	2.49	2.64	2.55	2.71	2.63	0.048	0.046	0.007	0.272
TE to the milk (g/100g)												
C18:2 *trans*-10, *cis*-12	2.26	5.30	2.61	5.67	3.01	4.61	4.04	4.81	0.443	0.081	<0.001	<0.001

PPO, CLA emulsion containing 20 mg Tonalin oil per ml potato tuber peel extract and 20 mM 4-methylcatechol; LUT, a commercially available protected product (Lutrell Combi); SEM, standard error of the mean; S, effect of supplement; T, effect of time; FA, fatty acid; TE, transfer efficiency

3.2.3 Daily evolution in milk composition

As a tendency for interaction between supplement and time was found for milk fat content (Table 9.5) and these were only depressed compared with the pre-treatment period for LUT (Table 9.4), milk fat was compared between the pre-treatment days (day -4 and -3) and individual measurements from day 1 to 10 (Figure 9.2). A delay in MFD was observed when CLA emulsions were fed to the animals compared with LUT. The latter induced MFD as compared with the pre-treatment days from day 4 of supplementation onwards, but with the CLA emulsions this was only the case from day 5 onwards. Milk fat remained at the lowest level until day 7, meaning there was still an effect of the supplements two days after ceasing their administration. A wash-out effect for both supplements was observed from day 8 onwards, as differences with the pre-treatment period were getting smaller compared to the days before. However, at each individual day of measurement, milk fat contents were not significantly different between both supplements ($P>0.642$). In a similar way, *trans*-10, *cis*-12 C18:2 in milk fat was compared between the pre-treatment days and individual measurements from day 1 to 10 (Figure 9.3). A significant increase in *trans*-10, *cis*-12 C18:2 proportions in milk fat compared with the pre-treatment period was observed for both supplements from the first day of supplementation onwards. However, milk fat of cows contained higher proportions of *trans*-10, *cis*-12 C18:2 when being fed LUT compared with CLA emulsions, with significant differences in FA proportions between both supplements at each individual measurement from day 2 until 6 ($P<0.037$). Feeding cows LUT resulted in a fast increase in *trans*-10, *cis*-12 C18:2, but a more gradual increase over time was found when the CLA emulsions were administered. At the end of the supplementation period (day 5), transfer efficiencies of the CLA emulsions were 16 % lower than those obtained by LUT. An immediate wash-out effect for both supplements was found when administration of the supplements was stopped (day 6). At day 10, proportions of *trans*-10, *cis*-12 C18:2 were diminished to the original negligible levels for both supplements.

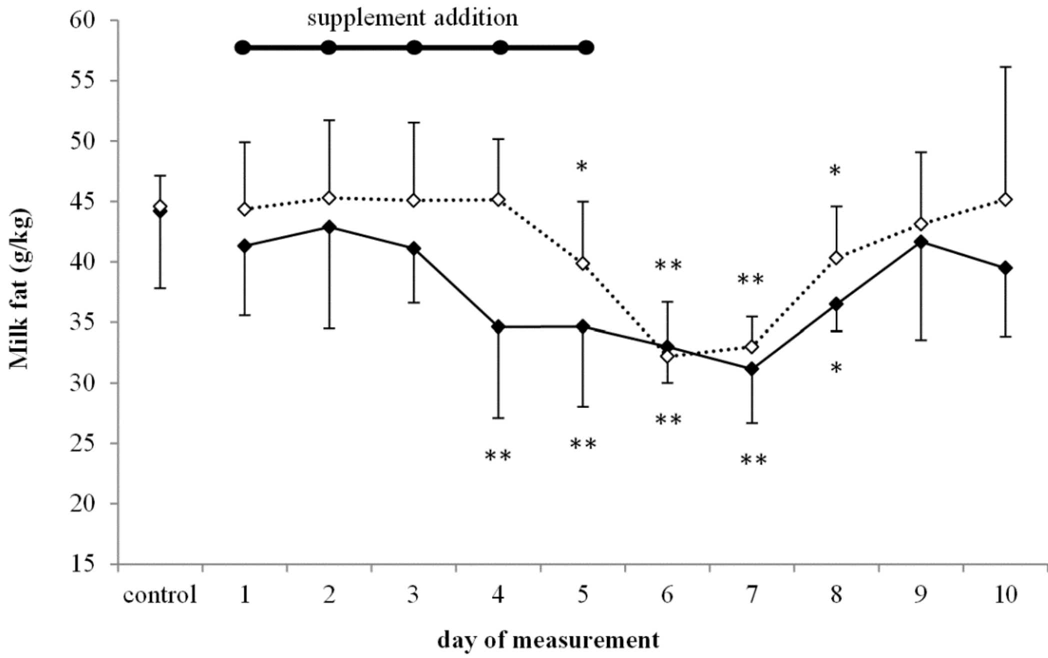

Figure 9.2 Milk fat depression was induced by 5-day addition of 7 g *trans*-10, *cis*-12 C18:2 as a CLA emulsion made with a polyphenol oxidase rich extract of potato tuber peels and treated with 20 mM 4-methylcatechol (white diamonds, dotted line) or as a commercially available protected product (black diamonds, solid line). Data points marked with * and ** are significantly different from the control (day -4 and -3) at respectively P<0.05 and P<0.01 (n=4)

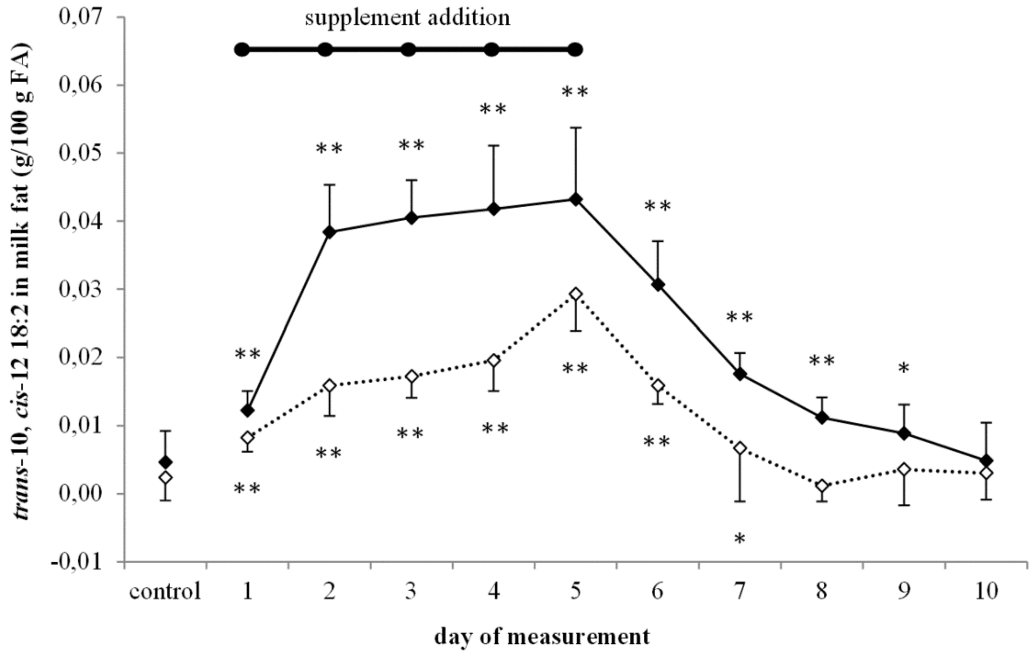

Figure 9.3 Proportions of *trans*-10, *cis*-12 C18:2 in milk fat increased by 5-day addition of 7 g *trans*-10, *cis*-12 C18:2 as a CLA emulsion made with a polyphenol oxidase rich extract of potato tuber peels and treated with 20 mM 4-methylcatechol (white diamonds, dotted line) or as a commercially available protected product (black diamonds, solid line). Data points marked with * and ** are significantly different from the control (day -4 and -3) at respectively P<0.05 and P<0.01 (n=4)

3.3 Fecal Fatty Acids

Concentrations of CLA in fecal grab samples were analyzed to assess the potential overprotection of both supplements. As the supplements contained both *cis*-9, *trans*-11 C18:2 and *trans*-10, *cis*-12 C18:2 in equal proportions, an increase in both FA was expected if overprotection occurred. Higher concentrations for both FA (P<0.001) were found in samples taken during the treatment period with CLA emulsions and LUT compared with the period before (pre-treatment) or after (post-treatment) administration Figure 9.4. This indicates some of the administered FA remained in the intestinal tract, but after the supplementation period all CLA was washed out of the body. Further, no supplement effect was observed in the case of *cis*-9, *trans*-11 C18:2 (P=0.432), meaning both supplements showed a similar behavior. However, the interaction effect between supplement and period for *cis*-9, *trans*-11 C18:2 (P=0.038) and *trans*-10, *cis*-12 C18:2 (P<0.001) revealed only *cis*-9,

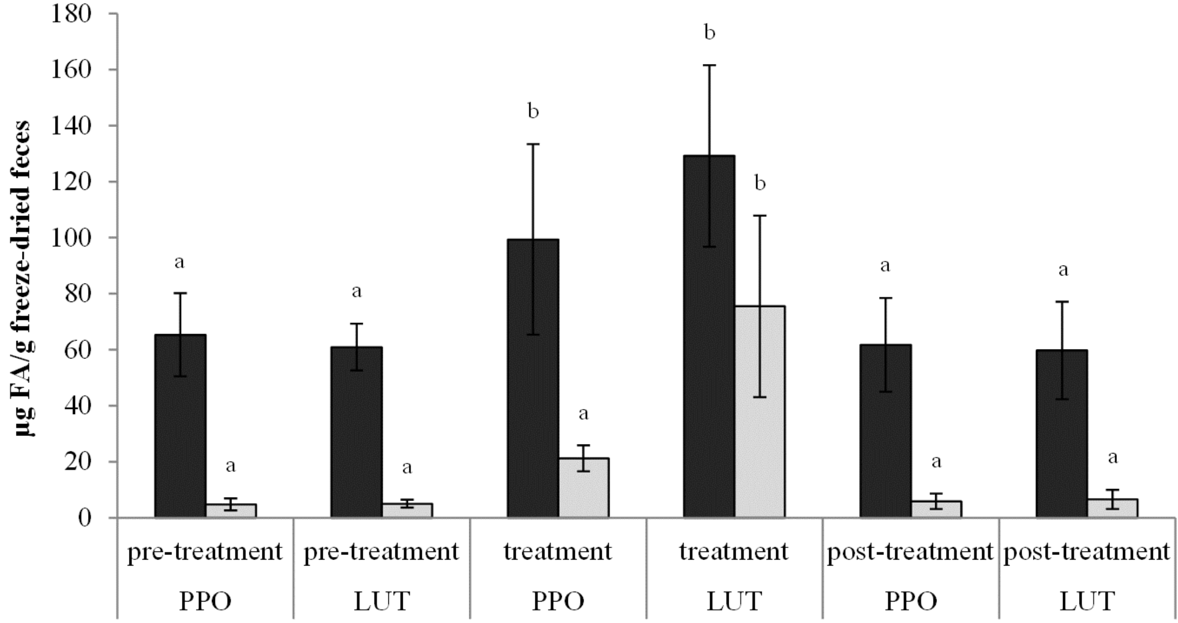

Figure 9.4 An increase in cis-9, trans-11 C18:2 in freeze-dried feces (dark bars) was observed by 5-day addition of 7 g cis-9, trans-11 C18:2 and 7 g trans-10, cis-12 C18:2 as a CLA emulsion made with a polyphenol oxidase rich extract of potato tuber peels and treated with 20 mM 4-methylcatechol (PPO) or as a commercially available protected product (LUT) compared with the pre- or post-treatment period, but trans-10, cis-12 C18:2 (light bars) was only increased during LUT treatment (n=4; characters above bars indicate differences within FA at P<0.05)

trans-11 C18:2 but not *trans*-10, *cis*-12 C18:2 was significantly increased during CLA emulsion supplementation, which was in contrast with the situation of LUT administration where both FA were increased.

4 Discussion

Emulsions which are rich in PUFA, stabilized with a PPO-rich protein extract of potato tuber peels as emulsifier and treated with 4-MC to induce interfacial cross-linking, have been proposed to be protected against ruminal BH. However, both at ruminal (Gadeyne *et al.*, 2015; Gadeyne *et al.*, 2016c) (Chapter 4 and Chapter 5 of PART II A) and post-ruminal level (Chapter 8 of PART II B) only *in vitro* data have yet been provided. The aim of this study was therefore to validate the PPO-based technology with *in vivo* results and compare this with a commercially available product. *In vivo* validation of the PPO-based technology was done by assessing the transfer from the diet to the milk. Rumen protection of PPO-based emulsions in previous experiments (Gadeyne *et al.*, 2015; Gadeyne *et al.*, 2016d) (see Chapter 4 and Chapter 5 of PART II A) was created using linseed oil, containing mainly linoleic and linolenic acids. As concentrations of these FA in milk could largely vary depending on the basal diet (Khiaosa-ard *et al.*, 2015; Sterk *et al.*, 2012a), it was more appropriate to evaluate the transfer of a FA which is present in minor and less varying amounts in milk fat under non-supplemented circumstances, such as *trans*-10, *cis*-12 C18:2. Moreover, *trans*-10, *cis*-12 C18:2 is a potent inhibitor of milk fat synthesis (Baumgard *et al.*, 2000), so monitoring of the milk fat content could be used as an alternative measure for the transfer from the diet to the milk. Indeed, abomasal infusions at limited doses (5 to 10 g/d) of *trans*-10, *cis*-12 C18:2 are known to be sufficient to observe differences in both *trans*-10, *cis*-12 C18:2 in milk fat as well as milk fat yields (de Veth *et al.*, 2004). Therefore, by protecting this particular FA, small amounts could be supplemented. Besides, this was an essential practical prerequisite within this trial, as the current laboratory scale procedures only allowed the production of small volumes of protected product. Critical labscale production factors within this protocol included

a 20 mg/ml oil-in-extract emulsion, large volumes of acetone to precipitate the potato tuber peel proteins and the use of a microfluidizer for emulsion preparation. Indeed, it was chosen to keep production circumstances the same as for the previous *in vitro* experiments (Gadeyne *et al.*, 2015; Gadeyne *et al.*, 2016e) (see Chapter 4 and Chapter 5 of PART II A), in order to effectively provide *in vivo* evidence of the previously described technology.

4.1 *In vivo* evidence for the PPO-based protection technology

The transfer of encapsulated CLA emulsions was proven as levels of *trans*-10, *cis*-12 C18:2 in milk fat were higher compared with the periods before or after supplementation (Figure 9.3). Similarly, MFD was observed upon supplementation (Figure 9.2), suggesting sufficient transfer of *trans*-10, *cis*-12 C18:2 to the mammary gland to induce changes in milk fat. The change in milk fat between the pre-treatment an treatment period was not accompanied with a change in milk yield. Indeed, no differences in milk yield are normally observed between control and treatment in studies with rumen-protected CLA supplements (Gervais *et al.*, 2005; Perfield *et al.*, 2002) or abomasal infusion of CLA (Chouinard *et al.*, 1999; Baumgard *et al.*, 2001). Administration of a commercially available rumen-protected product (LUT) to the animals also resulted in increased proportions of *trans*-10, *cis*-12 C18:2 in milk fat as well as MFD (Figure 9.2 and Figure 9.3). A similar set-up of consecutive control and treatment periods was also recently used to demonstrate the increase in specific milk FA by administering lipid-encapsulated Echium oil (Bainbridge and Kraft, 2016).

Although similar reductions in milk fat concentration were found for both treatments after 5 days of supplementation (Figure 9.2), proportions of *trans*-10, *cis*-12 C18:2 in milk fat generally were lower for the CLA emulsions than for LUT, particularly during the first supplementation days (Figure 9.3). Indeed, differences between both treatments became smaller on the fifth supplementation day. A possible explanation for the delayed increase of *trans*-10, *cis*-12 C18:2 in milk fat in the case of the CLA emulsions, which was also reflected in a delayed MFD (Figure 9.2), might be related to variation in rumen protection among the

different batches of the CLA emulsions. Indeed, batches administered in the beginning of the week showed lower *in vitro* protection efficiencies as compared with the batches given at the end of the week (Figure 9.1A). Remarkably, less protected emulsions showed the highest specific PPO activities in the original extracts, which was also reflected in larger total peak areas in Figure 9.1B. This might suggest increasing specific PPO activity beyond a certain threshold in potato tuber peel extracts could impede the creation of protected emulsions. Further investigation is needed to confirm this apparently negative link between emulsion protection and specific PPO activity.

