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The digesta passage rate among dairy cows with different abilities to consume large quantities of roughage

- its impact on the milk production, and the reliability of the chemical fibre marker

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Fodrets passagehastighet hos mjölkkor med olika förmåga att konsumera stora mängder grovfoder
- Dess påverkan på mjölkproduktionen och tillförlitligheten hos den kemiska fibermarkören

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Abstract

The objectives of the present master thesis were to investigate the passage rate among lactating Holstein and Swedish red dairy cows to investigate the hypotheses of: 1) The cows' individual total mean retention time (TMRT) has a negative relationship to the cows' individual forage intake capacity. 2) A reduced TMRT contributes to a reduced feed efficiency, but a higher milk yield. 3) The chromium (Cr) mordanted fibre marker used in the present study is a reliable substitute for rumen evacuations when measuring TMRT for the solid digesta fraction.

The study was divided into two parts, one marker method study, and one evaluation study. The marker method study was performed on 30 cows in mid lactation. The cows had free access of forage and a concentrate treatment of either 5.25 kg DM/day (5kgC) or 10.5 kg DM/day (10 kgC). The study was performed in four different study sessions of one week each, where each cow was included in one session only. A pulse dose of orally administered marker of 2 or 4 g of chromium (Cr) to measure the TMRT of the solid digesta fraction (TMRTCr), and 10 g of cobalt- ethylenediaminetetraacetic acid, to measure the TMRT of the liquid digesta fraction (TMRTCo), was given to each cow at h=0. This was followed by a scheduled faecal sampling session during a 164-hour period where a total of 24 faecal samples were collected from each cow. After completion of the four study sessions, 16 randomly chosen cows were selected for further analysis. The faecal samples got freeze dried, milled and analysed for Cr and cobalt (Co) concentrations. A plot of the marker concentrations was made for each cow and marker. A two-compartment, and age-independent model (G1G1) was thereafter used to make a curve fit and to predict TMRTCr and TMRTCo for each cow. Statistical analyses were performed by using SAS 9.4 MIXED procedure, proc REG and proc CORR. Statistical analyses was only performed on TMRTCr since the TMRTCo data turned out being unreliable.

The Cr mordanted fibre marker was evaluated in a separate study session of ruminal evacuations performed on four ruminally fistulated Swedish red cattle. Indigestible neutral detergent fibre (iNDF) was used as an internal marker. Three ruminal evacuations were performed on all cows at different days and different times to compensate for diurnal variation in rumen iNDF-pool. Feeds and rumen contents were analysed for iNDF whereafter daily iNDF intake and rumen iNDF-pool was calculated. The intake-based TMRT (TMRTin) was thereafter calculated and compared to TMRTCr data which was achieved by a separate marker method session for the ruminally fistulated cows. Proc REG and proc CORR was used to analyse the data.

A positive and significant relationship was shown considering feed efficiency and TMRTCr ($P<0.05$; $R^2=0.56$), but no significant relationship was observed between TMRTCr and kg milk ($P=0.87$; $R^2=0.00$), and neither between TMRTCr and kg energy corrected milk ($P=0.22$; $R^2=0.18$). There was no positive relationship between the cows' individual TMRTCr and forage DMI intake. The evaluation study could neither verify the Cr mordanted fibre marker as a reliable method to measure TMRT of the solid digesta phase. There was no significant relationship between TMRTCr and TMRTin ($P=0.16$, $R^2=0.71$). TMRTCr was shown to underestimate the passage rate compared to the rumen evacuation method, which is different compared to other studies regarding this subject. Further research to evaluate the marker method is needed to get a better understanding of the reliability of this less invasive method. Further studies are also requested to further investigate the TMRT's relation to milk production and feed efficiency among cows with different abilities to consume large quantities of roughage.

Keywords: chemical markers, chromium, chromium mordanted fibre, cobalt, concentrate level, EDTA, ethylenediaminetetraacetic acid, forage consumption, iNDF, indigestible neutral detergent fibre, liquid digesta phase, mean retention time, MRT, rumen evacuation, solid digesta phase, TMRT, total mean retention time.

Sammanfattning

Syftet med denna masteruppsats var att undersöka passagehastigheten hos lakterande holsteinkor och svensk röd och vit boskap (SRB). Detta gjordes för att pröva följande hypoteser: 1) Kons totala medelretentionstid (TMRT) av digesta har ett negativt samband med kons individuella foderintagskapacitet. 2) En lägre TMRT medför en sämre fodereffektivitet men bidrar trots detta till en högre mjölkavkastning. 3) Den krommärkta fibermarkören som används i denna studie är ett tillförlitligt substitut för våmtömningsmetoden som används när man mäter TMRT av den fasta digestafasen.

Studien var indelad i två delstudier, en markörstudie och en utvärderingsstudie. Markörstudien genomfördes på 30 kor i mittlaktation. Korna hade fri tillgång på grovfoder och hade en kraftfodergiva på antingen 5,25 kg torrsubstans (ts)/dag (5kgC) eller 10,5 kg ts/dag (10kgC). Studien var indelad i fyra likadana sessioner, där varje ko endast ingick i en av dessa sessioner. En pulsdos av oralt tillsatt markör av 2 eller 4 g krom (Cr), i form av krommärkt fiber, för att mäta den fasta digestans TMRT (TMRTCr), samt 10 g av cobolt-etylendiamintetraättiksyra för att mäta TMRT av digestans vätskefas (TMRTCo), gavs till korna vid tiden h=0. Detta följdes av en schemalagd träckprovtagningssession, vilken varade i 164 timmar där totalt 24 träckprover togs per ko. Efter genomförandet av samtliga fyra sessioner, valdes 16 slumpmässiga kor ut för vidare analys. Träckproverna frystorkades, maldes och analyserades för Cr- och kobolt (Co)-koncentrationer. En graf på markörkoncentrationerna gjordes för varje ko och markör. En tidsoberoende tvåpoolsmodell (G1G1) användes för att göra kurvanpassningar, samt för att estimera TMRTCr och TMRTCo för varje ko. Statistiska analyser genomfördes i SAS 9.4 genom MIXED procedure, proc REG och proc CORR. Inga statistiska analyser genomfördes på TMRTCo, då denna data visade sig vara icke tillförlitlig.

Den krommärkta fibermarkören var utvärderad i en separat studie där våmtömningar genomfördes på fyra våmfistulerade SRB-kor. Onedbrytbart NDF (iNDF) användes som en intern markör. Tre våmtömningar genomfördes på samtliga kor vid tre separata dagar och tidpunkter för att kompensera för dygnsvariationen i våmmens iNDF-pool. Det dagliga iNDF-intaget och våmmens iNDF-pool mättes och analyserades. Intagsbaserade TMRT (TMRTin) beräknades därefter, följt av statistisk jämförelse med TMRTCr-data vilken tagits fram i en separat markörmetodssession för dessa kor. Proc REG och proc CORR användes för den statistiska analysen.

Studien visade en positiv och signifikant samband mellan fodereffektivitet och TMRTCr ($P<0,05$; $R^2=0,56$), men ingen signifikant samband visades mellan TMRTCr och kg mjölk ($P=0,84$; $R^2=0,00$) och kg energikorrigerad mjölk ($P=0,22$; $R^2=0,18$). Inget positiv samband fanns heller mellan kornas individuella TMRTCr och deras ensilageintag, angivet i kg ts. Utvärderingsstudien kunde inte verifiera den krommärkta fibermarkören som en tillförlitlig metod att estimera TMRT av den fasta digestafasen. Det fanns ingen signifikant samband mellan TMRTCr och TMRTin ($P=0,16$; $R^2=0,71$). TMRTCr underestimerade passagehastigheten i jämförelse med våmtömningsmetoden. Detta skiljer sig från andra studier på området. Vidare studier för att utvärdera ovan nämnda markörmetoder behövs för att öka förståelsen av tillförlitligheten hos denna mindre invasiva metod. Ytterligare studier efterfrågas för att vidare undersöka TMRT:s relation till mjölkproduktion och fodereffektivitet hos kor med olika förmåga att konsumera stora mängder grovfoder.
Nyckelord: kemiska markörer, krom, krommärkt fiber, kobolt, kraftfodergiva, EDTA, etylendiamintetraättiksyra, grovfoderkonsumtion, iNDF, onedbrytbart NDF, digestans vätskefas, medelretentionstid, MRT, våmtömning, digestans fasta fas, TMRT, totala medelretentionstiden.

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Abbreviations

10kgC	The 10.5 kg DM concentrate diet treatment group
5kgC	The 5.25 kg DM concentrate diet treatment group
aNDFom	NDF analysis performed with heat resistant amylase, result presented without ash.
DM	Dry matter
DMI	Dry matter intake
dNDF	Digestible NDF
EDTA	Ethylenediaminetetraacetic acid
FSG	Functional specific gravity
iNDF	Indigestible NDF
K_p	Passage rate (commonly %/h)
MRT	Mean retention time
NDF	Neutral detergent fibre
OM	Organic matter
pdNDF	Potentially digestible NDF
ROO	Reticuloomasal orifice
TMRT	Total mean retention time
TMRTCo	TMRT of the liquid phase, calculated using Cobalt-EDTA as a marker
TMRTCr	TMRT of the solid phase, calculated using Chromium mordanted fibre as a marker
TMRTin	Intake-based TMRT of the rumen evacuated cows, calculated from rumen evacuations.

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1 Introduction

Ruminants have the unique ability to digest and utilize energy from cell wall materials. The microbial flora in the rumen makes it possible for cows to convert for humans' indigestible materials, such as grasses, into nutritionally available nutrients in milk and meat. However, the increased milk yield among the Swedish dairy cows over the last 60 years (Cattle Statistics, 2018), is partially explained by improved breeding and feeding (Simm, 2010). The availability of high nutrient density feed is a basic prerequisite for a higher milk yield (Spörndly & Kumm, 2010) and today's high producing Swedish dairy cows are fed approximately 50 % forage and 50 % concentrates on dry matter (DM) basis (Patel, 2012; Henriksson *et al.,* 2014). The high concentrate percentage has thus limited the usage of the cow's fibre converting ability, something which might be questioned from an environmental point of view.