In this experiment, the choice was made to include a commercially available protected product. Such a comparison is of paramount importance during the development of a new technology, allowing to position the latter against current best practices. Indeed, comparison of both the CLA emulsions as well as LUT with the non-supplemented pre- and post-treatment periods reveals a significant increase in *trans*-10, *cis*-12 C18:2 in milk fat of dairy cows. However, the transfer efficiency from the diet to the milk of *trans*-10, *cis*-12 C18:2 after 5 days of supplementation in this study using protected CLA emulsions remains generally low (4.04 ± 0.35 g/100 g), but results were comparable with the commercially available rumen lipid-encapsulated product (4.81 ± 0.63 g/100 g). Transfer efficiencies of protected CLA in this experiment are in the range of the ones which have been reported before (Table 3.1): 2.4 g/100 g – 7.9 g/100 g for lipid-encapsulated CLA, coated with hydrogenated vegetable fats (Castaneda-Gutierrez *et al.*, 2007b; Moallem *et al.*, 2010; Odens *et al.*, 2007; Pappritz *et al.*, 2011; Perfield *et al.*, 2004; Schwarz *et al.*, 2009), 1.9 g/100 g - 7.2 g/100 g for calcium salts of CLA (de Veth *et al.*, 2005), 7.1 g/100 g for amide-protected (Perfield *et al.*, 2004) and 6.9 g/100 g – 8.6 g/100 g for formaldehyde-protected CLA (de Veth *et al.*, 2005; Gulati *et al.*, 2006c). Furthermore, in previous experiments from our group, daily *in vivo* supplementation of 10 g of unprotected *trans*-10, *cis*-12 C18:2 (with an *in vitro* BH of 0.950; Dehkordi *et al.*, 2008) to dairy cows resulted in proportions in milk fat of 10 mg of *trans*-10, *cis*-12 C18:2 per 100 g FA which corresponds to a transfer efficiency of 1.5 g/100 g and did

not induce MFD (Prof. V. Fievez, unpublished data). Preliminary *in vitro* rumen incubation of *trans*-10, *cis*-12 C18:2 in unprotected Tonalin emulsions revealed a BH for this FA of 0.950 (results not shown). Based on this ruminal BH of *trans*-10, *cis*-12 C18:2 and a predicted transfer of 21.75 g/100 g from the abomasum to the milk (Figure 5 in de Veth *et al.*, 2004), a calculated transfer efficiency of 1.1 g/100 g for an unprotected CLA emulsion was expected, which would probably have resulted in minimal changes in *trans*-10, *cis*-12 C18:2 in milk fat. Concomitantly, no decrease in milk fat yield would be expected too (Figure 4 in de Veth *et al.*, 2004). Comparison with previous experiments in which unprotected FAME of *trans*-10, *cis*-12 C18:2 was supplemented to cows also showed low transfer efficiencies (0.77 g/100 g), when much higher dietary amounts (144 g/d) of *trans*-10, *cis*-12 C18:2 were given (Dohme-Meier and Bee, 2012). Similarly, Hawley *et al.* (2001) demonstrated that the transfer of unprotected CLA into milk fat was less than half of that of protected CLA, but still rather large dietary amounts were used (as cited in de Veth *et al.*, 2004, and Dohme-Meier and Bee, 2012). Comparable experiments with small ruminants which were given relatively large dietary proportions of the same unprotected FAME also resulted in rather small transfer efficiencies of 1.18 g/100 g to 1.68 g/100 g for dairy goats (Baldin *et al.*, 2013; Fernandes *et al.*, 2014) and 3.20 g/100 g for ewes (Oliveira *et al.*, 2012). All of this indicates that the present transfer efficiencies exceed those of unprotected products. However, comparison with studies in which large amounts of dietary *trans*-10, *cis*-12 C18:2 were supplemented is somewhat biased, as transfer efficiencies generally decrease considerably when large amounts of FA are infused into the abomasum or duodenum of dairy cows (Chilliard *et al.*, 2000). Nevertheless, at omasal infusion doses below 10 g/d of *trans*-10, *cis*-12 C18:2 a positive and linear relationship between *trans*-10, *cis*-12 C18:2 in milk fat and abomasal infusions has been observed (de Veth *et al.*, 2004). To our knowledge, however, no studies are published which deal with low dietary levels of unprotected *trans*-10, *cis*-12 C18:2 (i.e. up to 10 g/d) instead of post-ruminal infusions.

4.2 Rumen protection versus overprotection at intestinal level

As only minor amounts of dietary *trans*-10, *cis*-12 C18:2 are transferred to the milk, the question remains what happened with the rest of this FA. *In vitro* BH assessment revealed high protection against ruminal breakdown, but potential overprotection at the level of the small intestine was not assessed *in vitro*. In case of overprotection, CLA isomers were hypothesized to be mainly found in fecal material of the animals, as transfer efficiencies of dietary protected *trans*-10, *cis*-12 C18:2 to peripheral tissues were shown before to be only marginal (von Soosten *et al.*, 2013a). Indeed, in this study increased levels of both dietary *cis*-9, *trans*-11 C18:2 and *trans*-10, *cis*-12 C18:2 were found in feces at the end of the supplementation period for both additives. The highest concentrations of CLA were found for the LUT treatment, although this additive resulted in higher levels of *trans*-10, *cis*-12 C18:2 in milk fat. An increase in fecal *trans*-10, *cis*-12 C18:2 has been observed before in bulls upon addition of dietary rumen protected methyl esters of CLA (Cesaro *et al.*, 2013) and in dairy cows upon addition of a similar supplement as the commercially available product in the current experiment (von Soosten *et al.*, 2013b).

Integration of *in vitro* results, milk fat and fecal *trans*-10, *cis*-12 C18:2 levels of both additives on the one hand suggested protection against rumen BH of CLA emulsions was better than LUT (Figure 9.1A), at least for the emulsions given during the last three supplementation days. However, *trans*-10, *cis*-12 C18:2 transfer from the diet to the milk was lower for the CLA emulsions and an apparent lag time was observed (Figure 9.3). Although this lag time could be related to lower protection efficiencies of emulsion batches administered during the first two days of the experiment, the considerably higher transfer efficiencies of *trans*-10, *cis*-12 C18:2 of LUT are striking. Indeed, *in vitro* protection efficiency of the latter did not exceed that of the least protected CLA emulsions, which had been administered during the first two days of the experiment. Accordingly, combination of *in vitro* results and milk fat *trans*-10, *cis*-12 C18:2 concentrations could be interpreted as more effective protection of the CLA emulsions at the rumen level, but overprotection post-ruminally as FA were less efficiently

transferred to the milk. However, lower recoveries of *trans*-10, *cis*-12 C18:2 in the feces of animals supplemented with CLA emulsions contradict the latter interpretation. Potentially, this discrepancy might have been caused by differences in rumen outflow kinetics of the (solid) commercially available product and the (liquid) CLA emulsion. Indeed, differences in outflow kinetics are not captured during *in vitro* experiments and a 5-day *in vivo* supplementation period could have been too short to obtain steady state conditions.

4.3 Limitations of the current study and recommendations for further investigation

We are fully aware that this first *in vivo* experiment contained some flaws. Indeed, the main objective of this chapter was reached, i.e. to illustrate the potential for rumen BH, post-ruminal release and transfer to the mammary gland of dietary PUFA protected using the PPO technology. The increase in *trans*-10, *cis*-12 C18:2 in milk fat and induction of MFD upon supplementation of PPO-protected CLA emulsions were used as an indicator here. However, these indicators as well as the design of the current experiment show some limitations which have to be taken into account for future investigations. Because of the limitations of the current study, we consider this experiment therefore preliminary.

One of the first limitations of the current *in vivo* trial was related to the control. Indeed, animals were given either the encapsulated test CLA emulsions or a commercially available protected product. Future experiments should also include a negative control, i.e. a non-protected CLA product. In this case, for example, the incorporation of an unprotected CLA emulsion or pure Tonalin oil as treatment could have shown unequivocally that protection was indeed needed to obtain the transfer efficiencies which were now observed.

Another key element for future experiments is related to sample size and treatment period. Now, only 4 animals per treatment were used for only 5 consecutive days. The choice of sample size and length of treatment were in this preliminary experiment mainly related to the fact that it was decided to produce the protected emulsions according to the same

procedures as for previous *in vitro* experiments, rather than to invest time and resources for optimization of a larger scale prototype production. Hence, only small volumes of protected CLA emulsion could be prepared in a reasonable amount of time. Instead of the present design, a more powerful Latin square design should be used too, but then, much more CLA emulsion would be needed, as such experiment would last much longer. Therefore, future research first has to focus on pilot scale production of the PPO-protected emulsions to produce larger volumes. A longer experiment would also allow steady state conditions to be reached, for example after 10 to 14 days of supplementation, in order to deal with possible differences in rumen outflow dynamics, which also might have accounted for the differences between the CLA emulsions and LUT in this experiment.

Further, it should have been better if the ten batches of CLA emulsion were mixed prior to administration. This would have prevented differences in protection efficiencies between batches and, presumably, no delay in increase of *trans*-10, *cis*-12 C18:2 in milk fat or MFD upon administration of CLA emulsions would have been found. However, due to practical limitations, it was not possible to monitor the protection efficiency of all batches prior to supplementation and to mix them to eliminate the impact of variation of individual batches.

Finally, fecal output should have been measured. Now, spot samples were taken to get an idea of the potential overprotection of the administered supplements. Unfortunately, the current set of results of the *in vitro* assessments and milk fat composition on the one side and the fecal output on the other side led to contradictory conclusions on ruminal protection and post-ruminal overprotection of the supplements. Therefore, it is necessary to establish a mass balance to determine the fate of CLA in the cow's body, which requires information on the total amount of fecal output and preferentially also on the extent of rumen protection under *in vivo* conditions. Determination of acid detergent lignin in feed and faeces could have helped to estimate faeces production in the current experiment.

5 Conclusions

The present study confirmed that CLA in an oil emulsified with a PPO rich protein extract of potato tuber peels and treated with 4-MC could be protected against ruminal breakdown *in vitro* and was released from its protected shell during gastro-intestinal passage, absorbed post-ruminally and transferred to the mammary gland. *In vivo* proof of concept for the bioavailability of CLA, protected using the described technology, was given as increasing proportions of *trans*-10, *cis*-12 C18:2 were found in milk fat of cows for both the CLA emulsions as well as the commercially available protected product, which both induced MFD. However, small levels of *trans*-10, *cis*-12 C18:2 in milk fat and the high extent of the same FA in fecal material indicates further research is needed to improve the PPO-based protection technology.

6 Acknowledgements

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PART II C

PROTECTION OF EMULSIFIED FATTY ACIDS:

PROCESS UPSCALING

Chapter 10

First actions towards upscaling of the polyphenol oxidase protection technology

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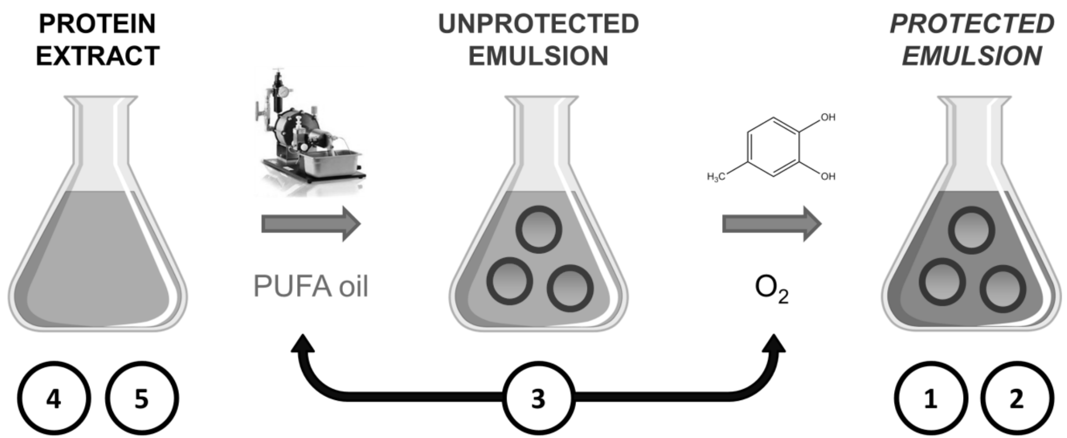

First actions towards upscaling of the protection technology

1. Experiment 1: freeze-drying of emulsions
2. Experiment 2: high-temperature pelletizing
3. Experiment 3: reversed protocol
4. Experiment 4: sidestreams from the potato processing industry (Agristo & Lutosa, BE)
5. Experiment 5: sidestreams from the potato processing industry (Kemin, USA)

Partially redrafted after Gadeyne F., De Neve N., Vlaeminck B., Claeys E., Van der Meeren P. and Fievez V., 2016, Polyphenol oxidase containing sidestreams as emulsifiers of rumen by-pass linseed oil emulsions: interfacial characterization and efficacy of protection against *in vitro* ruminal biohydrogenation, Journal of Agricultural and Food Chemistry, 64, 3749-3759.

Chapter 10

First actions towards upscaling of the polyphenol oxidase protection technology

Abstract. A new encapsulation technology has been proposed based on the properties of the naturally occurring enzyme polyphenol oxidase (PPO). Previous research has demonstrated the potential to protect polyunsaturated fatty acids (PUFA) against rumen biohydrogenation (BH) and the concomitant post-ruminal release, both *in vitro* and *in vivo*. Several experiments were performed to explore the potential upscalability of the protection process. Emulsions created using a PPO-containing protein extract and treated with 4-methylcatechol (4-MC) could be freeze-dried or steam conditioned in order to obtain a dry and easily manageable product, although the protection against ruminal BH was lost to some extent. Further, it was possible to protect emulsions using a reversed protocol, i.e. cross-linking of proteins before emulsification, which is of interest as the “ballast” continuous phase could be re-used. Finally, the possibility to use extracted proteins from industrial potato processing sidestreams has been shown, which creates possible valorization opportunities by upgrading these PPO- and protein-rich sidestreams.

1 Hypothesis and objective

The aim of this chapter was to put the first steps towards upscaling of the protection technology. In the first experiment, it was hypothesized that emulsions could be lyophilized without detrimentally affecting the protection against ruminal BH. Second, emulsions were subjected to steam conditioning to simulate the process of vapor addition and temperature increase during pelletizing and to assess any possible loss of protection. Third, the possibility was assessed to reverse the protocol while maintaining the protection efficiency: i.e., first,

adding a diphenol to the protein extract to create cross-linked protein molecules and only second, emulsifying oil in this cross-linked protein solution. Finally, sidestreams from the potato processing industry were hypothesized to be a useful PPO-rich protein source for the protection protocol.

2 Materials and methods

In all experiments, proteins were extracted using acetone, extracts used to emulsify linseed oil (20 mg per ml protein extract) and emulsions treated with 4-MC to induce protein cross-linking, unless stated otherwise. Methods and measurements of emulsions were as described before in section 2.2 of Chapter 4. Measurements of the protein extracts were as described in section 2.3.1 of Chapter 5, except for experiment 1 and 3a (vide infra), which were assessed as described in section 2.2 of Chapter 4. *In vitro* batch incubations simulating rumen metabolism were performed to evaluate the protection of PUFA against ruminal BH according to the protocol described in section 2.4 of Chapter 4. Fatty acids in incubation fluid were transesterified and analyzed using gas chromatography to assess ruminal BH as described in section 2.5 of Chapter 4. The background, research questions and specific characteristics of the different experiments within this chapter are described in the following subparts.

2.1 Experiment 1: freeze-drying of emulsions

In the first experiment, it was assessed whether a dry protected product could be obtained without loss of protection. Therefore, emulsions (n=2) were made with protein extracts of red clover (*Trifolium pratense* L.) (n=2) and contained a final 4-MC concentration of 0, 12.5, 25 or 50 mM. Comparison was made between fresh emulsions, emulsions which were frozen at -20°C and emulsions which were further freeze-dried.

Results were analyzed with the MIXED procedure of SAS (SAS Enterprise Guide 7, SAS Institute Inc., Cary, North Carolina, USA). Analytical replicates were averaged prior to

statistical analysis. The following model was used: $Y_{ij} = \mu + T_i + D_j + T_i \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, T_i the fixed effect of treatment (i = fresh, frozen or freez-dried emulsions), D_j the fixed effect of diphenol concentration (j = 0, 12.5, 25 or 50 mM 4-MC) and ε_{ij} the residual error. Differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

2.2 Experiment 2: high temperature pelletizing

Compared with freeze-drying of emulsions, a cheaper and more practical application could be to spray emulsions on a carrier material, followed by pelletizing of this mixture in order to obtain a dry and easily manageable product. Therefore, the idea of experiment 2 was to assess the effect of a short and fast rise in temperature in the presence of water vapor on the protection of the emulsified PUFA against ruminal BH, as during the process of pelletizing steam conditioning is performed for a few seconds, resulting in a fast rise in temperature (Skoch *et al.*, 1981).

Emulsions (n=2) were made with protein extracts of potato (*Solanum tuberosum* L.) tuber peels (n=2) and contained 20 mg Tonalin® (TG80, BASF-AG, Ludwigshafen, Germany) per ml of protein extract and had a final 4-MC concentration of 20 mM. Tonalin is a triacylglyceride mixture which mainly contains *cis*-9, *trans*-11 C18:2 and *trans*-10, *cis*-12 C18:2 (see section 2.1 of Chapter 9). For comparative reasons, Lutrell Combi® (LUT; BASF-AG, Ludwigshafen, Germany) was included in the trial as a commercially available rumen-protected product which contains a methyl ester mixture of the same FA (see also section 2.1 of Chapter 9). Next, emulsions were concentrated by centrifugation (see section 3.7 of Chapter 4 - Experiment 7) and the fat fraction was mixed with soybean meal to obtain a solid product including the protected emulsion. This mixture of soybean meal and protected concentrated emulsion was calculated to contain similar levels of *trans*-10, *cis*-12 C18:2 as in LUT.

In order to mimic the increased temperature conditions in the presence of water vapor during pelletizing, 50 g of solid product was weighed and brought into a plastic container with a thermometer. Steam was added during either 10 or 15 seconds. After steam addition, the container was weighed and the added amount of steam was calculated based on the weight difference due to the condensation of the steam into the feed mixture. After steam addition, the product was kept for another 30 seconds in the container. Containers were shaken manually. After this, the feed was brought on a 50 µm sieve and placed in an air cooling system to lower the temperature back to maximally 20°C within two minutes. The 50 µm sieve has been selected because it assures a quick cooling (air passes) without loss of material. The average temperature for the 10 seconds steam treatment was 66°C and moisture increased with 7.9 % (w/w), while the mean temperature for the 15 seconds steam treatment was 82°C and moisture increase was 11.8 % (w/w). These temperatures simulate the minimum and maximum temperatures during feed pelletizing at moderate conditioning, but increases in moisture were higher than in reality under practical conditions (prof. Mia Eeckhout, personal communication; Skoch *et al.* (1981)).

Similar amounts of FA (20 to 24 mg per incubation flask) and *trans*-10, *cis*-12 C18:2 (2.3 to 3.1 mg per incubation flask) across the different treatments were incubated to assess the level of BH and results analyzed with the MIXED procedure of SAS (SAS Enterprise Guide 7, SAS Institute Inc., Cary, North Carolina, USA). Analytical replicates were averaged prior to statistical analysis. The following model was used: $Y_{ij} = \mu + P_i + S_j + P_i \times S_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, P_i the fixed effect of product (i = protected emulsion mixed with soybean meal or LUT), S_j the fixed effect of steam addition (j = 0, 10 or 15 seconds) and ε_{ij} the residual error. Differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

2.3 Experiment 3: reversed protocol

When the general procedure as described in section 2.2 of Chapter 4 is applied, stable emulsions are created before diphenol is added. Results from all previous experiments indicated diphenol addition is required when attempting to create rumen protected emulsions. In this experiment, it was assessed whether the protocol could be reversed: i.e., first, adding diphenol to the protein extract and incubating for 24h at room temperature to create cross-linked protein molecules and only afterwards, emulsifying oil in this cross-linked protein solution. The concept of such a reversed protocol is depicted in Figure 10.1. The potential to use this reversed protocol to create protected emulsions is of interest towards upscaling. Indeed, with the original protocol, a large amount of cross-linked protein is remaining in the continuous phase, which is unneeded to obtain the protective effect, as results described in section 3.7 of Chapter 4 showed no loss of protection when emulsions were ‘washed’, i.e. removing the continuous phase. A successful reversed protocol would allow to re-use the remaining continuous phase.

Experiment 3a was set up using a red clover protein extract (n=1) to compare the effect of cross-linking the protein extract before or after emulsification (n=1). In case of cross-linking after emulsification, the general procedure of section 2.2 of Chapter 4 was applied using increasing concentrations of 4-MC (0, 1.25, 2.5, 5, 10, 20 or 40 mM). On the other hand, to assess the cross-linking before emulsification, i.e. the “reversed protocol”, the protein extract was first cross-linked for 24h at room temperature using the same concentrations of 4-MC. Second, this cross-linked protein solution was used to emulsify 20 mg linseed oil per ml of red clover protein extract.

Further, in experiment 3b a potato tuber peel protein extract was made and the same conditions were applied as in experiment 3a, but using a larger range and higher concentrations of 4-MC (0, 5, 10, 20, 40, 80, 160, 320 or 640 mM) when applying the reversed protocol.

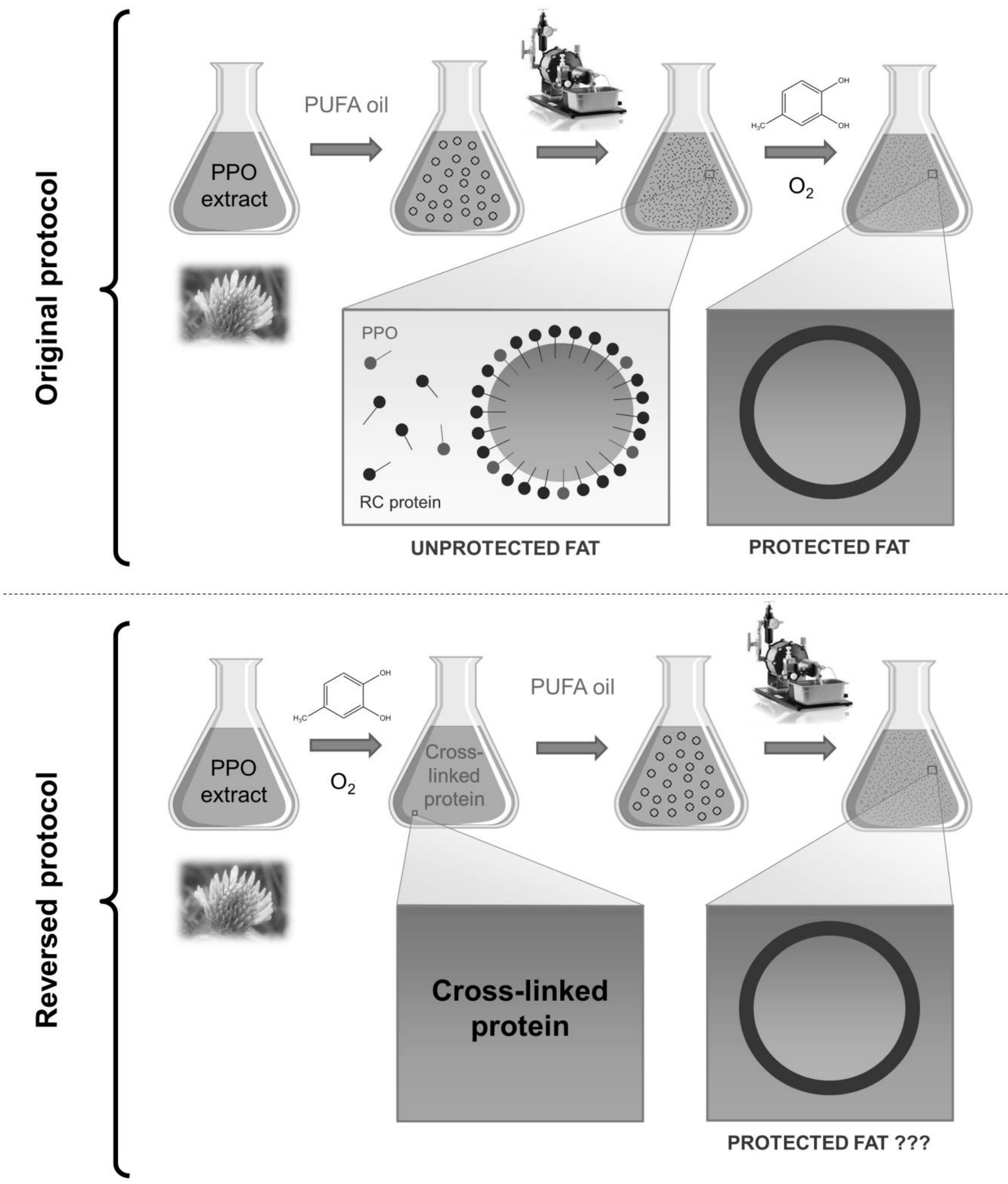

Figure 10.1 Concept of the reversed protocol: first, addition of diphenol to the protein extract and incubating for 24h at room temperature to create cross-linked protein molecules and only second, emulsifying oil in this cross-linked protein solution

2.4 Experiment 4: sidestreams from the potato processing industry (Agristo and Lutosa, BE)

In this experiment, sidestreams of the production process of two potato processing companies were collected. Samples of industrial potato processing sidestreams were kindly offered by Agristo (Harelbeke, Belgium) and Lutosa (Leuze-en-Hainaut, Belgium). Both solid (cutter scraps and slivers) as well as liquid sidestreams after steam peeling were collected, but all samples were taken before any blanching step was performed. Samples from both potato processing facilities were taken at similar places in the production process, kept as replicates throughout the experiment (n=2) and stored at -80°C until further use. Extraction procedures of solid and liquid sidestreams aimed at obtaining protein concentrations of at least 1 mg per ml solution. Therefore, extraction of solid sidestreams was as described before (see section 2.2 of Chapter 4). Volumes of liquid byproducts needed for extraction in 0.1 M sodium phosphate buffer with ascorbic acid were determined based on preliminary analysis of the protein content. Final 4-MC concentrations of emulsions (n=2) were 0 or 20 mM 4-MC.

Results were analyzed with the MIXED procedure of SAS (SAS Enterprise Guide 7, SAS Institute Inc., Cary, North Carolina, USA). Analytical replicates were averaged prior to statistical analysis. The following model was used: $Y_{ij} = \mu + P_i + D_j + P_i \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, P_i the fixed effect of protein source (i = solid or liquid sidestreams), D_j the fixed effect of diphenol concentration (j = 0 or 20 mM 4-MC) and ε_{ij} the residual error. Differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

2.5 Experiment 5: sidestreams from the potato processing industry (Kemin, USA)

As for the previous experiment, sidestreams of the potato processing industry were used as source of protein and PPO. This time, samples were kindly offered by Kemin (Des Moines, Iowa, USA).

Four protein sources were considered in experiment 5a. Liquid sidestreams of one single batch (batch 1) of potatoes were taken at three different locations along the production process and stored at -20°C until further use. Samples were anonymized for confidentiality reasons and further referred to as sidestream 1, 2 or 3. Peels of the raw potatoes used in the production process from the same batch 1 were also incorporated in the experimental set-up. Raw potatoes were stored at 4°C until peeling. Extracts (n=1) from potato tuber peels or sidestreams were diluted to obtain protein concentrations of 1 mg per ml solution, which allowed comparison across protein source. Therefore, extraction of potato tuber peels was as described before (see section 2.2 of Chapter 4) and volumes of liquid sidestreams needed for extraction in 0.1 M sodium phosphate buffer with ascorbic acid and dilution factors were determined based on preliminary analysis of the protein content. Emulsions were made in triplicate and contained final 4-MC concentrations of 0 or 20 mM.

Next, in experiment 5b only a protein extract of sidestream 1 from a second batch (batch 2) was made and the same conditions were applied as in experiment 5a, but with a larger range of 4-MC concentrations (0, 10, 20 or 40 mM).

Results were analyzed with the MIXED procedure of SAS (SAS Enterprise Guide 7, SAS Institute Inc., Cary, North Carolina, USA). Analytical replicates were averaged prior to statistical analysis. The following model was used for experiment 5a: $Y_{ij} = \mu + P_i + D_j + P_i \times D_j + \varepsilon_{ij}$, with Y_{ij} the variable of interest, μ the overall mean, P_i the fixed effect of protein source (i = potato tuber peels, sidestream 1, sidestream 2 or sidestream 3), D_j the fixed effect of diphenol concentration (j = 0 or 20 mM 4-MC) and ε_{ij} the residual error. Only the effect of

diphenol (j = 0, 10, 20 or 40 mM 4-MC) was included in experiment 5b. Differences were assigned at the 0.05 significance level and differences among least squares means were evaluated using Tukey's multiple comparison test.

3 Results and discussion

3.1 Experiment 1: freeze-drying of emulsions

In this first experiment it was examined whether freezing and the subsequent freeze-drying step had a detrimental effect on the protection efficiency against ruminal BH. A red clover extract (n=1) was used and contained 1.62 g protein per liter and had a specific PPO activity of 1052 ΔA/min/mg protein, resulting in emulsions (n=2) with a surface-weighted mean diameter D_{32} of 0.87 µm and a specific surface area SSA of 4.42 m² per g oil. Results for BH of C18:3*n-3* are shown in Figure 10.2. Emulsions showed differences in BH upon supplementation of 4-MC (P<0.001), for the different treatments (P<0.001) or the combined effects (P<0.001). In the case of fresh emulsions, a large drop in BH was observed from 12.5 mM 4-MC onwards (P<0.001), but no further decrease in BH was found for higher 4-MC concentrations (P=1.000). Some loss of protection is observed for emulsions frozen at -20°C compared with fresh emulsions (P<0.001). Levels of frozen and freeze-dried emulsions were similar (P=0.217). Some loss of protection of frozen or freeze-dried emulsions could have been expected, as emulsions generally destabilize upon freezing and thawing (Ghosh and Rousseau, 2009). Indeed, a network of crystalline fat droplets first forms during freezing and second the network collapses and the droplets coalesce during droplet melting (Cramp *et al.*, 2004). Different parameters of freezing and drying as well as the ingredients of the formulation could also affect the stability of freeze-dried emulsions (Morais *et al.*, 2016). This might result in an improved accessibility of PUFA for the ruminal microbes to perform the process of lipolysis followed by BH. Nevertheless, when emulsions contained the lowest concentration of 12.5 mM 4-MC, more than 50 % of *in vitro* protection was found compared with emulsions without 4-MC. Remarkably, the highest concentrations of 4-MC resulted in

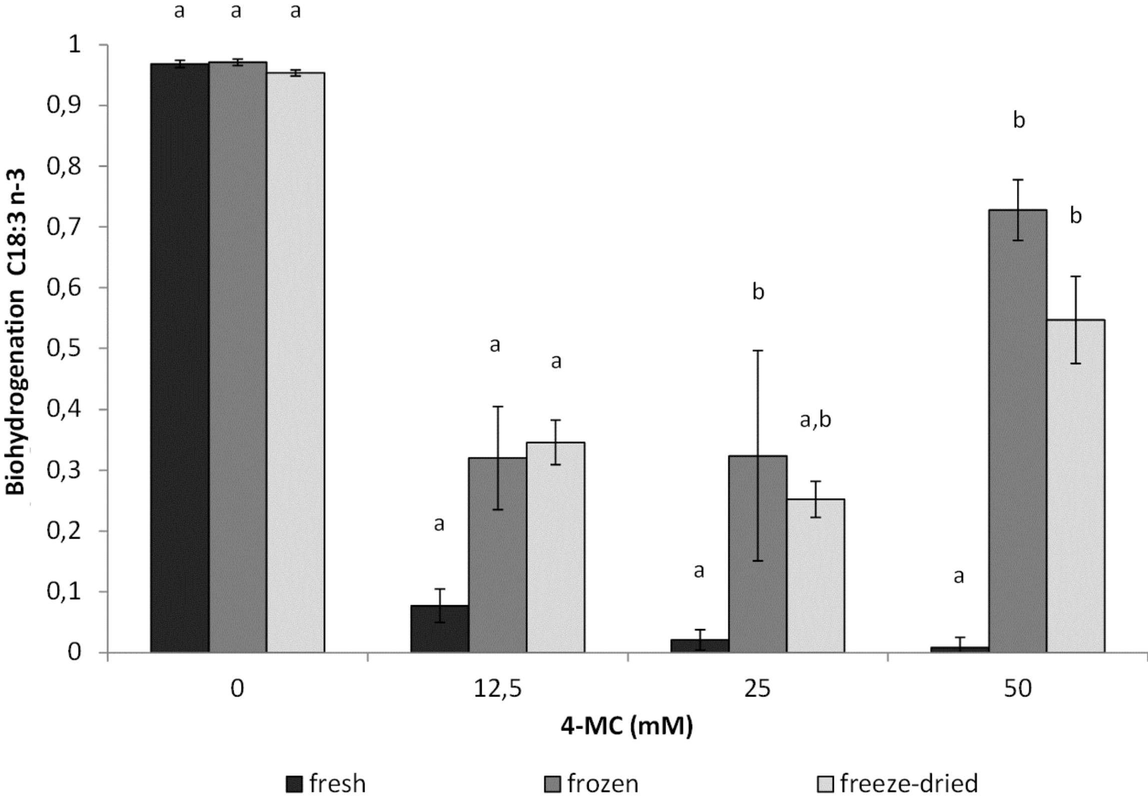

Figure 10.2 Biohydrogenation of C18:3*n-3* in fresh, frozen or freeze-dried emulsions containing 20 mg linseed oil per ml red clover protein extract and increasing concentrations of 4-methylcatechol (4-MC). [a,b] indicates differences in biohydrogenation between fresh, frozen or freeze-dried emulsions within 4-MC concentration at P≤0.05 (experiment 1; error bars represent standard deviations of 6 analytical incubation replicates, i.e. 3 analytical replicates of 2 original emulsions)

the lowest level of protection after freeze-drying, with the 50 mM 4-MC emulsions being more hydrogenated than the ones containing 25 mM 4-MC for both the frozen (P=0.003) and freeze-dried emulsions (P=0.030). Consequently, lyophilizing allowed to produce a more concentrated protected product, but losses of protection compared with fresh emulsions were observed.