The increased interest in organic products in recent years has however increased the amount of organic dairy farmers in Sweden (KRAV, 2017; von Unge *et al.,* 2017). This put other demands on the feed rations, with a minimum level of 50 % forage in the feed ration during three months of early lactation and 60 % thereafter (National guidelines for organic production, 2017; KRAV, 2018). Feed costs are also big expenses for the dairy farmers (von Unge *et al.,* 2017) and an increased proportion of forage in the feed rations can, depending on the market prices and farm size, reduce the total feed costs on the farm (Patel, *et al.* 2013). High quality forages, and a high forage utilization among the cows, is therefore of importance for today's dairy farms for many reasons.

Even though an increased proportion of roughage may be eligible, a study by Lawrence *et al.* (2015) showed that the milk yield, body condition score and energy balance was negatively affected if the average individual concentrate allowance was 4 kg DM per day, compared to 7 kg DM during free access of forage in early lactation. However, not only the concentrate:forage ratio affects the dairy cattles' productivity: The roughage nutrient quality also has an impact on the dairy cattles' productivity at different concentrate:forage ratios (Patel, 2012). In a Swedish study by Patel *et al.* (2013) the average dietary DM inclusion of 51 or 62 % of high-quality forage during lactation showed no significant differences in dry matter intake (DMI) and milk yield between the diets (Patel, 2012; Patel *et al.,* 2013). However, the cows given the average forage ration of 70 % DM had a significantly lower productivity. The average daily DMI did however not differ significantly from the other treatment groups (Patel, 2012; Patel *et al.,* 2013).

There are however individual variations in the dairy cows' ability to consume large quantities of roughage (Patel, 2012) and it is not known what causes these different intake abilities. This individual variation might make some cows more suitable to high forage diets compared to others. Research using rumen evacuation techniques or slaughter experiments to measure digesta passage show that the passage rate, or retention time, may increase, respectively decrease, with increased feed intake (Paloheimo & Mäkelä, 1959; Robinson *et al.,* 1987; Huhtanen & Kukkonen, 1995). It may as well be reversely hypothesised that it is the passage rate itself that affects the cows' ability to ingest larger volumes of forage, something which also has been suggested as a contributing factor in a research by Stensig *et al.* (1998a). The passage rate, together with the feed intake would thus play an important role in the forage utilization among dairy cows, since a short retention time in the gastrointestinal tract (GIT) can lead to improperly digested feed (McDonald *et al.,* 2011), while a long retention time may result in a sub-optimal nutrient intake and uptake due to a slow passage rate of digesta through the reticuloomasal orifice (ROO).

A lot of research has been performed on digesta passage rate throughout the years to better understand the nutritional physiology in different environments and production stages, with different feed rations and feed qualities. Methods of slaughter (Paloheimo & Mäkelä, 1959), ruminal evacuations (Krizsan *et al.*, 2010) and gastrointestinal administration of chemical markers (Lund *et al.,* 2006) have been used and developed throughout the years. Ruminal evacuations are considered a reliable method (Minde & Rygh, 1997; Volden & Larsen 2011; Huhtanen *et al.,* 2008) and can, unlike slaughter experiments, be performed with direct access to the reticulorumen digesta on live animals. Methods like the marker method, which is the least invasive method, has however rarely been evaluated (Stensig *et al.,* 1998b; Huhtanen *et al.,* 2008) and further research to better understand the method's reliability is requested (Huhtanen *et al.,* 2008). To further investigate the individual animal's impact of the passage rate, where feed intake was especially mentioned, has also been requested (Huhtanen *et al.,* 2008). Both of these points are incorporated into the present master thesis.

The present study, which was a part of a bigger research project (REF O-16-23-762, "Finding key parameters for improved forage utilization and lowered methane emissions in dairy cows"), analysed the passage rate of digesta among 16 cows in mid lactation to investigate the individual variation of the total mean retention time (TMRT) and milk production among cows with different voluntary consumption of roughage. The study was performed with orally administered markers to measure TMRT of the liquid and solid digesta fractions. The marker method of the solid digesta fraction was also evaluated by a dynamic rumen model performed by rumen evacuations of four ruminally fistulated cows. The dynamic rumen model used indigestible neutral detergent fibre (iNDF) as digesta marker.

The hypotheses of the present thesis are:

1) The cows' individual TMRT has a negative relationship to the cows' individual forage intake capacity

2) A reduced TMRT contributes to a reduced feed efficiency, but a higher milk yield

3) The chromium (Cr) mordanted fibre marker used in the present study is a reliable substitute for rumen evacuations when measuring TMRT for the solid digesta fraction

2 Literature study

The physiology of the GIT is complex, and its function is dependent on the volume, shape and chemical properties of the ingested feed, of solid as well as liquid kind. The body's microbial populations and enzymatic and hormonal functions are also central in the function of the alimentary tract. This literature study will further investigate the physiology of feed intake, rumen dynamics and passage rate among healthy dairy cattle. Different methodologies to measure the passage rate will also be examined to give a view of how different studies have been performed.

"Passage rate" (The speed a particle leaves a certain site of the GIT) and "mean retention time" (MRT) (The average time a particle spend at a certain site of the GIT) will be used continuously throughout the present thesis and refers to the same process, only expressed in two different ways.

2.1 Measuring the passage rate and total mean retention time

There are different ways to measure the passage rate of digesta throughout the GIT. Indigestible materials, such as plastic beads, can be used to measure the passage (desBordes & Welsh, 1984) and TMRT (Clauss *et al.,* 2011) from mouth (or rumen) to rectum (desBordes & Welsh, 1984; Clauss *et al.,* 2011), also stained materials were earlier used to measure the passage rate (McDonald *et al.,* 2011). The passage rate of solid or liquid digesta can be measured by the adhesion of chemical markers to a specific analytical fraction of the animals' feed, followed by collection and analysis of the animals' faeces (Udén *et al.,* 1980).

The rumen evacuation technique is another method of measuring the passage rate. This method is invasive, since it requires ruminally cannulated animals. Some experimental designs also require fistulas at additional sites along the GIT to measure the retention time at different compartments along the alimentary tract, or by performing the measurements in different ways (Minde & Rygh, 1997; Lund *et al.,* 2007). Total or partial digesta evacuations are thereafter performed, depending on the purpose of the study (Minde & Rygh, 1997; Lund *et al.,* 2007). The method involves internal markers, such as neutral detergent fibre (NDF) (Minde & Rygh, 1997; Lund *et al.,* 2007) and sometimes also external markers of chemically marked digesta (Lund *et al.,* 2007) to make it possible to estimate the retention time and passage rate (Minde & Rygh, 1997; Lund *et al.,* 2007).

The TMRT measured by 1) oral administration of chemical markers, and 2) the rumen evacuation technique, using internal digesta markers, will be further reviewed ahead after some extended information about the plant cell wall and NDF, since these structures are central in the techniques of measuring passage rate and TMRT of the solid digesta fraction.

2.1.1 Cell wall analysis

Neutral Detergent Fibre is a well-used analytical fraction in animal feeds that may be used to describe *"... "fibre", "cell wall", "dietary fibre", "cell wall carbohydrates" or "structural carbohydrates"..."* (Lund, 2002) even though some of these structures, by definition, are not the exact same thing.

The NDF fraction contains the cell wall structures of cellulose, hemicellulose, lignin, silica, lignified nitrogen and proteins that are bonded to these mentioned structures (McDonald *et al.,* 2011). Since the NDF fraction contains digestible, as well as indigestible components, which also have different kinetic features (Huhtanen *et al.,* 2008), NDF may be divided into two fractions; "digestible NDF" (dNDF) and "indigestible NDF" (iNDF) (Minde & Rygh, 1997; Huhtanen *et al.,* 2007). Since the dNDF fractions might not be fully digested, for example due to a higher passage rate compared to digestion rate, dNDF-particles may end up in the cattles' faeces. The dNDF fraction may therefore be called "potentially digestible NDF" (pdNDF).

The two fractions of NDF (iNDF and dNDF or pdNDF) may be analysed and separated *in vitro* by the usage of rumen fluid and buffer (Krizsan *et al.,* 2010) or *in situ* by the *in sacco* method (Stensig *et al.,* 1998b; Åkerlind *et al.* 2011). The *in sacco* method is performed by ruminal incubation of nylon bags containing the samples that will be analysed. Incubations are normally performed during at least 96 hours (Krizsan *et al.,* 2010) and the method assumes that everything that disappears from the nylon bag is digested (NRC, 2001).

It is known that the pure NDF analysis methods (Udén *et al.,* 2005), as well as the (p)dNDF and iNDF analysis methods (Lund, 2002) may differ between studies (Lund, 2002; Udén *et al.,* 2005). This will reduce the comparability of (p)dNDF (Lund, 2002) iNDF and NDF between different studies. This may also lead to misunderstandings due to misused terminology (Udén *et al.,* 2005).

Acid detergent fibre (ADF) is another way of analysing the cell wall structure. The ADF covers cellulose, silica, lignin and lignified nitrogen (McDonald *et al.,* 2011) and may also be divided into an indigestible fraction (iADF) (Park *et al.,* 2011) and a digestible fraction (dADF).

2.1.2 Rumen evacuation technique

Rumen evacuation is considered a reliable method to measure the passage rate and digestibility of solid digesta (Minde & Rygh, 1997; Volden & Larsen 2011; Huhtanen *et al.,* 2008). It is based on removal of ruminal contents from cannulated animals, followed by measurement of the rumen digesta iNDF content, faecal iNDF content and feed iNDF content (Huhtanen *et al.,* 2007). The method is, unlike the marker method, able to measure the passage rate with rumen digesta accessed from the living animal. The method is however costly, time-consuming (Huhtanen, *et al.,* 2008) and invasive.