3.2 Experiment 2: high temperature pelletizing

In the second experiment it was assessed whether increasing temperature in the presence of water vapor, which occurs during pelletizing, had a detrimental effect on the protection against ruminal BH. A potato tuber peel extract (n=2) was used and contained 4.97 g protein per liter and had a specific PPO activity of 253 nkatal per mg protein, resulting in emulsions

(n=2) with a surface-weighted mean diameter D_{32} of 1.86 µm and a specific surface area SSA of 3.60 m² per g oil. Results for BH of *trans*-10, *cis*-12 C18:2 are shown in Figure 10.3. Ruminal BH differed between the commercial and the emulsified products (P=0.007), was affected by the duration of steam addition (P=0.023), as well as the interaction between both (P=0.033). No differences in BH upon steam conditioning were observed in case of the commercially available protected product, meaning levels of BH were similar with or without the addition of steam. However, in case of emulsions protected by the PPO-based protection method, an increase in BH upon steam addition and the concomitant rise in temperature was observed. Despite the loss of protection upon steam conditioning of the PPO-based protected product, similar levels of protection were found for both products. To our knowledge, no results on the specific effect of steam conditioning on BH of bypass fat supplements has been reported before.

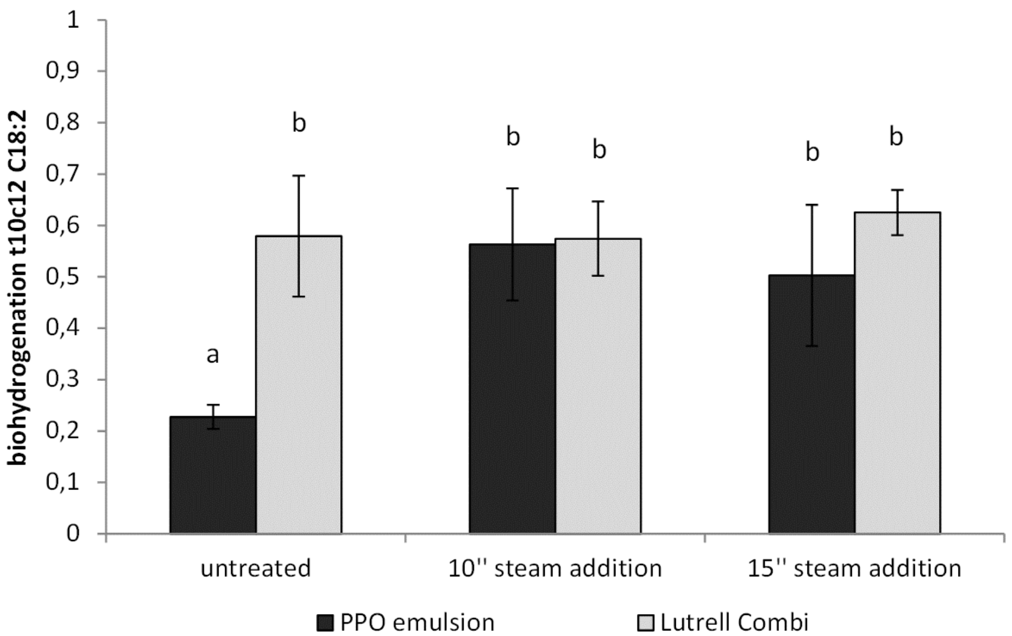

Figure 10.3 Biohydrogenation of *trans*-10, *cis*-12 C18:2 in emulsions containing 20 mg Tonalin oil per ml potato tuber peel protein extract and a final concentration of 20 mM 4-methylcatechol (PPO emulsion) was affected by steam treatment, but this wasn't the case for *trans*-10, *cis*-12 C18:2 in a commercially available protected product (Lutrell Combi). [a,b] indicates differences in biohydrogenation at P≤0.05 (experiment 2; error bars represent standard deviations of 6 analytical incubation replicates, i.e. 3 analytical replicates of 2 original emulsions)

In conclusion, the *in vitro* protection of *trans*-10, *cis*-12 C18:2 in the commercially available product seems to be unaffected by steam conditioning, but this wasn't the case for the PPO-based protected product. Nevertheless, ruminal BH of the untreated commercial product was considerably higher than the PPO emulsion. In case the resistance of the protected emulsion against increased temperature in combination with water vapor could not be improved, spraying of emulsions on a pelleted product could be considered for future applications, as is done for heat-labile compounds such as enzymes or vitamins (Misbah *et al.*, 2014; Lamichhane *et al.*, 2015; Abdollahi *et al.*, 2013).

3.3 Experiment 3: reversed protocol

The third experiment examined whether a reversed protocol could be applied to obtain protected emulsions, whereby cross-linking of proteins is performed before emulsification. The red clover extract (n=1) of experiment 3a contained 1.94 g protein per liter and had a specific PPO activity of 1144 ΔA/min/mg protein, resulting in emulsions (n=1) with a surface-weighted mean diameter D_{32} of 1.73 μm and a specific surface area SSA of 3.72 m² per g oil for the original protocol (cross-linking after emulsification). A D_{32} of 0.59 μm (average across all 4-MC concentrations, with a standard deviation of 0.06 μm) and a SSA of 11.0 m² per g oil (average across all 4-MC concentrations, with a standard deviation of 1.09 m² per g oil) was found for the reversed protocol (cross-linking before emulsification). Results for BH of C18:3*n-3* are shown in Figure 10.4. At low 4-MC concentrations (< 10 mM) only a very slight reduction in BH and concomitant increase in protection was observed upon addition of increasing amounts of 4-MC with both protocols. At higher 4-MC concentrations (≥ 10 mM) a steep decrease in BH was observed for the original protocol, while this was much less with the reversed protocol. Nevertheless, in case of cross-linking before emulsification, protection against BH still gradually increased upon increasing 4-MC, but to a much lower extent than for cross-linking after emulsification. Accordingly, it was hypothesized that the improved protection efficiencies could have been obtained using the reversed protocol when applying higher concentrations of 4-MC.

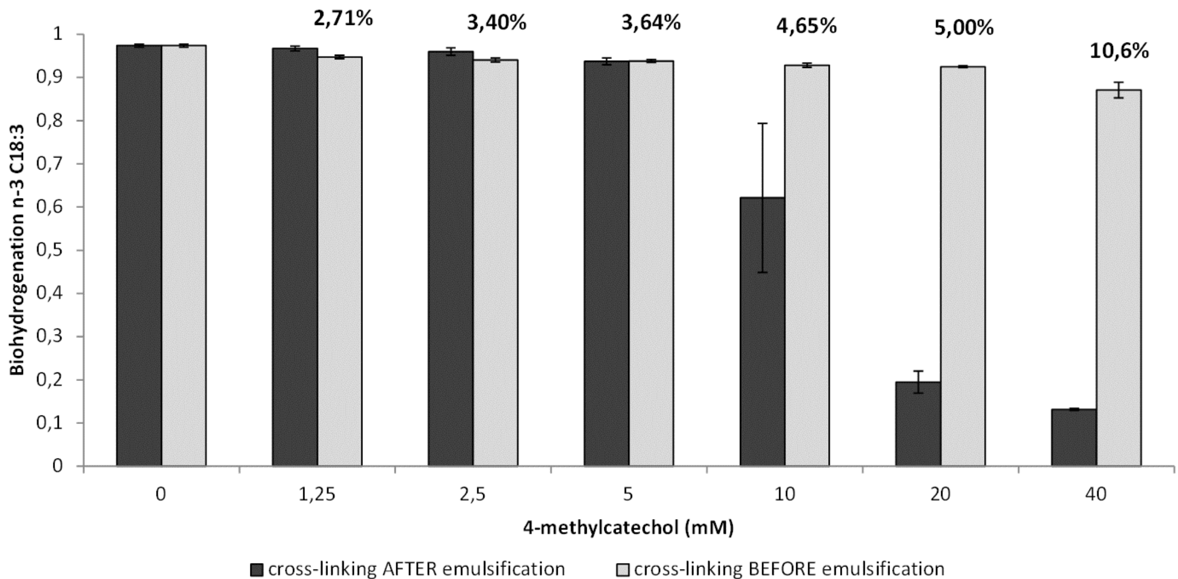

Figure 10.4 A steep decrease in biohydrogenation was observed for emulsions containing 20 g oil per liter red clover protein extract and increasing concentrations of 4-methylcatechol (up to 40 mM) when applying the general protocol (dark grey bars), whereas only a minor decrease was observed when the reversed protocol was applied (light grey bars). Percentages above columns represent the corresponding protection efficiencies of the reversed protocol and error bars represent the standard deviation over three analytical replicates (experiment 3a; 1 original emulsion)

Therefore, experiment 3b was performed, in which 4-MC concentrations ranged up to 640 mM in case of cross-linking before emulsification. The potato tuber peel extract (n=1) of experiment 3b contained 2.60 g protein per liter and had a specific PPO activity of 76.4 nkatal per mg protein, resulting in emulsions (n=1) with a surface-weighted mean diameter D_{32} of 1.56 µm and a specific surface area SSA of 4.03 m² per g oil for the original protocol (cross-linking after emulsification). A D_{32} of 4.54 µm (average across all 4-MC concentrations, with a standard deviation of 2.07 µm) and a SSA of 1.75 m² per g oil (average across all 4-MC concentrations, with a standard deviation of 0.71 m² per g oil) was found for the reversed protocol (cross-linking before emulsification). Results for BH of C18:3*n-3* in Figure 10.5 confirmed the hypothesis that appropriate levels of protection could be reached with the reversed protocol when applying higher levels of 4-MC, in case of 160 and 640 mM. The large extent of BH at 320 mM 4-MC is remarkable, however, this might be related to the incomplete solubilization of 4-MC which was observed in this treatment.

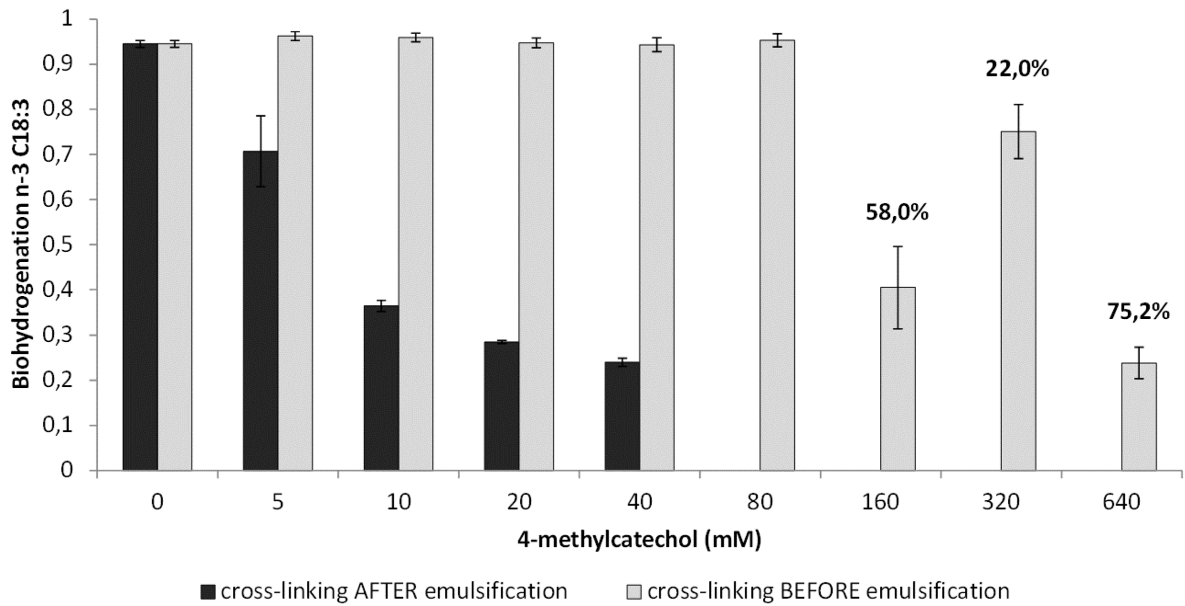

Figure 10.5 A decrease in biohydrogenation was observed for emulsions containing 20 g oil per liter potato tuber peel protein extract and increasing concentrations of 4-methylcatechol when the reversed protocol was applied (light grey bars; up to 640 mM), but at higher concentrations as compared with the general protocol (dark grey bars; up to 40 mM). Percentages above columns represent the corresponding protection efficiencies of the reversed protocol and error bars represent the standard deviation over three analytical replicates (experiment 3b; 1 original emulsion)

In conclusion, it was possible to protect emulsions against BH using a reversed protocol, which gives interesting perspectives towards upscaling, as the ballast continuous phase could potentially be re-used. However, the diphenol concentrations to reach an appropriate level of protection had to be much greater than in the original protocol. Finally, it has to be remarked no statistical analyzes were performed during this experiment due to a lack of statistical replicates, meaning the outcome of these reversed protocol experiments has to be considered preliminary.

3.4 Experiment 4: sidestreams from the potato processing industry (Agristo and Lutosa, BE)

As the potential of potato tuber peels to create protection against BH was shown in Chapter 5, proteins extracted from sidestreams of the potato processing industry were used as emulsifiers. Protein solutions contained 1.39 and 1.50 mg per ml extract for solid and liquid industrial sidestreams, respectively, but low concentrations of protein-bound phenols (< 34.1

µg Tyr-eq/mg protein) and specific PPO activity (< 83.5 nkatal/mg protein) were observed. Ultimately, protected emulsions were created with protein extracts of potato processing sidestreams (with D_{32} equaling 12.1 and 2.12 µm for protein extracts of solid and liquid byproducts, respectively) and 20 mM 4-MC, resulting in protection efficiencies against ruminal BH of 0.110 and 0.557, respectively. Despite these large numerical differences in protection efficiency, protected emulsions created through either of the two sidestreams did not differ (P=0.289). This was particularly due to large differences in protection efficiency between the liquid byproducts, obtained from the two companies (0.258 and 0.856). Besides, proteins extracted from steam peels didn't result in any protection at all (results not shown), most probably because of the inactivation of the PPO enzyme during steam treatment of the potatoes. This means a short heat treatment during steam peeling didn't affect the activity of PPO deeper inside the potato tuber.

Some byproducts from the potato industry are nowadays disposed as a (protein-rich) animal feed resource, particularly solid sidestreams such as steam peelings or slivers, whereas other sidestreams (particularly liquid ones) are discarded, although they still might contain nutritionally valuable molecules. Indeed, results of this experiment confirmed the possibility to use extracted proteins from industrial potato processing sidestreams. Nevertheless, this experiment has to be considered preliminary given the limited number of replicates and large variation between observations using samples from two different companies. Still, this preliminary study shows scope to upgrade sidestreams with the proposed rumen bypass technology, which creates possible valorization opportunities.