In the rumen evacuation procedure, the rumen pool size is measured by total rumen evacuations (Stensig *et al.,* 1998b). Indigestible neutral detergent fibre, which is a natural and voluntarily ingested feed component, that passes through the GIT unaffected, may be used as an internal marker (Other internal markers such as iADF (Park *et al.,* 2011) may also be used). Representative rumen samples are also taken followed by iNDF analysis. The passage rate is thereafter calculated by dividing the iNDF-intake with the rumen iNDF-pool or by dividing the faecal iNDF-pool with the rumen iNDF-pool, as described by Huhtanen *et al.* (2007).

Feed intake may be measured using different techniques, such as individual food distribution and weighing of the daily orts (Rezaei *et al.,* 2015), or using scales and a computer program that registrates intake for individual cows' (Holtenius *et al.,* 2018; Biocontrol, 2013). Which method

that is used depends on what is practically possible. Knowledge of the dry matter feed intake and iNDF analyses of the feeds makes it possible to calculate the daily iNDF intake.

The daily iNDF output may also be used for calculations. Different ways of measuring and predicting faecal output, and thus directly or indirectly the faecal iNDF output, will be further explained below.

2.1.2.1 Faecal output

The faecal output may be measured by complete collection of faeces (Huhtanen *et al.,* 2007; Morris *et al.,* 2018), or by administering external markers to the GIT throughout several days at specific times to reach steady state of the output of the marker (Ferret *et al.,* 1999). Steady state can however only be reached if the marker administration is invariable in time and quantity, and if feed intake is invariable (Owens & Hanson, 1992) since the passage rate changes with different feed intakes (Robinson *et al.,* 1987). A similar estimation can also be made by using internal markers, such as iNDF or acid insoluble ash (AIA), to estimate the faecal output (Morris *et al.,* 2018). This method requires AIA or iNDF analysis of several faecal samples distributed at different times of the day, as well as AIA analyses of the feed (Morris *et al.,* 2018).

A pulse dose of marker may also be used to estimate the faecal output (Susmel *et al.,* 1996). Research performed on ruminally fistulated lambs showed that a pulse dose of ytterbium marked forage was a reliable way of estimating the faecal output among sheep in metabolism stalls (Krysl *et al.,* 1985). The study showed no statistical differences ($p<0.05$) when comparing total faecal collection and the dose marker estimations of faecal output among twelve lambs. Susmel *et al.* (1996) who performed a study on cattle, did however request further research to better evaluate the method to be able to use it for reliable digestibility calculations.

2.1.2.2 Assumptions used for the rumen evacuation method

The rumen evacuation technique assumes that the practical procedures of the rumen evacuations has no impact on the passage rate (Huhtanen *et al.,* 2007). The method also requires a steady state rumen pool-size (Huhtanen *et al.,* 2007). Huhtanen *et al.* (2007) showed that the time of rumen evacuation had a significant impact on the rumen pool size among cows fed two or 18 times per day. The diurnal variations are also hard to control among cows with free access to feed (Huhtanen, *et al.,* 2008). The diurnal variation of the rumen pool size may be adjusted for by the performance of several rumen evacuations distributed on several days at different times of the day to get a reliable mean value of the rumen pool size (Robinson *et al.,* 1987; Lund *et al.* 2007; Huhtanen *et al.,* 2007).

2.1.3 Chemical marker methods

Elements such as Cr (Udén *et al.,* 1980; Lee & Hristov, 2014), ytterbium (Yb) (Lund *et al.,* 2006; Huhtanen *et al.,* 2007), cerium (Ce) (Udén *et al.,* 1980; Combs *et al.,* 1992), dysprosium and erbium (Ahvenjärvi, *et al.,* 2010) may be used as external markers to measure the passage rate of solid digesta (Udén *et al.,* 1980; Lund *et al.,* 2006; Ahvenjärvi, *et al.,* 2010; Lee & Hristov, 2014). Udén *et al.* (1980) who investigated the Cr- and Ce fibre mordant markers, explained that the markers can form complexes, based on ligand attractions, together with predominantly cell

wall materials. These complexes are then used to measure the passage rate of the solid digesta fraction.

Chromium (Udén, *et al.* 1980), and cobalt (Co) can also be used to measure the passage rate of the liquid phase of digesta (Udén, *et al.* 1980; Park *et al.,* 2011; Fraley *et al.,* 2015). These elements can adhere to the ligand ethylenediaminetetraacetic acid (EDTA) that can dissolve in water (Udén *et al.,* 1980; Krämer *et al.,* 2013; Nationalencyklopedin, 2019). Polyethylene glycol (PEG) may also be used as a liquid marker (Stensig *et al.,* 1998a; Ahvenjärvi *et al.* 2018).

2.1.3.1 Procedures for measuring mean retention time with markers

To measure the MRT, the marker complexes can be administered to the GIT through the mouth (Wang *et al.,* 2018), or through rumen cannulas (Lund *et al.,* 2006; Ahvenjärvi *et al.,* 2018). The markers will later end up in the faeces which is collected according to a predetermined schedule (Ahvenjärvi *et al.,* 2018). The schedule is prepared to make sure that enough samples, within the right time, are collected to catch the excretion curve of the marker. The first post-dose faecal sample are generally collected within a few hours after the administration of the marker (Lund *et al.,* 2006; Huhtanen *et al.,* 2007). This is generally followed by further collections for some days (Lund *et al.,* 2006; Huhtanen *et al.,* 2007). After the faecal samplings, the marker concentration is analysed in each sample (Ahvenjärvi *et al.,* 2018) and the passage rate and MRT is calculated and estimated by the usage of a mathematical model (Minde & Rygh, 1997).

2.1.3.2 Requirements of the chemical markers

The marker method is less invasive compared to for example the rumen evacuation technique (Owens and Hanson, 1992). It is however important that the mordanted fibre fraction and the EDTA complex is stable and passes through the GIT unaffected; The marker should not be digested or get released from its mordant during its passage through the digestive tract (Udén *et al.,* 1980). Neither should the environment of the GIT, nor the microbial flora, be affected by the marker addition (Owens & Hanson, 1992). However, no marker method of measuring the passage rate is complete (Owens & Hanson, 1992).

Udén *et al.* (1980) made early investigations of (inter alia) Co-EDTA and Cr mordanted fibre. The study concluded that 99.5 % of the Cr was recovered in the faeces within a ten-day period, and that negligible amounts of Cr were found in the urine. Cobalt, which was used to measure the liquid digesta fraction, had a faecal recovery of 90 % within 82 hours after ruminal administration. About 3 % of Co was detected in the urine which means that parts of the marker got absorbed from the GIT. Despite that, Co-EDTA was still considered suitable to use for digesta kinetic measurements (Udén *et al.,* 1980). Ytterbium and Ce has however been criticized due to its tendency to migrate from its marked fraction: An *in vitro* experiment performed on marked bromegrass hay incubated in rumen fluids showed that Ce, as well as Yb, migrated to other particles present in the solution (Combs *et al.,* 1992). Also Udén *et al.* (1980) observed that 55 % of the Ce-fibre bindings were destroyed when incubated in ruminal fluid. When considering Yb-labelled grass silage which was used in a study by Stensig *et al.* (1998b), an underestimation of the rumen MRT was observed when compared to the rumen evacuation technique. The authors did suggest it was caused by the above mentioned migration of Yb from the marked forage.

Ehle *et al.* (1984) concluded that an increased Cr marker concentration (marked percentage of cell wall) increased the cell wall density of the marked alfalfa. The increased cell wall density (from 1.21 to 1.33 and 2.08) did in turn linearly increase the ruminal turnover rate estimated from faecal samples. The same study also performed the calculations based on ruminal samples, where the increased particle density showed a quadratic relationship to turnover rate: the turnover rate increased from particle density 1.21 to 1.33, followed by a decrease to particle density 2.08. Correspondingly, a study by Lirette and Milligan (1989), who used the radioactive isotope Cr-51 as a marker, showed that the digestibility of the marked fibre fraction was significantly reduced when using 5 g Cr/kg DM, compared to 0.1 g Cr/kg DM which showed no reduction of digestibility. The study which based its TMRT calculations on data from faecal samplings could not find any significant effects on TMRT depending on level of mordant (Lirette & Milligan, 1989). However, when making calculations based on ruminal samplings, the turnover time tended ($P<0.1$) to be increased at the higher level of mordant (Lirette & Milligan, 1989). The above demonstrates that the level of mordanting may have an effect on the particles transport through the GIT.

Minde and Rygh (1997) investigated different mathematical models; two compartment models of gamma and exponential types, where they concluded that the ruminal retention time, as well as passage rate were largely affected by the choice of model. Krizsan *et al.* (2010) also hypothesised that the iNDF-based rumen evacuations are more reliable than the marker method. The hypothesis referred to comparisons between studies which (according to Krizsan *et al.* (2010)) showed that the marker method had a noteworthily shorter retention time compared to (reliable) slaughter experiments.

The National Research Council (NRC) and Cornell Net Carbohydrate and Protein System (CNCPS) has compiled prediction equations of the passage rate of different feed types, such as forages, concentrate and water, where the data got achieved from marker methods (Fox *et al.,* 2004; NRC, 2001). These predictions are for example used to further estimate the rumen degradable protein (NRC, 2001), metabolizable energy in the feed ration and the nutrient absorption in the small intestines (Fox *et al.,* 2004). In the meta-analysis by Krizsan *et al.* (2010), data obtained from ruminal evacuations was compared to the predictions from NRC and CNCPS. The analysis concluded that the marker-based predictions overestimated the ruminal passage rate of solid digesta compared to the rumen evacuation method based on NDF (Krizsan *et al.*, 2010). Additionally, some researchers have mentioned that experiments using marker methods rarely have been evaluated by comparing them to results achieved by for example rumen evacuation techniques or other *in vivo* methods considering digestibility (digestibility can be calculated from the digesta passage rate) (Stensig *et al.,* 1998b; Huhtanen *et al.,* 2008). The lack of evaluations adds uncertainties to the method.