3.5 Experiment 5: sidestreams from the potato processing industry (Kemin, USA)

Similar to experiment 4, sidestreams from another potato processing plant were used in order to create protection of PUFA against BH. Extracts (n=1) of the sources from batch 1 used in experiment 5a contained similar protein concentrations after dilution (1 mg/ml), but

differed in specific PPO activity. Extracts of potato tuber peels and sidestream 1 showed a specific PPO activity of 529 and 23.5 nkatal per mg protein, respectively, but no PPO activity was detected in case of sidestream 2 and 3. Low concentrations of protein-bound phenols were found in extracts of potato tuber peels and sidestream 1 (0.156 and 0.181 mg Tyr-eq/mg protein, respectively), but concentrations were much higher in extracts of sidestream 2 and 3 (1.17 and 1.51 mg Tyr-eq/mg protein, respectively). Emulsions (n=3) made with extracts of 1 mg/ml showed a surface-weighted mean diameter D_{32} of 2.54, 2.35, 6.67 and 0.699 µm for potato tuber peels and sidestream 1, 2 and 3, respectively, after addition of sodium dodecyl sulphate to prevent droplet flocculation during measurement. Ruminal BH of C18:3*n-3* (Figure 10.6) differed between protein source (P<0.001), was affected by the addition of diphenol (P=0.002), as well as the interaction between both (P=0.002). BH was reduced only when 4-MC was present in the emulsions and either potato tuber peels or sidestream 1 were used as protein source. Sidestream 2 and 3 did not result in protection

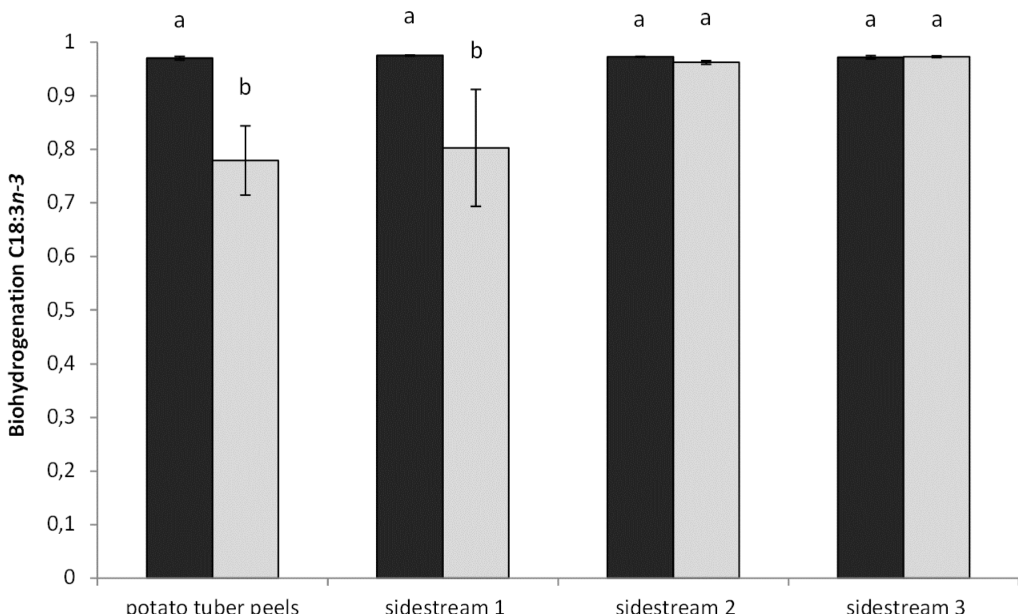

Figure 10.6 Biohydrogenation of C18:3*n-3* in emulsions of experiment 5a containing 20 mg linseed oil per ml protein extract (potato tuber peels or sidestream 1, 2 or 3 from batch 1 in a potato processing facility of Kemin, USA) and containing 0 (dark bars) or 20 mM (light grey bars) of 4-methylcatechol was reduced only when potato tuber peels or sidestream 1 were used. [a,b] indicates differences between treatments at P≤0.05 and error bars represent standard deviations of 3 original emulsions

against BH upon addition of 4-MC. However, protection efficiencies were rather small compared with previous experiments and calculated as 0.197 and 0.177 for potato tuber peels and sidestream 1, respectively. The low protection efficiencies might have been linked with the amount of protein or 4-MC used in the current experiment. Indeed, protein extracts were diluted and only 20 mM 4-MC was used as mediator of cross-linking.

A second experiment 5b was performed to assess the importance of the latter two parameters, i.e. whether high protection could be obtained at higher protein and 4-MC concentrations. This time, a protein extract was made using sidestream 1 from batch 2 only. The extract (n=1) contained 15.0 mg protein per ml extract, 0.488 mg Tyr-eq/mg protein and had a specific PPO activity of 127 nkatal/mg protein, resulting in emulsions (n=3) with a surface-weighted mean diameter D_{32} of 3.34 µm after addition of sodium dodecyl sulphate to prevent droplet flocculation during measurement. Increasing diphenol concentrations resulted in a decrease in BH ($P<0.001$; Figure 10.7) and, concomitantly, protection efficiencies of 0.738, 0.969 and 0.970 for 10, 20 or 40 mM 4-MC, respectively.

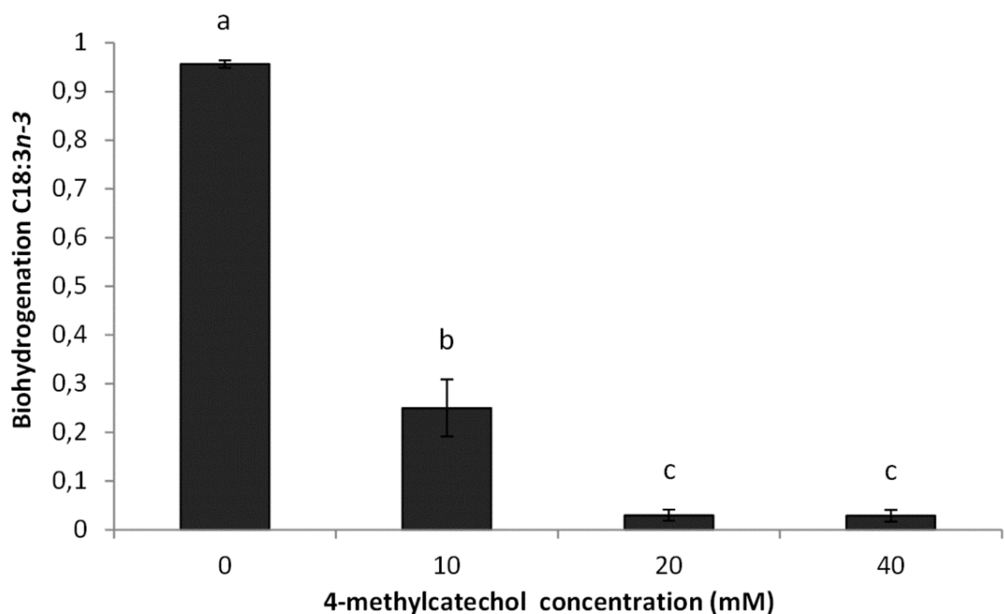

Figure 10.7 Biohydrogenation of C18:3*n-3* in emulsions of experiment 5b containing 20 mg linseed oil per ml protein extract of sidestream 1 from batch 2 in a potato processing facility of Kemin (USA) was reduced upon increasing concentration of 4-methylcatechol. [a,b,c] indicates differences between treatments at $P\leq0.05$ and error bars represent standard deviations of 3 original emulsions

Protection efficiencies at 20 mM 4-MC, found in experiment 5b, were considerably greater than in experiment 5a. This could be related with a higher protein concentration or a higher PPO activity in batch 2 used in experiment 5b compared with batch 1 in experiment 5a or a combination of both factors. As dilution of the protein extract of sidestream 1 from batch 2 to 1 mg/ml resulted in similarly low protection efficiencies as obtained with sidestream 1 from batch 1 (results not shown), the protein concentration is suggested to be the largest contributor to the differences between experiment 5a and 5b. Moreover, it was observed acetone extraction from sidestream 1 was no prerequisite to obtain a protein extract with good emulsifying properties, as also a simple watery extract without prior acetone extraction could be used to prepare protected emulsions (results not shown).

In conclusion, sidestreams from the potato processing industry could be a valid source of PPO and proteins for the PPO-based rumen lipid bypass technology, depending on the sampling location of the sidestream. This illustrates again the potential of upgrading sidestreams by valorizing protein to protect PUFA against ruminal BH.

4 Conclusions

Several experiments were performed as a first action towards the upscaling of the current PPO protection process. PPO-protected emulsions could be freeze-dried or steam conditioned in order to obtain a dry and easily manageable products, but the protection against ruminal BH was lost to some extent. Furthermore, a reversed protocol could be applied which generates perspectives for upscaling as the ballast continuous phase could be re-used. Finally, the valorization potential of the process has been demonstrated, as proteins from industrial potato processing sidestreams could be used to protect PUFA against ruminal BH.

5 Acknowledgements

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GENERAL DISCUSSION

General discussion

Increasing the dietary polyunsaturated fatty acid (PUFA) intake is one of the major challenges to achieve the recommendations of the World Health Organization to obtain a healthier human diet (WHO, 2008). This work aimed at developing a feed technology which could allow to increase the PUFA content in milk and meat of ruminant products, which is challenging due to microbial saturation of dietary PUFA in the rumen, a process called biohydrogenation (BH). A plethora of such protection technologies have been developed over the years to overcome this undesirable BH, but issues of concern are related to safety, cost-effectiveness and degree of protection or overprotection. A new rumen lipid protection technology was proposed and explored throughout this thesis by use of a natural and omnipresent cross-linking enzyme: polyphenol oxidase (PPO). This enzyme is most commonly known for the browning of fruits such as bananas or apples, blackspot bruising of potatoes and the formation of melanin in skin melanocytes or dark hair. For this technique, lipid was emulsified in a plant protein extract, containing the PPO enzyme, and proteins adsorbed at the oil-extract interface were cross-linked upon addition of a diphenol as mediator of the reaction. It is hypothesized that cross-linked protein forms a protective interfacial barrier that prevents ruminal bacteria from hydrolyzing and hydrogenating the emulsified lipids.

Ruminal protection of emulsions and cross-linking hypotheses

A natural protection of plant lipids seems to be present in forages such as red clover. Indeed, fatty acids in conserved red clover seemed to be partially protected against rumen BH, as shown in Chapter 2 of the current thesis and previous experiments (Halmemies-Beauchet-Filleau *et al.*, 2013; Lee, 2014; Van Ranst *et al.*, 2011). This has been related to the high PPO activity of red clover and was postulated to be most likely caused by thylakoid lipid entrapment within a matrix of protein-bound phenols (Lee *et al.*, 2010; Van Ranst *et al.*,

2011). Based on this observation, an encapsulation technology was developed as potential competitor for currently existing and commercially available rumen lipid bypass products. Results from part II A indicate that the proposed protection method, i.e. emulsifying lipids in a PPO-rich protein extract of red clover and treating with a diphenol to cross-link interfacial protein, seems a worthy candidate, as *in vitro* results demonstrated PUFA could be protected against ruminal BH. Four factors seemed to be of importance to protect PUFA-rich oils from microbial metabolism: protection could be obtained with red clover extracts containing PPO (1) and sufficient levels of protein (2), which emulsified relatively low amounts of lipid (3) into stable emulsions with small droplet size in the presence of enough diphenolic mediator (4), in this case 4-methylcatechol (4-MC). Indeed, protection against ruminal BH seemed to be insufficient when too much oil was emulsified, probably because not enough protein as emulsifier was present, but also a certain threshold of 4-MC was needed, most probably to obtain sufficient cross-linking of proteins at the interface of the oil and the aqueous extract. Besides red clover, (parts of) other plants, such as potato tuber peels, cauliflower florets, broccoli wastes or spinach leaves, could also be used to protect PUFA against *in vitro* BH. Some straightforward relationship between oil or 4-MC content and protection was generally observed, i.e. increased oil concentrations lead to lower protection and higher 4-MC concentrations lead to an increased protection. However, this was not the case for PPO activity, as major differences between plants in PPO activity and isoforms did not correlate with the degree of rumen bypass. Gel electrophoretic analysis also suggested that the PPO enzyme did not necessarily had to be part of the emulsion interface. Besides the presence of PPO during the preparation of the bypass emulsions, the origin and concentration of the protein extract seemed to be largely correlated with ruminal protection efficiency. Combination of these observations could imply stable emulsions, with sufficiently small oil droplet size, in combination with 4-MC and some (undefined) level of PPO activity are a prerequisite for protection, but once these conditions are met the protein concentration of the extract is the most determining factor to acquire protection against BH. This might be related to differences in emulsifying properties of proteins or in amino acid profile which might affect

polymerization properties. Further, first tests showed this protocol also allowed protection of PUFA against (pre-ingestive) oxidation during storage, which illustrates a wider application potential of the PPO-based encapsulation technology.

Despite the identification of some key factors for protection as discussed in the previous paragraph, the precise working mechanism remains unknown. Cross-linking of interfacial proteins has been suggested as the major working hypothesis of the proposed PPO protection technology, but experiments of PART II A did not provide definite evidence for this. Nevertheless, all results which are currently described point into the direction of an interfacial cross-linking of the emulsions. Indeed, protection was not obtained, for example, when adding 4-MC to unstable emulsions with high loads of unemulsified oil, if PPO and its substrate 4-MC were not both present or when levels of oil and emulsifying protein were not matched to obtain sufficiently small and stable droplets. Particularly, the fact that the 4-MC treated continuous phase of PPO-rich protein extract-oil emulsions could be removed without losing protection against BH and maintaining similar droplet size distributions supports the plausibility of this cross-linked interface hypothesis.

In order to answer unequivocally the question how protection was obtained, several interfacial cross-linking working mechanisms and influencing factors could be considered. First, the thickness of the interfacial shell might have a major impact on the eventual protection of lipid against degradation. To emulsify, only a thin layer of a few nanometers of protein is needed to cover lipid droplets, in the micrometer diameter range. However, it is unknown whether the thickness of a cross-linked layer is of importance to reach effective protection, as illustrated in Figure D.1A. Possibly, a minimum thickness has to be reached, as protection is obtained after a few hours of exposure to 4-MC of PPO-containing emulsions. Further, the porosity of the cross-linked shell could be of importance too. Rather than the thickness of the interfacial layer, a certain degree of cross-linking and polymerization of proteins as protein-bound phenol complexes might influence the level of protection. Indeed, an interfacial network is probably formed by PPO-induced quinone

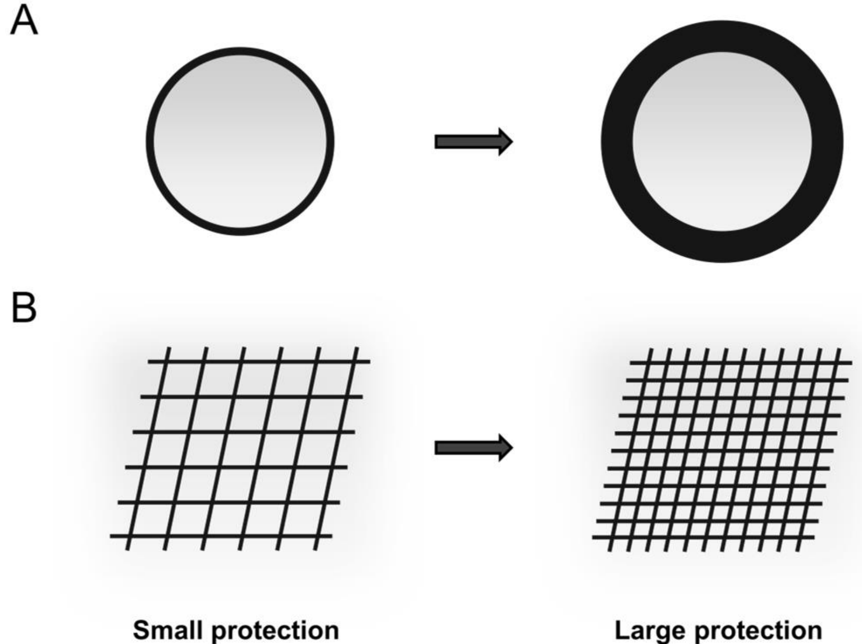

Figure D.1 Illustration of possible influencing factors on interfacial cross-linking and protection

formation, but it remains unclear if appearance of protection is linked with an increased closeness, permeability, rigidity or abundance of cross-linked polymers at the interface of the emulsion. In this perspective, the cross-linked layer could be seen as a fishing net with differing hole sizes (Figure D.1B), allowing molecules such as lipolyzing or hydrogenating enzymes to move inwards or lipids to move outwards below a certain threshold of cross-linking. Related to this, there could be differences in chemical composition, size or number of the protein-phenol polymers, formed due to PPO-induced quinone formation, which might influence the level of protection. As in this study almost exclusively 4-MC was used, and the resulting 4-methylbenzoquinone, the affinity of this quinone for differing nucleophilic compounds in plant proteins might be a factor of interest too. Besides these first factors, the speed of reaction could be seen as a fourth element: to what extent does this formation of cross-linked polymers have to go fast in order to entrap PUFA sufficiently? Finally, it is known that PPO induces the formation of quinones which are responsible for the creation of cross-linked polymers, but it should be elucidated where this quinone formation takes place. Quinones could either be formed in proximity of the interface where they can bind immediately with the adsorbed proteins (1), or they can be formed and react with proteins in

the continuous phase before migrating to the interface where they could displace proteins which were already adsorbed at the interface (2). Indeed, as adsorption of proteins is dynamic, molecules which are more surface-active could displace the ones initially occupying the interface (Dickinson, 1999). Protein-phenol polymers are surface-active, as pre-emulsification formed polymers can also be used to obtain protection with the reversed protocol. In case of competition between (cross-linked) proteins to adsorb at the interface, the speed of displacement should be elucidated too. Moreover, not only the quinones, but also the PPO enzyme itself could be required in proximity of the interface.

With the current set of experiments, the exact working mechanism cannot be elucidated, nor whether a combination of mechanisms and to what extent they are defining rumen protection. New experiments have to be designed to clarify this. Possible experiments and equipment for this sake include the use of several microscopic, spectroscopic or chromatographic techniques to identify differences in porosity or degree of cross-linking of protein-phenol complexes (Chen *et al.*, 2011; Czubinski and Dwiecki, 2017) at interfaces of particles, adsorption on polystyrene beads to assess the thickness of cross-linked protein layers (Mackie *et al.*, 1991; Partanen *et al.*, 2013) or the analysis of adsorption kinetics by the pendant drop technique (Maldonado-Valderrama *et al.*, 2015; Beverung *et al.*, 1999; Macierzanka *et al.*, 2012).