2.2 Rumen dynamics

Depending on the ingested feed particle's size, shape and weight it will sediment to different levels in the rumen. Gasses that are produced as fermentation products of the microorganisms will also affect the particles ability to sediment, since gas might get trapped inside or under the ingested feed particle, as discussed by Robinson *et al.* (1987). Large particles that need to be

ruminated ends up more dorsal in the reticulorumen compared to small particles with high density. Fibrous materials stimulate rumination through nerve endings around cardia (Sjaastad *et al.,* 2010).

Functional specific gravity (FSG) can be applied to describe a particles tendency to sediment in the reticulorumen (Seo *et al.,* 2009). The higher FSG a particle has, the more ventral in the reticulorumen the particle will tend to appear (*Figure 1*) (Seo *et al.* 2009). The FSG is central in the ingested particles ability to pass through the GIT (desBordes & Welsh, 1984; Hristov *et al.,* 2003, Seo *et al.,* 2009). Hristov *et al.* (2003) showed that digesta with a FSG higher than the study's target value of 1.02 had a faster passage rate compared to particles with lower FSG than 1.02. Also desBordes & Welsh, (1984) investigated the passage of cylindric plastic beads of different gravities (desBordes & Welsh, 1984) where Van Soest (1994) further investigated the data and concluded that the optimal gravity for passage out of the rumen was around 1.2.

Objects with high densities and large sizes tend to get trapped in the reticulum (Hall & Silver, 2009; Parish *et al.* 2017). This is for example taken advantage of considering the case of boluses that are supposed to stay in the reticulorumen (Fallon & Rogers, 2001). A study by Fallon and Rogers (2001) showed that electronical boluses of the size of 20x86 mm and density of 1.75 was lost among 67 % and 78 % of cattle getting the boluses administered at 6 months, and 18 months of age, respectively. If increasing the density to 2.35, the boluses was lost among 2 % of the cattle within a 150-day interval after administration (Fallon & Rogers, 2001). The reported data of boluses gives further understanding to the rumen function and the densities impact on passage rate.

If turning back to rumen dynamics of the feed, sedimentation and an increased FSG for further passage through the ROO is achieved through rumination and microbial fermentation (Sjaastad *et al.,* 2010). Functional specific gravity is the most crucial parameter to predict the destination of a particle (Seo *et al.,* 2009).

2.2.1 Intrinsic and extrinsic factors

Another way to describe the particles movements and digestibility in the rumen and the rest of the GIT are by considering the digesta particles extrinsic (Krämer *et al.,* 2013) and intrinsic factors (Ahvenjärvi *et al.,* 2010). These two factors refer to the current feed particle's inner (Ahvenjärvi *et al.,* 2010) and outer properties and environment (Huhtanen *et al.,* 2008). The intrinsic factors cover the feedstuff's FSG, shape, content, and at what rate the particle size decreases (Krämer *et al.,* 2013), while the extrinsic factors cover the feed ration and characteristics related to the alimentary tract of the cow (Huhtanen *et al.,* 2008). The extrinsic factors, such as ruminal environment, are independent of the intrinsic factors (Huhtanen *et al.,* 2008).

2.2.2 Rumen contractions

A study on sheep showed that the particle passage though the ROO got altered when impairing the reticular contractions by the addition of a weight to the reticulum (Kaske & Midasch, 1997). The changes in reticular contractions resulted in enlarged particles in the faeces, but also an altered reticulorumen MRT among high density plastic markers which stayed significantly longer in the reticulorumen (Kaske & Midasch, 1997). The reticulorumen MRT of low-density

plastic markers was however unaffected or shortened depending on whether data was compared to when the sheep had *ad lib.* or controlled feed intake during times without the weights (Kaske & Midasch, 1997). This indicates the impact of reticular contractions in the passage through the ROO (Kaske & Midasch, 1997). A similar study using weights placed in the rumen of steers showed that the duration of reticular contractions around the feeding was significantly greater compared to the steers without weights in the rumen (Okine *et al.,* 1989). The frequency of contractions was however lower during and after feeding among these steers compared to steers without weights (Okine *et al.,* 1989). The increased duration of the reticular contraction was thought to be the reason for the shown increased passage rate of solid digesta, as well as liquid digesta in the study, since the second phase of the reticular contraction is performed simultaneously with the opening of the ROO (Okine *et al.,* 1989; Ohga *et al.* 1965 in Okine *et al.,* 1989). The ROO is also central in the passage out of the reticulorumen (Mathison *et al.,* 1994), and is involved in the previous examined characteristics of rumen dynamics. However, the ROO will not be further examined in the present thesis.

2.2.3 Rumen dynamics in passage rate calculations

The previous paragraphs demonstrates that the passage rate out of the reticulorumen is selective; particles that enter the reticulorumen first does not necessarily pass to the omasum first. These characteristics may be corrected for in the mathematical models used for the passage rate estimations performed by pulse dosed chemical markers (Seo *et al.,* 2009), and (to some extent) for the rumen evacuation technique (Lund *et al.,* 2007). Different studies use self-developed models, since research has tried to apply different mathematical models to the marker method (Huhtanen *et al.,* 2008). To include the rumen dynamics into the mathematical models, the reticulorumen may be divided into different compartments or pools (Seo *et al.,* 2009). These compartments can follow different assumptions of residence time (Pond *et al.,* 1988).

In a *one-compartment model*, the rumen is considered as a single functional unit. This model may be used for the elemental rumen evacuation method when measuring the total rumen pool size (Lund *et al.,* 2007), as well as the marker method (Pond *et al.,* 1988). A *two-compartment model*, which divides the GIT or rumen into two pools, can also be used (Pond *et al.* 1988; Lund *et al.,* 2007). The two compartments may refer to the "inescapable-" and the "escapable pool" of the rumen (Lund *et al.,* 2007). The inescapable pool covers the dorsal half of the rumen content and is assumed to contain particles that need to reduce their size and increase their FSG before leaving the reticulorumen (Seo *et al.,* 2009). The escapable pool covers the ventral half of the rumen content and is assumed to cover the small particles, of higher FSG, that are susceptible to pass through the ROO after entering the reticulum (Seo *et al.,* 2009). The model may also follow the principle that particles that has entered the escapable pool, cannot go back to the inescapable pool (Seo *et al.,* 2009). The two compartments are visualized in *Figure 1*.

Despite the above description about the escapable- and inescapable pools, the compartments may not always be as distinct. According to the models used by Pond *et al.* (1988), the distributions of the two compartments are not specified. This is in agreement with Van Soest, (1994) who mentioned that there sometimes is ambiguity whether the two pools, including its mathematical model, refers to the reticulorumen, or if one pool or parts of a pool refer to the post reticulum part of the GIT.

Beyond the one- and two-compartment models, the GIT may also be divided into three, four (France *et al.,* 1985) or more compartments (Dhanoa et al., 1985) in different constellations. If using many compartments in the model, it may be called a multi compartment model (Dhanoa *et al.,* 1985). All these different models may be used in different ways. For example, Lund *et al.* (2007) used the two-compartment model and divided it into separate iNDF and dNDF fractions when using the rumen evacuation technique in combination with chemically marked digesta. This gave four separate passage rates through the two compartments.

When the digesta marker has escaped from the reticulorumen it will pass through the remaining part of the digestive tract before it leaves the animal in the faeces. The lag phase that is shown in the rumen after ingestion of feeds refers to the time of colonisation of microbes, and microbial activity as well as moistening of solid particles (Huhtanen *et al.,* 2008). The lag phase may also be the mathematical formulas' analogue for the transit time of digesta through the post-ruminal parts of the GIT (Aikman *et al.,* 2008).

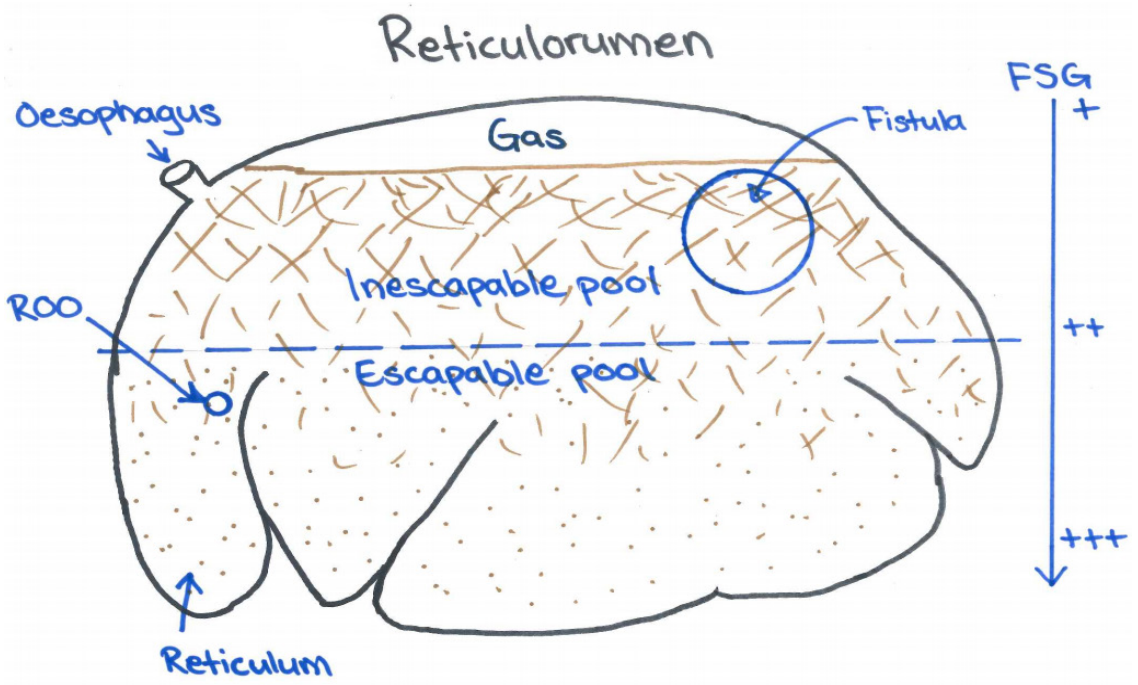

Figure 1. The reticulorumen and its content where large, fibrous materials are placed dorsal in the reticulorumen. An increased functional specific gravity (FSG), and a reduced particle size result in a more ventral location of the particle. The increased FSG and its expected effect on digesta location is demonstrated by an arrow together with different amounts of plus signs. Oesophagus, reticuloomasal orifice (ROO), reticulum and the potential location of a ruminal fistula are pointed out. The two-compartment model, with its "inescapable pool" and "escapable pool", are also marked out. The figure is modified from Seo et al. (2009) and Sjaastad et al. (2010).