Post-ruminal release and overprotection

Obviously, obtaining protection against ruminal BH is only of interest when post-ruminal overprotection can be prevented, which is another major challenge. Therefore, properties of an efficient rumen lipid bypass product include protection efficiency against breakdown in the rumen as well as efficiency of release from its protective shell and post-ruminal bioavailability. This post-ruminal availability of rumen protected PPO-based emulsions was examined in PART II B, both by means of a mice trial, an *in vitro* method as well as a preliminary *in vivo* trial with dairy cows. The idea of a 'monogastric' mice trial was to

simultaneously assess the release of lipid from (protected) emulsions during gastro-intestinal passage as well as true bioavailability, i.e. absorption into the circulatory system. However, results of the mice trial were inconclusive, as lipids from the protected emulsions did not seem to be fully recovered across sampled tissues. A release from the protective shell was observed with the *in vitro* trial, as triacylglycerides in protected emulsions were not lipolyzed (and PUFA subsequently not hydrogenated) during rumen incubation, but were released as free fatty acids during abomasal and small intestinal incubation. The *in vitro* release was, however, incomplete and some level of post-ruminal overprotection with the highest concentration of 4-MC and the concomitant highest protection against ruminal BH was observed. Although the overall balance of PUFA in the bio-available FFA fraction after a combination of rumen and post-ruminal digestion was still positive, the need remains for further experiments on the cross-linking of interfacial proteins and how this affects the protection or overprotection of emulsions. This was illustrated by the preliminary *in vivo* trial with dairy cows. Indeed, dietary conjugated linoleic acids (CLA) were at least partially protected against ruminal breakdown and at least partially released from its protected shell during intestinal passage, absorbed post-ruminally and transferred to the mammary gland, but the extent of transfer from the diet to the milk was still limited. The latter might be related to the choice of PUFA in this trial, as *trans*-10, *cis*-12 C18:2 is known to be transferred to the milk to a lower extent than for example C18:3*n-3*. The advantage of the use of this PUFA, however, is its complete absence in milk fat under normal (non-rumen-acidotic) conditions, when not supplemented. Because transfer of CLA was not as effective as a commercially available product in the current experiment, there is further need for improvements of this PPO-induced protection protocol. Indeed, transfer efficiencies were still below 5 %, being lower than many scientifically reported transfer efficiencies, meaning much PUFA was lost, either due to BH during ruminal passage or a too high extent of post-ruminal overprotection. Nevertheless, transfer efficiencies from the small intestine to the milk reported in post-ruminal infusion studies maximally reached 22 % for *trans*-10*, cis*-12 C18:2 (de Veth *et al.*, 2004). The transfer of protected PUFA such as linoleic and linolenic acids in milk fat is

potentially much higher, since transfer efficiencies up to 80 % for these FA were observed at low abomasal or intestinal infusion levels (Chilliard et al., 2000). Further, it must be remarked that there were many limitations to the set-up of the current 'preliminary' *in vivo* trial, such as the labscale production of emulsions, the short period of supplementation, a rather small sample size and power, the lack of a negative control, etc. Therefore, before any future *in vivo* trials can be performed pilot scale production of the PPO-protected emulsions should be envisaged. Increased volumes of protected products would make it possible to perform an *in vivo* study with linseed oil, preferably using a more powerful Latin square design, which also guarantees steady state conditions to be reached (e.g. after 10 to 14 days of supplementation) in order to deal with possible differences in rumen outflow dynamics and to incorporate unprotected emulsions as negative control.

Lipids should be released from its protective shell to be bio-available, but the exact mechanism of release is not elucidated yet. Information about the factors mentioned before about interfacial cross-linking working mechanisms, might be of interest to elucidate critical factors to avoid overprotection. Indeed, the thickness, porosity or chemical composition of the cross-linked interface might influence the release from its protective shell. The most logical supposition about post-ruminal release is that the chemical composition of the cross-linked interfacial proteins is changed by proteolytic cleavage of the protein-phenol interface during exposure to pepsin under abomasal or trypsin under intestinal conditions. Because of this alteration in interfacial structure upon post-ruminal digestion, the idea is that the thickness of the shell is reduced, the porosity of the interfacial barrier is increased and/or the interfacial membrane is more permeable compared with the situation under 'protected' ruminal conditions, resulting in an increased bioavailability. It remains also unclear whether a full post-ruminal bioavailability means that the protective shell is completely disrupted or molecules such as enzymes are simply able to move inwards or lipids to move outwards once cross-linked protein-phenol polymers underwent some proteolysis.

Process upscaling

Based on the results of part II A and B, the PPO-based protection technology might become a worthy alternative for currently existing rumen lipid bypass products. The next hurdle which has be taken towards practical application of this protocol is related to process upscaling. Indeed, emulsions which were protected in this thesis only contained minor amounts of oil. For example, in most experiments only 2 % (w/v) of oil was emulsified and encapsulated, meaning 98 % of the emulsion was ballast. Hence, a first factor of interest is increasing the amount of encapsulated and protected oil. Factors which are related to this are the protein content, as emulsifier of the oil, and 4-MC concentration, as mediator of cross-linking. Likewise, the droplet size of the emulsion is of interest, as larger droplets have a smaller droplet surface and concomitantly less interfacial cross-linked protein is needed. Further, a dry and easily manageable product should be obtained, which is preferred under practical conditions instead of a wet emulsion, which is more difficult to store. However, first experiments in PART II C illustrated some loss of protection against ruminal BH by freeze-drying or steam conditioning, the latter simulating the process of vapor addition and temperature increase during pelletizing. Next, related to the large 'ballast' continuous phase which remains in the emulsions, preliminary experiments have shown a reversed protocol could be applied, in which proteins were first cross-linked and only afterwards oil was emulsified. This generates perspectives for upscaling, as the ballast continuous phase could be re-used. Besides these aforementioned factors for upscaling, one of the most promising outcomes of this thesis is the fact that proteins from differing plant resources, including industrial potato processing sidestreams, could be used to protect PUFA against ruminal BH. Indeed, sidestreams from the potato industry, some of which are nowadays disposed as a protein-rich animal feed resource, might still contain other valuable molecules. This creates possible valorization opportunities with the PPO-based rumen bypass technology. A major factor which should still be addressed in this perspective includes a cost-effective extraction and purification of protein from some of these sidestreams.

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ADDENDUM A

Addendum A

Addendum A.1 Composition of the major fatty acids (g per 100 g detected fatty acids) and total fatty acids (mg per g dry material) of fresh and wilted red clover which was undamaged, crushed or frozen/thawed before wilting (n=3)

Stage	Treatment	C16:0 (g/100g FA)	C18:0 (g/100g FA)	C18:1*n-9* (g/100g FA)	C18:2*n-6* (g/100g FA)	C18:3*n-3* (g/100g FA)	Total FA (mg/g DM)
Fresh	-	11.0	2.65	1.67	13.2	65.6	20.8
Wilted	ND	13.9	3.16	2.44	13.7	59.9	15.1
	CR	16.0	3.00	2.47	16.6	57.0	17.2
	FT	19.2	3.47	3.71	18.2	49.9	14.8
SEM (df = 6)		1.03	0.075	0.236	0.94	1.83	2.36
P-value							
Treatment		0.031	0.011	0.014	0.041	0.020	0.737

FA, fatty acid; DM, dry material; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom

Addendum A.2 Composition of the major fatty acids (g per 100 g detected fatty acids) and total fatty acids (mg per g dry material) in red clover which was undamaged, crushed or frozen/thawed before wilting and ensiled with molasses or formic acid as additive for 2, 4, 10 or 60 days (n=3)

Stage	Treatment	C16:0 (g/100g FA)		C18:0 (g/100g FA)		C18:1*n-9* (g/100g FA)		C18:2*n-6* (g/100g FA)		C18:3*n-3* (g/100g FA)		Total FA (mg/g DM)	
		MOL	FOR	MOL	FOR	MOL	FOR	MOL	FOR	MOL	FOR	MOL	FOR
2-days silage	ND	19.2	21.2	4.01	4.42	3.71	3.10	15.3	18.3	51.2	47.5	11.1	13.0
	CR	19.2	20.6	4.02	4.51	3.36	3.64	17.7	18.3	49.8	47.9	11.0	13.8
	FT	20.5	20.3	4.08	3.80	3.65	3.94	18.6	20.0	47.9	46.7	15.1	16.6
4-days silage	ND	18.8	17.8	4.11	3.71	2.94	2.82	15.8	16.2	49.3	50.6	16.0	17.4
	CR	18.2	17.9	4.23	3.49	4.05	2.73	15.4	17.6	49.5	52.3	18.5	16.1
	FT	20.5	21.0	3.99	4.45	3.46	5.65	17.8	19.7	42.8	40.8	16.1	15.9
10-days silage	ND	18.1	19.3	3.72	4.03	3.28	2.80	17.6	18.4	51.6	50.7	15.8	13.6
	CR	18.3	19.7	3.69	4.24	3.26	3.43	17.0	19.0	52.6	49.2	15.3	12.1
	FT	20.8	21.0	3.90	4.30	3.80	4.17	18.8	19.2	48.0	46.7	15.1	13.0
60-days silage	ND	19.1	18.0	4.64	4.15	5.36	3.40	17.7	17.0	48.0	52.9	12.3	12.9
	CR	18.4	19.3	3.94	3.81	3.68	3.73	17.4	18.7	52.0	48.4	11.4	13.7
	FT	22.3	22.8	4.97	4.30	5.34	4.74	20.0	21.5	41.4	41.0	11.2	11.0
SEM (df = 48)		0.64		0.322		0.759		0.71		1.98		0.99	
P-value													
Stage (LC)		0.912		0.471		0.141		0.040		0.715		0.006	
Stage (QC)		0.007		0.107		0.087		0.059		0.330		<0.001	
Additive		0.083		0.946		0.686		<0.001		0.406		0.949	
Treatment		<0.001		0.398		0.091		<0.001		<0.001		0.852	
Additive × Stage (LC)		0.544		0.349		0.366		0.270		0.501		0.190	
Additive × Stage (QC)		0.619		0.443		0.258		0.916		0.829		<0.001	
Treatment × Stage (LC)		0.018		0.201		0.716		0.727		0.157		0.016	
Treatment × Stage (QC)		0.942		0.647		0.360		0.384		0.868		0.033	
Additive × Treatment		0.565		0.959		0.313		0.557		0.664		0.796	
Additive × Treatment × Stage (LC)		0.416		0.904		0.702		0.233		0.215		0.845	
Additive × Treatment × Stage (QC)		0.780		0.209		0.179		0.522		0.664		0.024	

FA, fatty acid; DM, dry material; MOL, molasses; FOR, formic acid; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom; LC, linear contrast; QC, quadratic contrast

Addendum A.3 Composition of the major fatty acids (g per 100 g detected fatty acids) and total fatty acids (mg per g dry material) of fresh, wilted and ensiled undamaged perennial ryegrass with molasses as additive for 4 or 60 days (n=3)

Stage	C16:0 (g/100g FA)	C18:0 (g/100g FA)	C18:1*n-9* (g/100g FA)	C18:2*n-6* (g/100g FA)	C18:3*n-3* (g/100g FA)	Total FA (mg/g DM)
Fresh	14.4	2.26	1.35	12.8	64.2	17.3
Wilted	14.3	2.33	1.25	13.4	64.4	16.2
4-days silage	19.5	3.95	2.81	13.0	54.2	20.4
60-days silage	19.2	2.70	1.61	14.5	58.7	18.4
SEM (df = 8)	0.73	0.728	0.733	0.47	2.39	0.63
P-value						
Stage	0.001	0.381	0.457	0.116	0.048	0.008

FA, fatty acid; DM, dry material; PL, polar lipid fraction; SEM, standard error of the mean; df, degrees of freedom

Addendum A.4 Distribution of total fatty acids in the polar lipid, free fatty acid or neutral lipid fraction (g/100g FA) and biohydrogenation of C18:2*n-6* and C18:3*n-3* after 24h *in vitro* rumen incubation of fresh and wilted red clover which was undamaged, crushed or frozen/thawed before wilting (n=3)

Stage	Treatment	PL (g/100g FA)	FFA (g/100g FA	NL (g/100g FA)	BH C18:2*n-6* (g/g)	BH C18:3*n-3* (g/g)
Fresh	-	80.9	2.90	16.2	0.875	0.802
Wilted	ND	62.7	7.24	30.1	0.893	0.768
	CR	46.2	8.28	45.5	0.884	0.748
	FT	38.2	19.1	42.7	0.904	0.686
SEM (df = 6)		6.25	1.331	6.16	0.0075	0.0091
P-value						
Treatment		0.079	0.002	0.248	0.247	0.002

PL, polar lipid fraction; FFA, free fatty acid fraction; NL, neutral lipid fraction; BH, biohydrogenation; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom

Addendum A.5 Distribution of total fatty acids in the polar lipid, free fatty acid or neutral lipid fraction (g/100g FA) in red clover which was undamaged, crushed or frozen/thawed before wilting and ensiled with molasses or formic acid as additive for 2, 4, 10 or 60 days (n=3)

Stage	Treatment	PL (g/100g FA)		FFA (g/100g FA)		NL (g/100g FA)	
		MOL	FOR	MOL	FOR	MOL	FOR
2-days silage	ND	26.6	24.5	36.3	36.2	37.0	39.3
	CR	18.9	25.6	36.3	31.8	44.8	42.6
	FT	30.8	26.5	37.2	23.9	32.0	49.6
4-days silage	ND	22.8	31.6	50.8	33.0	26.4	35.4
	CR	23.0	29.4	47.7	32.3	29.3	38.3
	FT	25.5	27.2	37.2	28.5	37.3	44.3
10-days silage	ND	20.2	26.2	49.3	39.8	30.5	34.0
	CR	17.2	24.7	50.4	43.1	32.4	32.2
	FT	20.7	23.8	42.2	40.3	37.1	35.9
60-days silage	ND	12.9	21.9	56.8	43.2	30.3	34.9
	CR	15.5	11.0	53.0	46.9	31.5	42.1
	FT	16.0	5.83	49.5	55.0	34.5	39.2
SEM (df = 48)		2.661		2.64		2.72	
P-value							
Stage (LC)		<0.001		<0.001		0.013	
Stage (QC)		0.001		0.376		0.001	
Additive		0.058		<0.001		<0.001	
Treatment		0.184		0.001		0.006	
Additive × Stage (LC)		0.520		0.398		0.664	
Additive × Stage (QC)		0.008		0.043		0.331	
Treatment × Stage (LC)		0.044		0.211		0.885	
Treatment × Stage (QC)		0.701		0.105		0.019	
Additive × Treatment		0.030		0.013		0.558	
Additive × Treatment × Stage (LC)		0.037		0.034		0.075	
Additive × Treatment × Stage (QC)		0.531		0.531		0.053	

PL, polar lipid fraction; FFA, free fatty acid fraction; NL, neutral lipid fraction; MOL, molasses; FOR, formic acid; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom; LC, linear contrast; QC, quadratic contrast

Addendum A.6 Distribution of total fatty acids in the polar lipid, free fatty acid or neutral lipid fraction (g/100g FA) and biohydrogenation of C18:2*n-6* and C18:3*n-3* after 24h *in vitro* rumen incubation of fresh undamaged perennial ryegrass which was wilted or ensiled with molasses as additive for 4 or 60 days (n=3)

Stage	PL (g/100g FA)	FFA (g/100g FA)	NL (g/100g FA)	BH C18:2*n-6* (g/g)	BH C18:3*n-3* (g/g)
Fresh	85.1	3.75	11.2	0.858	0.856
Wilted	76.4	4.76	18.8	0.880	0.826
4-days silage	19.9	39.7	40.4	0.858	0.861
60-days silage	7.13	62.8	30.1	0.798	0.876
SEM (df = 8)	1.488	2.913	1.73	0.0067	0.0105
P-value					
Stage	<0.001	<0.001	<0.001	<0.001	0.061

PL, polar lipid fraction; FFA, free fatty acid fraction; NL, neutral lipid fraction; BH, biohydrogenation; SEM, standard error of the mean; df, degrees of freedom

Addendum A.7 Biohydrogenation of C18:2*n-6* and C18:3*n-3* after 24h *in vitro* rumen incubation of red clover which was undamaged, crushed or frozen/thawed before wilting and ensiled with molasses or formic acid as additive for 4 or 60 days (n=3)

Stage	Treatment	BH C18:2*n-6* (g/g)		BH C18:3*n-3* (g/g)	
		MOL	FOR	MOL	FOR
4-days silage	ND	0.863	0.882	0.717	0.738
	CR	0.878	0.887	0.714	0.740
	FT	0.896	0.902	0.706	0.709
60-days silage	ND	0.857	0.861	0.715	0.685
	CR	0.867	0.861	0.681	0.654
	FT	0.868	0.874	0.622	0.636
SEM (df = 24)		0.0065		0.0094	
P-value					
Stage		<0.001		<0.001	
Additive		0.101		0.855	
Treatment		0.001		<0.001	
Stage x Additive		0.199		0.008	
Stage x Treatment		0.325		0.003	
Additive x Treatment		0.534		0.610	
Stage x Additive x Treatment		0.668		0.039	

BH, biohydrogenation; MOL, molasses; FOR, formic acid; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom

Addendum A.8 Volatile fatty acid production (µmol per incubation flask), proportion of acetic acid, propionic acid and butyric acid (g/100g of total volatile fatty acid production), methane and hydrogen production (µmol per incubation flask) after 24h *in vitro* rumen incubation of fresh and wilted red clover which was undamaged, crushed or frozen/thawed before wilting (n=3)

Stage	Treatment	Total VFA (µmol/flask)	Acetic acid (g/100g total VFA)	Propionic acid (g/100g total VFA)	Butyric acid (g/100g total VFA)	H_2 (µmol/flask)	CH_4 (µmol/flask)
Fresh	-	1052	55.3	31.2	7.53	0.487	391
Wilted	ND	1056	56.2	30.3	6.17	0.466	389
	CR	998.4	56.7	29.5	6.28	0.466	386
	FT	1030	58.8	28.2	7.04	0.456	402
SEM (df = 6)		28.5	1.04	0.87	0.090	0.0263	3.3
P-value							
Treatment		0.410	0.249	0.278	0.001	0.956	0.035

VFA, volatile fatty acid; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom

Addendum A.9 Volatile fatty acid production (μmol per incubation flask), proportion of acetic acid, propionic acid and butyric acid (g/100g of total volatile fatty acid production), methane and hydrogen production (μmol per incubation flask) after 24h *in vitro* rumen incubation of red clover which was undamaged, crushed or frozen/thawed before wilting and ensiled with molasses or formic acid as additive for 4 or 60 days (n=3)

Stage	Additive	Treatment	Total VFA (μmol/flask)	Acetic acid (g/100g total VFA)	Propionic acid (g/100g total VFA)	Butyric acid (g/100g total VFA)	H_2 (μmol/flask)	CH_4 (μmol/flask)
4-days silage	MOL	ND	1011	55.4	31.7	6.16	0.402	353
		CR	990.0	55.4	31.2	6.53	0.408	359
		FT	1031	55.6	30.6	7.43	0.458	385
	FOR	ND	1063	56.6	30.9	6.45	0.417	368
		CR	1035	57.1	29.6	6.41	0.431	384
		FT	1015	57.6	28.8	7.29	0.512	407
60-days silage	MOL	ND	1178	51.1	33.7	7.80	0.655	343
		CR	1184	52.3	32.7	7.52	0.667	343
		FT	1078	54.0	32.1	7.48	0.484	366
	FOR	ND	1126	51.2	34.6	7.78	0.783	311
		CR	1162	51.2	33.8	8.40	0.513	341
		FT	1093	56.2	30.3	7.68	0.499	375
SEM (df = 24)			45.4	0.75	0.53	0.268	0.0504	8.7
P-value								
Stage			<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Additive			0.897	0.027	0.045	0.255	0.678	0.263
Treatment			0.377	<0.001	<0.001	0.100	0.111	<0.001
Stage x Additive			0.380	0.175	0.024	0.278	0.548	0.015
Stage x Treatment			0.332	0.009	0.161	0.003	0.001	0.822
Additive x Treatment			0.977	0.214	0.058	0.651	0.265	0.127
Stage x Additive x Treatment			0.561	0.383	0.196	0.246	0.223	0.408

VFA, volatile fatty acid; MOL, molasses; FOR, formic acid; ND, undamaged; CR, crushed; FT, frozen/thawed; SEM, standard error of the mean; df, degrees of freedom

Addendum A.10 Volatile fatty acid production (μmol per incubation flask), proportion of acetic acid, propionic acid and butyric acid (g/100g of total volatile fatty acid production), methane and hydrogen production (μmol per incubation flask) after 24h *in vitro* rumen incubation of wilted and ensiled undamaged perennial ryegrass with molasses as additive for 4 or 60 days (n=3)

Stage	Total VFA (μmol/flask)	Acetic acid (g/100g total VFA)	Propionic acid (g/100g total VFA)	Butyric acid (g/100g total VFA)	H_2 (μmol/flask)	CH_4 (μmol/flask)
Fresh	1153	54.2	32.4	9.41	0.460	396
Wilted	1105	53.0	33.3	8.65	0.496	397
4-days silage	1304	54.5	32.9	7.81	0.423	379
60-days silage	1369	51.7	36.2	7.21	0.530	381
SEM (df = 8)	52.9	0.93	0.68	0.221	0.0290	7.5
P-value						
Stage	0.024	0.201	0.018	<0.001	0.130	0.260

VFA, volatile fatty acid; SEM, standard error of the mean; df, degrees of freedom

ADDENDUM B

Addendum B

Addendum B.1 Biohydrogenation of C18:2*n-6* and C18:3*n-3*, protection efficiency of C18:3*n-3* and formation of C18:0 after 24h *in vitro* ruminal incubation of emulsions containing 20 mg per ml protein extract of thirteen different plant sources and having a final diphenol concentration of 0, 10, 20 or 40 mM 4-methylcatechol (experiment 1; n=3)

protein source	4-MC	BH C18:2*n-6* *g/g*		BH C18:3*n-3* *g/g*		PE C18:3*n-3* *-/-*		Formation C18:0 *g/g*	
		AVG	SD	AVG	SD	AVG	SD	AVG	SD
apple (peel)	0	0.925	0.029	0.966	0.014	-	-	0.418	0.345
	10	0.912	0.040	0.960	0.016	0.007	0.006	0.454	0.350
	20	0.907	0.035	0.951	0.019	0.016	0.015	0.579	0.241
	40	0.899	0.030	0.941	0.028	0.026	0.030	0.477	0.238
artichoke (flower leaf)	0	0.897	0.020	0.932	0.019	-	-	0.482	0.237
	10	0.829	0.039	0.858	0.035	0.079	0.023	0.743	0.176
	20	0.770	0.026	0.802	0.019	0.139	0.024	0.797	0.190
	40	0.474	0.177	0.493	0.193	0.468	0.214	0.906	0.096
broccoli (stem)	0	0.934	0.018	0.972	0.008	-	-	0.497	0.147
	10	0.921	0.031	0.950	0.029	0.022	0.023	0.631	0.145
	20	0.765	0.156	0.805	0.152	0.173	0.151	0.714	0.163
	40	0.213	0.170	0.236	0.169	0.758	0.171	0.898	0.150
carrot (peel)	0	0.940	0.009	0.974	0.009	-	-	0.415	0.185
	10	0.935	0.017	0.968	0.012	0.007	0.008	0.445	0.177
	20	0.924	0.017	0.960	0.015	0.015	0.010	0.520	0.178
	40	0.867	0.074	0.901	0.070	0.075	0.069	0.452	0.138
cauliflower (floret)	0	0.920	0.013	0.957	0.006	-	-	0.56	0.126
	10	0.841	0.041	0.873	0.037	0.089	0.034	0.778	0.166
	20	0.401	0.050	0.436	0.062	0.544	0.063	0.890	0.130
	40	0.093	0.015	0.106	0.012	0.889	0.013	1.05	0.077
cauliflower (stem)	0	0.935	0.013	0.970	0.004	-	-	0.539	0.160
	10	0.914	0.043	0.946	0.039	0.025	0.036	0.598	0.133
	20	0.739	0.245	0.771	0.244	0.206	0.249	0.710	0.144
	40	0.276	0.151	0.308	0.161	0.683	0.165	0.879	0.109
cauliflower (leaf)	0	0.924	0.014	0.965	0.012	-	-	0.649	0.260
	10	0.924	0.010	0.961	0.006	0.004	0.012	0.698	0.181
	20	0.770	0.165	0.806	0.166	0.166	0.165	0.751	0.226
	40	0.298	0.082	0.332	0.090	0.657	0.091	0.950	0.142

4-MC, 4-methylcatechol; BH, biohydrogenation; PE, rumen protection efficiency; AVG, average; SD, standard deviation

Addendum B.1 Continued

protein source	4-MC	BH C18:2*n-6* *g/g*		BH C18:3*n-3* *g/g*		PE C18:3*n-3* *-/-*		Formation C18:0 *g/g*	
		AVG	SD	AVG	SD	AVG	SD	AVG	SD
eggplant (pulp)	0	0.925	0.010	0.968	0.006	-	-	0.310	0.161
	10	0.924	0.006	0.966	0.011	0.003	0.006	0.452	0.221
	20	0.908	0.013	0.954	0.013	0.015	0.011	0.655	0.193
	40	0.782	0.058	0.901	0.029	0.070	0.027	0.851	0.151
pineapple (peel)	0	0.838	0.040	0.959	0.010	-	-	0.410	0.222
	10	0.902	0.025	0.967	0.011	0.000	0.015	0.290	0.229
	20	0.878	0.010	0.959	0.010	0.000	0.016	0.285	0.241
	40	0.885	0.019	0.953	0.014	0.006	0.016	0.239	0.227
potato tuber (peel)	0	0.915	0.029	0.963	0.013	-	-	0.419	0.321
	10	0.330	0.025	0.340	0.030	0.646	0.035	0.957	0.186
	20	0.250	0.027	0.249	0.030	0.741	0.034	0.932	0.252
	40	0.182	0.049	0.175	0.052	0.817	0.056	0.954	0.341
red clover (stem & leaf)	0	0.925	0.013	0.966	0.006	-	-	0.375	0.161
	10	0.727	0.204	0.770	0.192	0.202	0.202	0.705	0.141
	20	0.605	0.336	0.639	0.344	0.337	0.358	0.755	0.298
	40	0.395	0.230	0.438	0.245	0.545	0.256	0.741	0.344
spinach (leaf)	0	0.913	0.021	0.958	0.009	-	-	0.583	0.280
	10	0.851	0.082	0.882	0.075	0.079	0.079	0.724	0.161
	20	0.529	0.230	0.561	0.238	0.416	0.244	0.842	0.091
	40	0.116	0.036	0.127	0.044	0.868	0.045	1.010	0.109
tomato (stem & leaf)	0	0.926	0.008	0.970	0.008	-	-	0.741	0.277
	10	0.800	0.169	0.840	0.162	0.134	0.170	0.835	0.169
	20	0.661	0.380	0.683	0.401	0.295	0.415	0.884	0.215
	40	0.577	0.365	0.601	0.392	0.380	0.404	0.787	0.297
P-value									
Protein source		<0.001		<0.001		<0.001		<0.001	
4-MC		<0.001		<0.001		<0.001		<0.001	
Protein source x 4-MC		<0.001		<0.001		<0.001		0.571	

4-MC, 4-methylcatechol; BH, biohydrogenation; PE, rumen protection efficiency; AVG, average; SD, standard deviation

CURRICULUM VITAE

Curriculum vitae

Brief resume

Frederik Gadeyne was born in Torhout (Belgium) on January 19th 1988. He graduated with distinction as a Master of Science in Bioscience Engineering: Agriculture from Ghent University in 2011. He performed his master thesis at the Laboratory for Animal Nutrition and Animal Product Quality under the guidance of prof. dr. ir. Veerle Fievez and investigated the potential of milk odd and branched chain fatty acids as biomarkers to mitigate nitrogen emissions from dairy cows. After his studies, he started his PhD as teaching assistant at the same laboratory of Ghent University. During these six years, he performed research on the protection of polyunsaturated fatty acids against ruminal biohydrogenation by use of emulsions and the naturally occurring and omnipresent browning enzyme polyphenol oxidase. He presented his work on several international conferences, both through oral and poster presentations. Frederik is author and co-author of multiple international peer-reviewed papers and is co-inventor of one patent application. He assisted in practical exercises and theoretical lectures of several national and international courses and supervised the bachelor's and master's theses of multiple students.

Education

2011 – 2017 PhD student (teaching assistant)

Laboratory for Animal Nutrition and Animal Product Quality

Department of Animal Production, Faculty of Bioscience Engineering

Ghent University, Ghent, Belgium

PhD thesis: Protection of polyunsaturated fatty acids against ruminal biohydrogenation using polyphenol oxidase

Promotor: Prof. dr. ir. Veerle Fievez

2006 – 2011 Master of Science in Bioscience Engineering: Agriculture

Ghent University, Ghent, Belgium

MSc thesis: Biomarkers in the milk: supporting instruments for the mitigation of nitrogen emissions from dairy cows?

Promotor: Prof. dr. ir. Veerle Fievez

2000 – 2006 Latin-Mathematics (8h)

Sint-Jozefsinstituut College, Torhout, Belgium

Scientific publications (Web of Science indexed)

Gadeyne F., De Neve N., Vlaeminck B., Van der Meeren P. and Fievez V., 2017, *In vivo* bioavailability of conjugated linoleic acid in dairy cows supplemented with rumen bypass emulsions created by polyphenol oxidase rich protein extracts from potato tuber peels. *[submitted to Animal Feed Science and Technology]*

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Conference contributions

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Patent applications

Fievez V., Gadeyne F. and Van Ranst G., 2015, Method to protect lipophilic nutrients against ruminal degradation, International Publication Number: WO 2015091840 A1 (also

published as US 20160316791 A1, CA 2934427 A1, EP 3091849 A1 and CN 106028830 A).

Doctoral schools courses

2016 - PhD Career Focus (transferable skills, cluster Career Management)
- Fostering Responsible Conduct of Research (transferable skills, cluster Research and Valorization)

2015 - 7th Training School on Microencapsulation (specialist course, Bioencapsulation Research Group, Strasbourg, France)

2013 - Populair wetenschappelijk schrijven (transferable skills, cluster Communication Skills)
- Basisassistententraining (transferable skills, cluster Career Management)

2012 - Colloïd- en oppervlakchemie (specialist course, Ghent University)
- Fatty acids in dairy cattle in relation to product quality and health (specialist course, Ghent University)

2011 - Sustainable Animal Husbandry (specialist course, Ghent University)

National and international experience

2017 - Facilitator of a Short Training Program in Dairy Nutrition
Mountains of the Moon University, Fort Portal, Uganda

2011 – 2017 - Involved in the exercises and practicals of the courses Animal Nutrition, Animal Production Biology, Organic Farming, Sustainable Animal Husbandry and Tropical Feed Resources (Ghent University)

2013 – 2016 - Involved in the exercises, practicals and contact sessions of the International Training Program in Dairy Nutrition (Ghent University)

2010 - Internship at Nutrition Sciences (part of the Nuscience Group)
Drongen, Belgium
Topic: Feed additives for the control of Salmonella: *in vitro* gastrointestinal simulation and *in vivo* assays
- Stay at the Friedrich Loeffler Institut for *in vivo* sampling for MSc thesis
Braunschweig, Germany

Supervision of bachelor and master dissertations and internships

Claes S., 2016, Laboratory internship at the Laboratory for Animal Nutrition and Animal Product Quality, student of the Erasmus University College of Brussels. Supervisors: ir. Frederik Gadeyne and dr. ir. Joris Michiels

Delva J., 2016, *In vivo* evaluatie van de pensbestendigheid van een geëmulsifieerde poly-onverzadigde vetzuurbron gestabiliseerd met behulp van polyfenoloxidase. Thesis to obtain the degree of MSc in Biosciences: Agriculture and Horticulture. Promotor: prof. dr. ir. Veerle Fievez

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SUMMARY – SAMENVATTING

Summary

Increasing the polyunsaturated fatty acid (PUFA) content of food products is an important aspect to obtain a healthy diet. PUFA are mainly ingested through plant or fish products, as terrestrial animal products contain rather poor levels of these healthy compounds. Improving the PUFA content of monogastric products such as pork meat, chicken meat or eggs can be achieved relatively easily by increasing their level of ingested PUFA-rich lipids. However, increasing PUFA in ruminant products such as beef or milk is much more challenging. Ruminants have the unique ability to digest fibrous rich materials such as grass due to the presence of a wide range of microorganisms in the rumen. However, a consequence of this symbiosis is the biohydrogenation (BH) of PUFA by ruminal bacteria, which limits its transfer from the diet to the milk or peripheral tissues. Therefore, the addition of PUFA-rich feeds or oils as a nutritional strategy to increase their bioavailability for the animal is hampered. Hence, this work focused on the protection of PUFA against this undesirable BH using a natural and omnipresent enzyme: polyphenol oxidase (PPO).

The objectives of this thesis were twofold. In the first (minor) part, the natural PPO-induced protection of PUFA against BH in roughages was further explored (PART I). Several experiments have showed before a reduction in BH of PUFA and increase in milk PUFA levels when including conserved red clover in the diet. This has been attributed to the PPO activity in red clover. In this thesis, it was assessed whether BH of PUFA in red clover was linked with conservation of polar lipids (PL) in (conserved) material (Chapter 2), as lipolysis is a prerequisite for BH. Remarkably, red clover PUFA seemed to be partially protected against ruminal BH, while disappearance of fatty acids from the PL fraction did not seem to be hampered. Further, microbial lipases in silages did not enhance FA disappearance from the PL fraction. Based on current and previously reported results and as postulated before, it was concluded that an encapsulation mechanism because of protein-bound phenol

formation, being induced by PPO, seems the be most probable hypothesis to explain red clover's increased flow of PUFA beyond the rumen.