The passage out of each compartment is calculated separately within the mathematical model. The retention time of the particles in each pool may be assumed to follow different residence time distributions:

Age-independent (G1) residence distributions follows the assumption that all particles in the compartment, independently of its residence time, has the same probability to leave the compartment (Pond *et al.,* 1988). The G1 assumption of the pulse dose of marker follows an exponentially declining residence time of the total dose of marker. This means that a constant proportion of the marker is leaving the compartment until it is all gone (first order kinetics). Particle residence time may also follow the *age-dependent* (G2) assumption, following the gamma distribution, where the assumption is that the probability of a feed particle to leave the compartment increases with its residence time. Higher distributions of gamma distributed, age-dependent models may also be used ((G2,) G3, …, G*n*) (Pond *et al.,* 1988).

2.3 Passage rate, feed ration and digestion

The reticulorumen constitutes about 85 % of the volume of the four stomachs in cattle (Dyce *et al.,* 2010) and contains around 100 to 110 kg of fresh weight (Wang *et al.,* 2018). Reticulorumen is also the site where the ingested particles stay for the longest time and was according to Paloheimo and Mäkelä's study (1959) accounting for around 70 % of the TMRT of lignin (Paloheimo & Mäkelä, 1959). Lund *et al*. (2006), who measured the passage rate on ruminally, duodenally, and ileally cannulated cows, had similar results where the pre-duodenal part of the GIT covered 82 % of the TMRT. In agreement with the retention time, the pre-duodenal part of the GIT could also explain 75 % or more of the digestion in a study by Okine and Mathison (1991).

2.3.1 Effects on digestion caused by slow and fast passage rate

The previous paragraph demonstrates that the passage rate is closely related to digestibility, and the relation between passage rate and digestion is generally described to function in a competitive way (Van Soest 1994; Lund, 2002). An increased digesta passage rate may impair the overall degradation (Fox *et al.,* 2004) since the cows' endogenous enzymes may not get access to nutrients wrapped inside non-fermented fibre materials passing through the ROO. An increased dilution rate (passage rate) of the liquid fraction has also been shown to reduce the degradation of digesta, and its fibre fractions (Meng *et al.,* 1999) as will be further examined below.

A fast passage rate of the liquid digesta fraction may result in flush out of rumen bacteria (McDonald *et al.,* 2011). An in vitro study performed by Meng *et al.* (1999) showed that an increased dilution rate (passage rate) of the liquid fraction increased the microbial efficiency (grams of microbial nitrogen/kg organic matter truly digested feed) and decreased the digestibility of organic matter and DM for the three used diets. The diet based on fibrous carbohydrates did also show a reduced digestibility of ADF and NDF (Meng *et al.,* 1999). The reason for the increased microbial efficiency was partly hypothesised to be due to an enlarged population of fast-growing microbes (Meng *et al.,* 1999). Additionally, these findings show that an increased passage rate of liquid affect the rumen environment.

If the passage rate through the reticulorumen is slow, the outflow of digesta to the omasum might be reduced to such extent that the overall nutrient intake and uptake is reduced and thus sub-maximal (Nilsson, 2017). This may happen to animals that are eating an unbalanced ratio of carbohydrates and proteins or other nitrogen compounds. Sampaio *et al.,* (2010) discussed and synthesised the effects of an increased level nitrogen in low-quality forage. This discussion can instead be applied on the reversed situation: A decreased level of nitrogen supplement to a low-quality forage may negatively affect microbial activity, since the microbial protein production could become reduced, which in turn decrease the rate of fibre degradation. These effects may also imply a reduced passage rate out of the reticulorumen (Sampaio *et al.,* 2010).

2.3.2 Effects of feed and water

A study by Fraley *et al.* (2015) showed that the dietary addition of potassium carbonate, which led to an increased water intake, was positively and linearly related to the passage rate of liquid digesta through the ROO. The positive effect was shown for up to nine hours after feed intake. Similar results have also been shown considering feed intake and passage rate of the solid digesta fraction; Okine and Mathison (1991) showed that an increased DMI increased the passage rate of solid digesta through reticulorumen, where the increased DMI also was connected to a reduced digestibility of DM, cell wall materials and organic matter. A reduced NDF digestibility at greater feed intake has also been observed by Robinson *et al.* (1987).

By comparing different studies, roughage has the longest retention time of the different feed types (Cherney *et al.,* 1991; Minde & Rygh, 1997; Lee & Hristov, 2014; Ahvenjärvi *et al.,* 2018), followed by the concentrate (Minde & Rygh, 1997), while the liquid has the shortest retention time (Ahvenjärvi *et al.,* 2018). Minde and Rygh (1997) did for example observe a ruminal MRT of 36.2 h and 19.8 h for silage and concentrate, respectively, using the Cr mordanted fibre marker administered as a pulse dose. Contrary, Ahvenjärvi *et al.* (2018) showed a mean TMRT of about 15.7 hours of the liquid marker PEG. This knowledge is for example applied on passage rate calculations used in the Nordic feed evaluation system (NorFor) (Volden & Larsen, 2011). Separate calculations are developed and used for different feed types and feed components (Volden & Larsen, 2011).

Published data has shown that different forage-types has different passage rates, where grass silage is shown to have a slower passage rate of iNDF compared to diets based on corn silage and alfalfa silage (Krizsan *et al.,* 2010). If considering the passage of liquid, Poppi *et al.* (1981) showed that the water passage through the ROO increased with 25 % if changing the diet from stem fractions of grasses to more leafy fractions. The same study also showed that the retention time of lignin in the rumen increased with 37 % if fed the stem fraction. Considering the physical appearance of the feed Teimouri Yansari & Primohammadi (2009) showed that a reduced cutting length from 9.13 to 4.51 and 1.20 mm of alfalfa increased the passage rate of the solid digesta fraction. Morphological differences of ingested particles can thus affect the passage rate, where Cherney *et al.* (1991) concluded that: *"…representative samples of all fractions fed to measure retention times may lead to a better understanding of [the] ruminal function…"*.

If considering smaller constituents in the feed, it was shown that an increased sucrose content from 17 to 30 % of DM in the feed, increased the rumen passage rate of NDF and decreased the ruminal NDF digestion rate as well as its total digestibility (Stensig, *et al.,* 1998a). The same

study also showed that an increased starch content from 15 to 26 % of DM decreased the rumen passage rate and digestion rate of NDF. As a consequence, this resulted in a non-affected ruminal digestibility of NDF (Stensig, *et al.,* 1998a). The authors thought that the increased passage rate of NDF with increased sucrose level could be connected to the numerically increased passage rate of liquid, which in turn was thought to be affected by an altered osmotic pressure in the rumen when the sucrose levels got elevated (Stensig, *et al.,* 1998a). The potential reason for de altered passage rate with changed starch level was however not clearly discussed.

2.4 Non-feed characteristics that (may) affect the passage rate

Many factors, environmental as well as individual, affect the passage rate of digesta. Some of these factors may also be closely linked to each other. This sometimes makes it hard to distinguish them from each other, but some of these non-feed characteristics will be presented below.

Aikman *et al.* (2008) showed that Jersey cows had a shorter TMRT and rumen MRT of Cr mordanted fibre compared to Holstein cows. It was also shown that DMI was greater among the Holstein compared to the Jersey, but not if considering DMI/kg BW. The breed difference was inter alia thought to be due to the Jerseys smaller body size and greater chewing and rumination per bite of feed (Aikman *et al.,* 2008), which indicates the breed impact on passage rate.

If considering different periods during the production cycle, a study on Holstein cattle performed from late lactation, throughout the transition period and early lactation, showed that the ruminal passage rate of solid digesta increased during the dry period even though the feed intake was reduced (Park *et al.,* 2011). However, the cattle had different feed rations along the production circle where the dry period had a higher forage DM content compared to the other periods (Park *et al.,* 2011). An earlier study performed on grazing Jersey cattle, showed that the rumen fill capacity was smaller among the pregnant, dry cattle compared to their lactating, monozygotic twins (Tulloh & Hughes, 1965). This was however not shown in the Holstein study, where instead a numerical increase of the rumen fill capacity was observed during this period (Park *et al.* 2011). As mentioned, the diet was changed during the study period in the Holstein study (diets for lactation, early dry period, late dry period and early lactation) which could describe some of the outcomes of passage rates (Park *et al.* 2011). However, the mentioned reduced feed intake, in combination with the increased passage rate of solid digesta during the dry period, was discussed and suggested to be primarily caused by "physiological changes" that happens during this period and accordingly not as much by the feed intake and particle size (Park *et al.,* 2011). The same study also showed a significant and linear increase of the ruminal passage rate of liquid digesta during the transition period, but not if observing the whole dry period together, nor if observing the early lactation period (up to 90 days in milk) (Park *et al.,* 2011).