Based on the observations of red clover PPO to protect forage PUFA, the objective of the remainder of this thesis (PART II) was to assess the protection of emulsified lipids as a new methodology to protect non-roughage PUFA in oils against BH, through the action of PPO. Indeed, numerous protection technologies have already been developed to overcome this undesirable BH, but research is still ongoing to develop new ways of rumen protection of dietary lipids and to extend the plurality of rumen bypass methods that currently exist. A review of currently existing rumen lipid bypass techniques is given (Chapter 3), illustrating the indistinctness about which technique is best. As possible alternative, a new rumen lipid protection technology was proposed and explored throughout this thesis by use of the cross-linking enzyme PPO. With this technique, lipids are emulsified in a plant protein extract, containing the PPO enzyme. As such, proteins which are adsorbed at the oil-extract interface are cross-linked upon addition of a diphenol as mediator of the reaction. It was hypothesized cross-linked protein forms a protective interfacial barrier that withholds ruminal bacteria to hydrolyze and hydrogenate the emulsified lipids.

The protection of PUFA using this new PPO-based rumen bypass technology was explored in part II A. *In vitro* results confirmed the hypothesis that PPO-rich protein extracts of red clover could prevent emulsified PUFA from ruminal BH through addition of the diphenol 4-methylcatechol (4-MC) (Chapter 4). Protection efficiency depended on both the amount of oil and protein in the emulsions and increased with increasing amounts of diphenol in the emulsion per unit interfacial surface area. Besides red clover, it was also possible to obtain protection against ruminal BH using protein extracts of (parts of) various other plant resources, such as cauliflower florets, spinach leaves, broccoli waste and potato tuber peels (Chapter 5). Differences in PPO activity between plant resources were also not correlated to the degree of rumen bypass. Moreover, PPO isoforms in vegetal sources, effectively protecting emulsified lipids, were diverse and their presence at the emulsion interface did not

seem essential. On the other hand, the origin and concentration of the protein extract seemed to determine to a large extent the ruminal protection efficiency. Combination of all these observations could imply stable emulsions, with sufficiently small oil droplet size, in combination with 4-MC and some (undefined) level of PPO activity are a prerequisite for protection. However, once these conditions are met the protein concentration of the extract is the most determining factor to acquire protection against BH. Furthermore, the hypothesis was made that the same process responsible for protection of PUFA against ruminal BH might also provide protection against pre-ruminal processes (Chapter 6). It was demonstrated that the PPO technology could simultaneously protect PUFA against ruminal BH as well as oxidation during storage and that the pre-ingestive oxidation of other co-emulsified lipophilic compounds such as vitamin E is hampered upon addition of 4-MC, which illustrates the wide application potential of the PPO-based encapsulation technology.

Post-ruminal availability of rumen protected PPO-based emulsions was assessed in PART II B. Indeed, rumen protected PUFA have to be liberated again during post-ruminal passage through the gastro-intestinal tract, otherwise the added value of protection is meaningless. Post-ruminal availability was first assessed by means of a mice trial (Chapter 7). The idea of a 'monogastric' mice trial was to simultaneously assess the release of lipid from (protected) emulsions during gastro-intestinal passage as well as true bioavailability, i.e. absorption into the circulatory system. However, results of the mice trial were inconclusive, as lipids from the protected emulsions did not seem to be fully recovered across sampled tissues. As an alternative to assess the release from the protective shell, an *in vitro* approach was set up (Chapter 8). Triacylgylcerols in protected emulsions were not lipolyzed (and PUFA subsequently not hydrogenated) during rumen incubation, but were recovered as free fatty acids after abomasal and small intestinal incubation. This was not the case for unprotected emulsions, which were hydrolyzed and hydrogenated to a large extent during the rumen simulation. However, the post-ruminal *in vitro* release from protected lipids was incomplete and there was some risk of post-ruminal overprotection at the highest levels of rumen

protection. Ultimately, proof of concept for the transfer of dietary PPO-protected PUFA to milk was given by a preliminary trial with dairy cows (Chapter 9). The experiment confirmed that dietary conjugated linoleic acids (CLA) in an oil emulsified with a PPO containing protein extract of potato tuber peels and treated with 4-MC could both be protected against ruminal breakdown *in vitro* and released from its protected shell during gastro-intestinal passage, absorbed post-ruminally and transferred to the mammary gland *in vivo*, as increasing proportions of *trans*-10, *cis*-12 CLA were found in milk fat, which induced milk fat depression.

Finally, first steps towards upscaling of the PPO-based protection technology were taken in PART II C (Chapter 10). It was found emulsions could be freeze-dried or steam conditioned, the latter simulating the process of vapor addition and temperature increase during pelletizing, which is helpful to obtain a dry and easily manageable product, but protection against BH was lost to some extent. Also a reversed protocol could be applied, which generates perspectives for upscaling as the 'ballast' continuous phase could potentially be re-used. However, one of the most promising outcomes of this thesis is the fact that proteins from various plant resources, including sidestreams of industrial potato processing plants, could be used to protect PUFA against ruminal BH. This creates valorization opportunities with the PPO-based rumen bypass technology.

In conclusion, a new rumen bypass technology was developed to protect lipids from the undesired turnover of dietary unsaturated 'healthy' fatty acids to more saturated 'unhealthy' forms in ruminants. Protection was made by use of emulsions and the naturally occurring and omnipresent browning enzyme polyphenol oxidase. This enzyme could be extracted from a wide variety of plant resources, including potato tuber peels. Results from this thesis demonstrate unsaturated fatty acids could be protected against ruminal breakdown and released post-ruminally from their protected shell, and to some extent transferred to the mammary gland. Healthier milk and meat could be obtained by this technology, whereby protein-rich sidestreams containing this enzyme can be upgraded and valorized.

Samenvatting

De verhoging van poly-onverzadigde vetzuur (POVZ) gehaltes in voedingsmiddelen is een belangrijk aspect voor het verkrijgen van gezondere voeding. POVZ worden in het menselijke dieet voornamelijk opgenomen door het consumeren van plantaardige en visproducten. Producten van landdieren bevatten immers slechts lage niveaus van deze gezonde stoffen. Het verhogen van POVZ in producten van éénmagigen, zoals varkensvlees, kippenvlees of eieren, kan relatief eenvoudig bereikt worden door het voederen van producten rijk aan POVZ, zoals lijnzaadolie. Het aanrijken van POVZ in producten van herkauwers, zoals rundvlees of melk, is daarentegen een veel grotere uitdaging. Herkauwers hebben dan wel het unieke vermogen om vezelrijke materialen zoals gras te verteren door de aanwezigheid van een groot aantal micro-organismen in de pens, een gevolg van deze symbiose is de biohydrogenatie (BH) van POVZ door ruminale bacteriën. Dit proces beperkt de transfer van deze gezonde vetzuren uit het rantsoen naar de melk of het vlees. De mogelijkheid om POVZ-rijke voeders of oliën te voederen aan herkauwers als strategie om de biobeschikbaarheid voor het dier te verhogen is daardoor gelimiteerd. Dit proefschrift onderzocht de bescherming van POVZ tegen deze ongewenste hydrogenering met behulp van een natuurlijk en alomtegenwoordig enzym: polyfenol oxidase (PPO).

De doelstellingen van dit onderzoek waren tweedelig. In het eerste (kleinere) deel werd de natuurlijke bescherming van POVZ in ruwvoeders tegen BH door PPO verder onderzocht (Deel I). Verschillende experimenten toonden eerder een verminderde BH van POVZ en verhoogde POVZ in de melk aan wanneer (gedroogde of ingekuilde) rode klaver deel uitmaakte van het rantsoen. Dit werd voornamelijk toegeschreven aan de hoge PPO activiteit in rode klaver. In dit proefschrift werd nagegaan of BH van POVZ in rode klaver gelinkt was aan de polaire lipide gehaltes in gedroogd of ingekuild materiaal (Hoofdstuk 2). Aangezien

lipolyse een voorwaarde is voor BH, werd verondersteld dat BH versterkt zou worden als het ruwvoeder lagere gehaltes aan veresterde vetzuren bevatte. POVZ in rode klaver bleek opmerkelijk genoeg wel gedeeltelijk beschermd tegen penshydrogenatie, maar het verdwijnen van vetzuren uit de polaire lipide fractie leek niet te worden belemmerd. Verder bleken microbiële lipasen in kuilen geen invloed te hebben op de verdwijning van vetzuren uit de polaire lipide klasse. Gebaseerd op deze en eerder gerapporteerde resultaten werd, zoals reeds eerder aangenomen, geconcludeerd dat een inkapselingsmechanisme door vorming van eiwitgebonden fenolen, geïnduceerd door PPO, de meest waarschijnlijke hypothese lijkt om de relatief hoge uitstroom van PUFA uit de pens bij het voederen van rode klaver te verklaren.

Op basis van deze waarnemingen in rode klaver was het doel van de rest van dit proefschrift (Deel II) om de bescherming te beoordelen van geëmulgeerde oliën als een nieuwe manier om POVZ te beschermen tegen BH op basis van de werking van het PPO enzym. Talrijke beschermingstechnologieën werden vroeger reeds ontwikkeld om deze ongewenste BH te verhinderen, maar onderzoek is nog steeds aan de gang om nieuwe manieren van pensbescherming voor vetten te ontwikkelen en het aantal bestaande methodes uit te breiden. Een overzicht van bestaande technieken ter bescherming van vetzuren tegen penshydrogenatie werd gegeven in Hoofdstuk 3, wat enkel de onduidelijkheid over welke techniek nu precies de beste is illustreerde. In dit proefschrift werd een nieuwe alternatieve pensbeschermingsmethode voorgesteld en onderzocht door gebruik te maken van het vernettingsenzym PPO. Bij deze techniek werden lipiden geëmulgeerd in een eiwitextract van planten dat het PPO enzym bevatte. Eiwitten geadsorbeerd aan het water-olie oppervlak werden vernet na toediening van een difenol als substraat voor de reactie. De hypothese was dat vernet eiwit een beschermende scheidingslaag vormt aan het oppervlak van de emulsies dat de pensbacteriën er van weerhoudt de geëmulgeerde lipiden te hydrolyseren en hydrogeneren.

De bescherming van POVZ aan de hand van deze nieuwe PPO-gebaseerde pensbeschermingstechiek werd onderzocht in Deel II A. *In vitro* resultaten bevestigden de hypothese en illustreerden de mogelijkheid om POVZ te beschermen tegen penshydrogenatie door toevoeging van het difenol 4-methylcatechol (4-MC) aan de emulsies, gecreëerd met behulp van PPO-rijke eiwitextracten van rode klaver (Hoofdstuk 4). De beschermingsefficiëntie was afhankelijk van zowel de hoeveelheid olie als eiwit in de emulsies en nam toe bij toenemende hoeveelheden aan difenol in de emulsie per eenheid emulsieoppervlak. Naast rode klaver was het ook mogelijk om bescherming tegen BH te verkrijgen met behulp van eiwitextracten van (delen van) verschillende andere plantenbronnen zoals bloemkoolroosjes, spinaziebladeren, broccoliafval en aardappelschillen (Hoofdstuk 5). Grote verschillen in PPO activiteit tussen plantenbronnen waren opmerkelijk genoeg niet gecorreleerd met de graad van pensbescherming. Bovendien waren isovormen van PPO in de plantenbronnen die resulteerden in pensbescherming heel divers en leek hun aanwezigheid aan het oppervlak van de emulsies niet essentieel. Ook de oorsprong en de concentratie van eiwit in het extract leek voor een groot deel de uiteindelijke efficiëntie van pensbescherming te bepalen. Combinatie van al deze elementen kon impliceren dat stabiele emulsies, met voldoende kleine oliedruppelgroottes, samen met 4-MC en een zeker (ongedefinieerd) niveau aan PPO activiteit een noodzakelijke voorwaarde zijn voor bescherming, maar dat het eiwitgehalte de meest doorslaggevende factor is wanneer aan deze voorwaarden voldaan is. Verder werd verondersteld dat hetzelfde proces naast bescherming van POVZ tegen penshydrogenatie ook bescherming tegen andere pre-ruminale processen kon bieden (Hoofdstuk 6). Er werd aangetoond dat de PPO technologie zowel POVZ kon beschermen tegen penshydrogenatie als tegen oxidatie gedurende opslag en dat pre-ruminale oxidatie van andere geëmulgeerde lipofiele componenten zoals vitamine E ook belemmerd kon worden door toediening van 4-MC. Dit illustreert eens te meer de wijde toepassingsmogelijkheden van de PPO-gebaseerde inkapselingstechnologie.

De beschikbaarheid na penspassage van pensbeschermde PPO-gebaseerde emulsies werd onderzocht in Deel II B. Pensbeschermde POVZ moeten logischerwijs vrijgesteld worden in de rest van het gastro-intestinaal stelsel na passage door de pens, anders is de toegevoegde waarde door bescherming nutteloos. Post-ruminale beschikbaarheid van POVZ werd eerst onderzocht door middel van een muizenproef (Hoofdstuk 7). Het idee van een 'éénmagige' muizenproef was om tegelijkertijd de vrijstelling van lipiden uit (beschermde) emulsies tijdens gastro-intestinale passage alsook de opname in de bloedsomloop (biobeschikbaarheid) te bepalen. De resultaten van deze proef waren echter niet eenduidig, aangezien vetzuren uit beschermde emulsies niet volledig terug gevonden werden over de verschillende bemonsterde weefsels heen. Vrijstelling uit het beschermende kapsel werd vervolgens beoordeeld aan de hand van een *in vitro* experiment (Hoofdstuk 8). Triacylglyceriden in beschermde emulsies waren niet onderhevig aan lipolyse (en werden als dusdanig niet gehydrogeneerd) gedurende pensincubatie, maar werden wel teruggevonden als vrije vetzuren na lebmaag- en dunne darmincubatie. Dit was niet het geval voor onbeschermde emulsies, waarbij de vetzuren vrijgesteld en gehydrogeneerd werden tijdens pensincubatie. De *in vitro* vrijstelling was echter onvolledig en er bleek een zeker risico voor overbescherming aanwezig te zijn bij de hoogste niveaus van pensbescherming. Uiteindelijk werd aan de hand van een preliminaire *in vivo* proef met melkkoeien aangetoond dat het voederen van PPO-beschermde POVZ kon leiden tot een verhoging in het melkvet (Hoofdstuk 9). Het experiment bevestigde dat toevoeging aan het rantsoen van geconjugeerde linolzuren (CLA) in oliën geëmulgeerd met behulp van een PPO-bevattend eiwitextract van aardappelschillen en behandeld met 4-MC leidde tot bescherming tegen *in vitro* BH en beschermd CLA in zeker mate *in vivo* vrijgesteld kon worden tijdens de verdere gastro-intestinale passage, opgenomen werd in het lichaam en in de melkklier terecht kwam. Toenemende proporties aan *trans*-10, *cis*-12 CLA werden immers teruggevonden in het melkvet, wat tevens leidde tot melkdepressie als extra bevestiging van de transfer uit het rantsoen naar de melk.

Tot slot werden de eerste stappen richting opschaling van de PPO-gebaseerde beschermingstechnologie gezet in Deel II C (Hoofdstuk 10). Er werd gevonden dat emulsies gevriesdroogd konden worden en geconditioneerd met stoom (een proces dat de toevoeging van stoom en stijging van temperatuur tijdens pelleteren simuleert), maar een deel van de bescherming ging verloren. Dergelijke processen kunnen echter helpen om een droog en makkelijk handelbaar product te bekomen. Verder kon ook een alternatief protocol gebruikt worden ter bescherming van POVZ, wat mogelijkheden biedt tot opschaling aangezien de huidige grote 'ballast' aan continue fase mogelijk hergebruikt kan worden. Eén van de meest veelbelovende resultaten van dit proefschrift omhelst echter dat eiwit van verschillende plantaardige bronnen, bijvoorbeeld reststromen uit de aardappelverwerkende industrie, gebruikt kunnen worden om POVZ te beschermen tegen BH in de pens. Dit creëert opportuniteiten tot valorisatie van reststromen aan de hand van de PPO-gebaseerde pensbeschermingstechnologie.

Samengevat, een nieuwe pensbeschermingstechnologie werd ontwikkeld om oliën te beschermen tegen de ongewenste omzetting van 'gezonde' onverzadigde vetzuren naar meer 'ongezonde' verzadigde vetzuren in de pens van herkauwers. Vetzuren werden beschermd aan de hand van emulsies en het natuurlijke en alomtegenwoordige enzym polyfenol oxidase. Dit enzym kon geëxtraheerd worden uit een wijde reeks van plantenbronnen, waaronder aardappelschillen. Resultaten van dit proefschrift tonen aan dat onverzadigde vetzuren beschermd kunnen worden tegen hydrogenering in de pens en vrijgesteld kunnen worden na penspassage, en althans gedeeltelijk kunnen toenemen in de melk. Gebruik van deze technologie kan leiden tot gezondere melk en vlees, waarbij eiwitrijke reststromen die dit enzym bevatten kunnen worden gebruikt en gevaloriseerd.

DANKWOORD

Dankwoord

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