2.5 Milk production and passage rate

Milk yield is affected by several animal as well as environmental characteristics, such as breed (Cattle Statistics, 2018), feed ration, parity, lactation week (Phillips, 2010), milking system (Wingren, 2018) and health status (Edwards & Tozer, 2004). On today's Swedish dairy farms, individual feed rations of concentrates are often practiced. It is therefore hard to distinguish whether the cows' individual ability to produce milk is due to the cow's genetics or their feed ration (Krizsan *et al.,* 2013). Cows can alter their milk yield by changes in energy intake (Nielsen

et al., 2007), something which may also be adjusted throughout the lactation by the farmer. Due to the multifactorial impact on milk production, a limited amount of studies comparing milk yield to passage rate was found suitable for this literature study. Available studies generally focus on other features such as; different diets in combination with passage rate and production caused by the diets (Rogers *et al.,* 1985), or differences between breeds, where the collected milk data therefore was statistically analysed for the effect of breed rather than for the effect of MRT (Aikman *et al*., 2008).

Milk yield is positively related to DMI (Hristov *et al.,* 2000, Nielsen *et al.,* 2007) and DMI was earlier mentioned to be positively related to the passage rate of NDF out of the reticulorumen (Okine & Mathison, 1991). The earlier examined competition between digestion rate and passage rate, and the passage rate's impact on the nutrient intake and uptake among cattle are central considering productivity. An early study by Shellenberger and Kesler (1961) investigated the passage rate among 12 Holstein cattle and showed that cows in the "high milk yield group" had a shorter TMRT compared to the "low milk yield group". Included cows were in different stages of lactation and had different feeding levels. Dry matter intake (g/kg BW) was significantly correlated to TMRT (Shellenberger and Kesler, 1961), which agrees with the recently presented research result considering the passage rate of NDF from the reticulorumen (Okine & Mathison, 1991). No relationship was however shown considering apparent digestibility in relation to passage rate (Shellenberger and Kesler, 1961). The higher milk yield with shorter TMRT found by Shellenberger and Kesler (1961) was however different compared to a sheep experiment, which showed that a shorter ruminal MRT of the liquid, as well as solid fraction of digesta, was linked to a lower milk yield (Goopy *et al.,* 2014).

3 Material and method

The present study was performed at the Swedish Livestock Research Centre, Uppsala, Sweden, from May 2017 to January 2018. The experiment was divided into two parts; the marker method study, and the evaluation study. The experimental procedures of these two parts will therefore be presented separately. All cows were treated according to the Swedish Animal Welfare policy SFS: 1988:534, and the studies were approved by the Swedish Animal research Commitée DNR KC99/16 for the marker method study and DNR 5.8.18-16360/2017 for the evaluations study.

3.1 The marker method study

3.1.1 Animals and housing system

The marker method study was a part of a forage intake capacity study (FIC). The FIC study included 39 cows housed in a loose housing system, with an automatic milking system (AMS) (DeLaval VMS^{TM}; DeLaval, Tumba, Sweden), throughout the whole lactation period. Based on lactation period and concentrate feed, 30 of these cows were selected for the present marker method study (13 Holstein and 17 Swedish red). The included cows were multiparous (parity two to seven) and the majority of the cows (16 cows) were in their second parity (*Table 1*). All cows were treated the same way throughout the whole lactation and study period. From May 8th to August 14th, all cows were let out on an exercise pasture where forage intake was restricted and considered as negligible.

Table 1. *The study distribution of parity and breeds, after the first selection of cows in the herd. Hol.=Swedish holstein, SR=Swedish red*

Parity	Number of hol.	Number of SR	Total
2	8	8	16
3	2	2	4
4	2	2	4
5	1	2	3
6	-	1	1
7	-	2	2
total	13	17	30

3.1.1.1 Roughage, concentrate and water

The intake of forage and concentrate (kg), water consumption (kg) and milk yield (kg) was all registered and coupled to the cows' individual neck transponders. Grass silage (On average; 60 % Timothy, 20 % Meadow fescue, 20 % perennial ryegrass) of first cut was offered *ad lib.* in 20 one-by-one-access scale containers where the individual cows' silage consumption was measured in kg fresh weight (BioControl, Rakkestad, Norway). Feed analyses were performed on samples pooled over two weeks throughout the whole study period (February 2017 to January 2018), except during the change of bunker silo, with an interval of 22 days between the analyses. The analyses of DM were performed at 60°C (Åkerlind *et al.*, 2011), ash and crude protein analyses followed the EC No. 152/2009. Neutral detergent fibre (aNDFom) was assayed with a heat-stable amylase and expressed exclusive of residual ash (Chai and Udén, 1998), organic matter digestibility was performed *in vitro* (VOS) (Lindgren, 1979) and megajoules of metabolizable energy (MJ ME) was estimated by calculations according to Lindgren (1983). The cutting length

of the grass was set to 2 cm, and its chemical composition is presented in *Table 2* together with the Swedish NorFor average grass silage of 2017 for comparison. Vitamins (3.75 g per kg DM; VM17, Vilomix, Staffanstorp, Sweden), minerals (164 g calcium, 10 g phosphorus, 120 g magnesium, 77 g sodium, 15 g sulfur, 3.75 g sodium chloride per kg DM) and trace elements were also added and mixed into the grass silage.

Table 2. *The average chemical composition of the first cut grass silage and by-product-based concentrate. The data is shown together with the average Swedish chemical composition of first cut grass silage of 2017 according to NorFor (Foderstatistik, 2017) for comparison (n=50). The standard deviation is presented in the brackets*

Feed	Grass silage 1st cut	Swedish NorFor grass silage 2017	Concentrate By-product-based
DM, g/kg	413 (60.9)	487 (183)	880
Ash, g/kg DM	88 (5.0)	71 (14.2)	91
CP[a], g/kg DM	165 (15.8)	133 (35.8)	164
aNDFom[b], g/kg DM	425 (19.9)	533 (59.4)	442
OMD[c], % of OM	79.9 (2.6)	70 (5.2)	-
MJ ME[d]/kg DM	11.5 (0.5)	10.9	12.3

[a]*CP=crude protein*
[b]*aNDFom=amylase neutral detergent fibre method*
[c]*OMD=organic matter digestibility*
[d]*MJ ME=Mega Joule of metabolizable energy, calculated as ((0.16*VOS)-1.91), where VOS=in vitro organic matter digestibility, VOS=(OMD+2,0)/90*

A conventional by-product-based concentrate (56.6 % unmolassed sugar beet pulp fibre, 12.0 % wheat bran, 9.4 % wheat middlings, 7.0 % distiller's dried grain Agrow feed™ 90, 7.0 % heat treated rapeseed meal Expro©, 2.6 % vegetable fat AkoFeed®Cattle, 2.3 % limestone, 2.0 % molasses of sugar beet, 0.9 % sodium chloride, 0.2 % mineral premix on a DM basis) was offered according to two different feeding plans. The two feeding plans (*Figure 2*) had fixed rations of 10.5 kg DM (10kgC) and 5.25 kg DM (5kgC) concentrate per day, respectively from lactation week 3 to 27. The marker method study was performed during the period of invariable concentrate allowance for all cows (*Figure 2*). The average concentrate ration throughout the whole lactation was 8.2 kg and 4.2 kg DM per day for the 10kgC and 5kgC diet, respectively. The concentrate intake was automatically registered (DelPro, DeLaval International AB) and was offered in four feed dispensers in the loose housing system (FSC400, DeLaval International AB, Tumba, Sweden) as well as in the AMS during milking. The chemical composition of the concentrate is presented in *Table 2* according to data from Lantmännen (IDMJSJV).

Water was offered *ad lib.* in eight water bowls equipped with flow meters by BioControl AS. The water flow was measured in kg and was connected to the cows' individual transponders. The water stations were calibrated in February 2017 and June to July 2018. Two of the water stations needed calibration in 2018, due to a deviation of -11.54% and +8.29%, respectively.

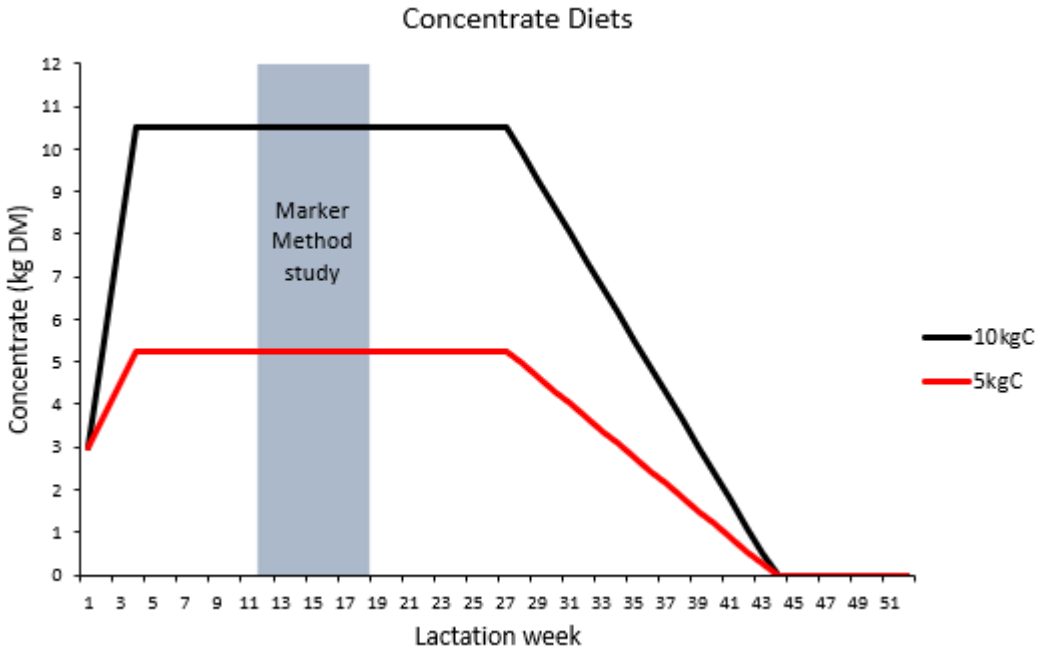

Figure 2. *The concentrate feeding plan for the two diets throughout the lactation. The grey area shows the lactation week distribution during the period of the marker method study, performed during one week for each cow. DM=dry matter, 10kgC=10.5 kg DM concentrate diet, 5kgC=5.25 kg DM concentrate diet.*

3.1.2 Experimental procedure, marker method study

The marker method study was performed during a seven-day period at four different sessions in May, June, August and October of 2017. During the faecal sampling sessions, the cows were in lactation week 12 to 19, with the median of lactation week 15. Each cow was used in one study session only.

3.1.2.1 Body weights, milking, feeding and drinking data

Feeding and drinking data from the earlier mentioned equipment was summarized during the sampling period with the start at 00:00 a.m. in study day one until 11:59 p.m. in study day seven. Milking data was also collected during the same period. An abnormally high forage intake was documented for cow 180 at one day during the faecal sampling session. The compilation of the daily intake was shown to be temporarily reset during one visit at the feed table and a silage intake of 81 kg fresh weight in less than five minutes was registered. This was corrected for by calculation of the mean eating rate (g/s) after removal of the outlier.

The cows' body weights (kg) were registered before entering the AMS at all milking occasions. The available body weight data was collected and summarized during the study week and two weeks ahead of the study. If no data was available for the intended period, due to technical problems, available data closest to the period was used. At the longest, a weight 15 weeks prior the intended period was taken (post calving weight).

Milk samples were automatically collected in the AMS every second week. The milk samples were analysed for fat, protein and lactose, by Fourier transform infrared spectroscopy (FTIR). Milk analysis was alternating between the laboratory of the Department of Animal Nutrition and Management, Swedish University of Agricultural Science, Uppsala, Sweden (CombiScope FTIR 300 HP, Delta Instruments B.V., Drachten, the Netherlands) and Eurofins, Jönköping, Sweden (Eurofins, 2016). If no milk analysis was performed during the sampling period, a mean value from the two closest analysis occasions was used for that cow.

3.1.2.2 ***Feed markers and faecal samplings***

Two different feed markers were used to measure the passage rate. The solid phase was marked with Cr mordanted fibre fractions of roughage prepared according to Udén *et al.* (1980). The liquid fraction was marked with the water-soluble Co-EDTA according to the same article by Udén *et al.* (1980). At study day one, h=0, the markers were inserted to the reticulorumen of the cow by oral administration of the solid marker, and ruminal addition of the liquid marker performed by an orally inserted tube. Ten grams of Co-EDTA and 2 grams of Cr was given, with an exception for the six cows in the first faecal sampling session, which were given 4 grams of Cr. The Cr-dose was evaluated after the first session, and the lower Cr administration of 2 grams was considered enough, which also could reduce the external markers potential impact of the digestibility and passage rate, as discussed by Minde & Rygh (1997). The administered quantity of marker was always held within non-toxic levels (NRC, 2001).

After the administration of the markers, a faecal sampling session was performed at 24 different occasions during a 164-hour period (target hours: 0, 12, 16, 20, 24, 28, 32, 36, 44, 52, 60, 68, 76, 84, 92, 100, 108, 116, 124, 132, 140, 148, 156, 164). If the cow did not defecate, a sample was collected through the rectum. Since the actual sampling time on average differed +/- 9 minutes from target time (the earliest sample was taken -64 minutes prior to target time and the latest sample was taken +113 minutes post target time), the exact time for each faecal sampling was registered and further corrected for before the statistical analyses were performed. All faecal samples were distributed into petri dishes and weighed (~150 g fresh weight) followed by storage at -20°C. After the completion of the first faecal sampling session, the faecal sampling timetable was evaluated. All samples from three randomly chosen cows (cows 369, 376 and 380) were analysed according to the method described below (see 3.1.2.3. “Further selection and sample analyses”). The evaluation resulted in an additional faecal sampling at h=8 in study sessions two, three and four to be able to better catch the excretion peak of the liquid fraction.

3.1.2.3 ***Further selection and sample analyses***

When the four faecal sampling sessions were performed, 13 cows, in addition to the three already analysed cows, were randomly picked for further analysis (*Table 3*). One cow was removed from the study prior to the selection due to health problems during the sampling week. In total twelve 5kgC and four 10kgC cows were randomly chosen. The samples from the collected cows were frozen at -80°C for ≥16 h, followed by freeze drying (≥72 h) and milling by a hammer mill through a 1 mm screen (Slagy 200, Kamas kvarn maskiner AB, Malmö, Sweden). All selected samples were sent to ALS Scandinavia AB (Luleå, Sweden) for Co and Cr concentration analysis performed by the Inductively Coupled Plasma Sector Field Mass Spectrometry (*ICP-SFMS*) method. Cobalt analyses were performed on the samples taken at h 0, 8, 12, 16, 20, 24, 28, 32,

36, 44, 52, 60, 68, 76, 84, 92, 100 and 108. Chromium analyses were performed on faecal samples at h 0, 12, 16, 20, 24, 28, 32, 36, 44, 52, 60, 68, 76, 84, 92, 100, 108, 124, 140 and 164. The selection of sampling hours for analysis was based on the timetable evaluation from three cows in an earlier stage of the study.

Table 3. *The 16 cows included in the analysis. ID=Identity, Hol=Swedish Holstein, SR=Swedish red, conc.=concentrate allowance, lact. w.=lactation week*

ID	Breed	Conc.	Parity	Median Lact. w.	Cr marker pulse dose (grams)	Co-EDTA marker pulse dose (grams)
357	Hol	5kgC	2	13	2	10
380	SR	5kgC	2	13	4	10
382	SR	5kgC	2	14	2	10
402	Hol	5kgC	2	18	2	10
406	SR	5kgC	2	16	4	10
444	SR	5kgC	2	15	2	10
274	SR	5kgC	3	18	2	10
48	Hol	5kgC	4	14	2	10
108	Hol	5kgC	4	14	2	10
979	Hol	5kgC	5	16	2	10
6524	Hol	5kgC	5	14	2	10
1572	SR	5kgC	6	18	2	10
369	SR	10kgC	2	18	4	10
376	Hol	10kgC	2	14	4	10
403	SR	10kgC	2	13	2	10
1518	SR	10kgC	7	15	2	10

3.1.3 Mathematical model derivations in marker method study

A two-compartment model was used to calculate the TMRT of the solid and liquid digesta (Pond *et al.*, 1988; Van Soest, 1994). As stated by Pond *et al.* (1988), the gastrointestinal distribution of the two compartments (pool 1, and pool 2) are not definite. Pool 1, as well as pool 2 follows the age-independent residence time distribution (G1G1). In accordance to Seo *et al.* (2009), the present model presumes that digesta particles that has entered pool 2 cannot go back to pool 1. The excretion curve-fit model follows this formula (Pond *et al.,* 1988; Van Soest, 1994):

$$Cr/Co_{curvefit} = A \cdot \frac{\mathrm{k1}}{k2-k1} \cdot \left(e^{-k_1 \cdot (t-L)} - e^{-k_2 \cdot (t-L)}\right) \quad (1)$$

$Cr/Co_{curvefit}$=Cr or Co concentration (mg/kg DM), A=the estimated marker concentration in pool 1 of the reticulorumen, k1=the estimated passage rate from pool 1 to pool 2 (h^{-1}), k2=the estimated passage rate from pool 2 (h^{-1}), L=lag phase (h), t=time (h). (Pond *et al.,* 1988; Van Soest, 1994).

Curve fitting was performed in the Excel® (2016) program by using the Solver add-in and its non-linear Generalized Reduced Gradient (GRG) engine. The method was also evaluated with a separate curve fitting program (Table curve2D®, Systat Software Inc., San Jose, California, USA) for the solid phase. Maximum and minimum levels for A, k1, k2 and L, were set to help the program to better fit the curve to the measured values. An adaption of $k_1<k_2$ was set according to model limitations established by Udén and Sutton (1993):

Solid phase: $1.000 \leq A \leq 2000$ (mg/kg DM), $0.00 \leq k_1 \leq 0.05$ (h^{-1}), $0.051 \leq k_2 \leq 2.00$ (h^{-1}), $0.00 \leq L \leq 20$ (h)

Liquid phase: $100 \leq A \leq 10000$ (mg/kg DM), $0.00 \leq k1 \leq 0.20$ (h^{-1}), $0.21 \leq k_2 \leq 5.00$ (h^{-1}), $0.00 \leq L \leq 20$ (h)

Total mean retention time was calculated in accordance to Lallès *et al.* (1991):

$$TMRT\ (h) = \frac{1}{k_1} + \frac{1}{k_2} + L \quad (2)$$

Total mean retention time for the solid digesta fraction was abbreviated TMRTCr, and TMRT for the liquid digesta fraction was abbreviated TMRTCo.

3.2 The evaluation study

To evaluate the marker method used in the present study, ruminal evacuations, which is considered a reliable method to measure passage rate (Minde & Rygh, 1997; Huhtanen *et al.,* 2008; Volden & Larsen 2011), were performed. The evaluation study included complete ruminal evacuations at three occasions per cow, followed by a separate marker method study with faecal samplings, identical to the above presented marker method study.

Three Swedish red cows were selected for ruminal fistulation and the evaluation study based on breed, fertility, lactation week and health. A previously ruminally fistulated cow was also included in the study. A fistula with a 10 cm inner diameter (Bar diamond, Inc. Parma, Idaho, USA) was fitted in the dorsal sac of the rumen. The fistulation surgery was at latest performed three months prior to the study.

The ruminally fistulated cows were kept in the same loose housing system as the cows in the marker method study and were offered the same forage and the by-product-based 5kgC diet (see *Table 2*). The cows had a 12-day adaption period to the feeding and housing system before the study.

3.2.1 Ruminal evacuations

Ruminal evacuations were performed in December 2017. Complete emptying of the rumen was performed at three occasions per cow, by following the time intervals of Lund *et al.* (2007) in order to account for diurnal variation in rumen content and digesta composition. Evacuations were performed at 08:00 *p.m.* in day 1, 02:00 *p.m.* in day 3, and 08:00 *a.m.* in day 5. The ruminal content was emptied by hand or by using a sponge or jar. All content was weighed, and a sample was prepared by placing every tenth grab or liquid removal into a separate container. After each complete emptying of the rumen, the non-sample rumen content was put back into the rumen. The whole process of removal, weighing and replacing rumen contents took about 30 to 40 minutes.

The collected sample was further prepared by separating the solid and liquid phases by using a strainer with approximately 0.8 cm openings. The liquid and solid phases were thereafter weighed separately, followed by calculations of the weight proportions of the two phases. Representative samples of the solid phase, and the liquid phase, were thereafter distributed into four petri dishes per cow and evacuation session. Each petri dish contained the calculated proportions of liquid and solid phase and contained 150 grams of ruminal content each. The samples where thereafter stored in -20°C.

Faecal samples, as well as samples of the roughage and concentrate was taken adjacent to each ruminal evacuation. The individual silage intake (BioControl, Rakkestad, Norway), concentrate intake and water intake (BioControl AS) was measured from 00:00 a.m. in day one, to 08:00 a.m. in day five.

3.2.2 Faecal sampling session

After the rumen evacuation sessions, marker-based faecal samplings were performed from late December 2017 to early January 2018. The practical procedure and the oral administration of Cr mordanted fibre and Co-EDTA was performed according to the marker method study described earlier in this thesis. Faecal samples were collected at h 0, 8, 12, 16, 20, 24, 28, 32, 36, 44, 52, 60, 68, 76, 84, 92, 100, 108, 116, 124, 132, 140, 148, 156, 164. Feed intake data and production data was collected and observed during the faecal sampling period but will not be presented in the present thesis.

3.2.3 Feed intake corrections

Cows 1565 and 451 had abnormally high forage intakes during the study period. This was supposed to be due to throwing of silage. According to earlier silage intake data from Lövsta research station, Uppsala, Sweden, 2015, cow's individual eating rate varied from about 3 g/s to 9 g/s (personal message Torsten Eriksson, Associate professor in feed science, Uppsala, Sweden, 2018-07-13). A feed intake higher than 10 g/s per visit at the feed table was therefore corrected for among these two cows at days ingesting >90 kg fresh weight of silage during the rumen evacuation period. The highest non-corrected daily silage intake among these two cows was 77 kg fresh weight, equal to 26 kg DM. Correction was made according to the individual cows' average intake rate (g/s) after removal of the DMI outlier meals. The real silage intake for the two cows might have become limited during three days for cow 1565, and one day for cow 451 during the marker method period. One day might also have become limited for cow 1565 during

the rumen evacuation period. This is because cows are not allowed to enter the feed table if the registered forage intake has reached 99 kg fresh weight (an intake level that cows normally does not reach).

3.2.4 Analyses, evaluation study

The pooled rumen content samples, faeces, silage and concentrate samples were dried in 60°C followed by milling by a hammer mill through a 1 mm screen (Slagy 200, Kamas kvarn maskiner AB, Malmö, Sweden). Analysis of NDF was performed with a heat-stable amylase and expressed exclusive of residual ash (Chai and Udén, 1998). Indigestible NDF was determined by *in-sacco* incubation for 288 h (Åkerlind *et al.*, 2011). Dry matter analysis was performed at 60°C (Åkerlind *et al.,* 2011), ash analysis followed the method of EC No. 152/2009.

Chromium and Co analysis from the marker-based faecal sampling period was prepared and performed according to the marker method study described above (see 3.1.2.3 "Further selection and sample analyses").

3.2.5 Model derivations, evaluation study

The Cr and Co concentrations from the faecal samples were mathematically handled by the same model as in the marker method study (see 3.1.3 "Mathematical model derivations in marker method study"). The mean iNDF intake per day was calculated for each cow. The mean ruminal iNDF pool (g) of the three ruminal evacuations was then calculated for each cow, followed by an intake-based calculation of TMRT (TMRTin) according to the mathematical model described in the following sections.

3.2.5.1 Intake-based calculations

The intake-based passage rate (% per h) was calculated in accordance to Huhtanen *et al.* (2007) by divisions of the mean iNDF intake/day (g) with the rumen iNDF pool (g). To get the passage rate denoted as percent per hour, the quota was divided by 24 (*calculation 3*):

$$Intake-based\ passage\ rate\ (\%\ per\ h) = \frac{\left(\frac{(mean\ iNDF\ intake/day,g)}{(Rumen\ iNDF\ pool,g)}\right)}{24\ h} \quad (3)$$

Where TMRTin (h) was achieved by calculating the inverse of the intake-based passage rate (percentage per hour) denoted in decimal form (*calculation 4*). The calculation followed a one compartment model:

$$TMRTin\ (h) = \frac{1}{Intake\ based\ passage\ rate\ (\%\ per\ h)} \quad (4)$$

4 Statistical methods

All statistical analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC, 2008). The min-, mean- and max values for the different treatment groups and silage data were calculated with Proc MEANS or by Excel® (2016).

TMRTCr values (n=14) for the two treatment groups (5kgC and 10kgC) in the marker method study was subjected to the MIXED procedure by using the model:

Yij = ForageDMI_BW + Treatment_i + $(\text{ForageDMI\_BW} \times \text{Treatment})_i + e_{ij}$

where the terms are: continuous effect of forage DMI per kg of BW; the fixed effect of the two concentrate diet treatment groups ($_j$ =2) and the random error, e_{ij}. Least square means were calculated using LSMEANS/PDIFF.

Proc CORR, Pearson correlation coefficients, were used to analyse all individual information about the cows in the 5kgC treatment group. In total 24 parameters were included in the analysis: sampling group, breed, parity, lactation week, BW (kg), DMI (kg/day), milk yield (kg/day), kg ECM (day^{-1}), drinking water (kg/day), TMRTCr (h), TMRTCo (h), feed efficiency calculated as kg ECM/DMI (kg/day), and more. The Proc CORR analyses were performed with focus on the combinations of parameters that were considered relevant for the present study. All 24 parameters are presented in *appendix I.* After the primary analyses using Proc CORR, data relevant for this master thesis were analysed using Proc REG. Proc REG analyses were performed to visualize eventual linear relationships and were indirectly used to get mean squared error (MSE) and the coefficient of determination (R^2).

A t-test was also performed in Excel® (2016) to compare breed in relation to milk yield within the 5kgC treatment group.

Proc CORR was similarly used for the evaluation study. TMRTCr and TMRTin were subjected towards each other. Proc REG was used to visualize the potential linear relationship and was indirectly used to get MSE and R^2.

Statistical significance in the present study was declared by $p \leq 0.05$.

5 Results

5.1 Marker method study results

The average TMRTCr for the 5kgC and 10kgC group was 52.8 and 48.9 h, respectively. The average TMRTCo was smaller than the solid phase with 12.8 h for the 5kgC and 11.1 h for the 10kgC treatment group. A compilation of the average values of intake data, production data and TMRT for the solid and liquid fractions of digesta for both treatment groups are presented in *Table 4.* Lactose was not included in the ECM calculation due to missing of data for four cows. Kg ECM was calculated according to Spörndly (2003).

Table 4. *Mean values and range (in brackets) for cow material, productive data, and total mean retention time for the solid and liquid digesta fraction (TMRTCr and TMRTCo) measured with two different chemical markers. The cows had free access of grass silage and were fed either 5 kg (5kgC) or 10 kg (10kgC) DM concentrates daily*

	5kgC (n=12)	10kgC (n=4)
Body weight, kg	713 (578-884)	677 (627-765)
Forage DMI, kg/day	19.8 (14.9-23.9)	16.8 (15.9-18.2)
Concentrate DMI, kg/day	5.1 (4.8-5.3)	10.4 (10.3-10.6)
Total DMI, kg/day	24.8 (19.9-28.8)	27.3 (26.3-28.7)
Drinking water intake, kg/day	113 (89-147)	121 (102-141)
Total water intake	139 (108-179)	141 (126-160)
Milk yield, kg/day	34.2 (27.3-39.9)	40.0 (35.0-46.7)
Milk yield, kg ECM/day	33.9 (27.1-40.5)	38.3 (35.1-44.6)
Kg ECM/kg DMI	1.4 (1.1-1.7)	1.4 (1.2-1.7)
Fat, %	4.1 (3.2-4.8)	3.8 (2.9-4.7)
Protein, %	3.2 (2.5-3.7)	3.2 (3.0-3.4)
TMRTCr, h	52.7 (46.7-63.2)[a]	48.9 (46.9-50.9)
TMRTCo, h	12.8 (9.1-15.1)[b]	11.1 (9.6-14.1)[c]

[a] *n=10,* [b] *n=5,* [c] *n=3.*

5.1.1 Solid phase

According to a control sample, the coefficient of variation of the Cr concentration analysis (*ICP-SFMS)* was 29 %. After the completion of the curve fit for each cow, the solid phase (Cr) data was removed for cow 274 and 382. This was made due to an R-value lower than the determined lowest limit of 0.90 between the predicted curve and the observed Cr concentrations at the different faecal sample occasions. Two additional cows (48 and 6524) had an R-value lower than 0.90 because of single outlier samples. These two cows were not removed from the data, neither the outlier samples. The average Cr concentration among the included cows (n=14) at h=0 was 1.66 mg Cr/kg faecal DM (range 1.08 - 3.51 mg/kg DM). Two randomly chosen excretion curves of the Cr mordanted fibre are shown in *Figure 3 a* and *b*.

The statistical analyses performed on the 5kgC and 10kgC treatment groups together (n=14) showed that there was no linear relationship between forage DMI per kg BW and TMRTCr (P=0.76) and that TMRTCr was not affected by concentrate allowance (P=0.72): LSmeans 52.8 ± 2.1 and 48.3 ± 9.4 hours for 5kgC and 10kgC, respectively. The DMI/kg BW for both 5kgC and 10kgC together were plotted against TMRTCr (*Figure 4*).

Breed (P=0.50) and BW (P=0.57) had no significant relationship to TMRTCr among cows in the 5kgC treatment group.

Figure 3 a.

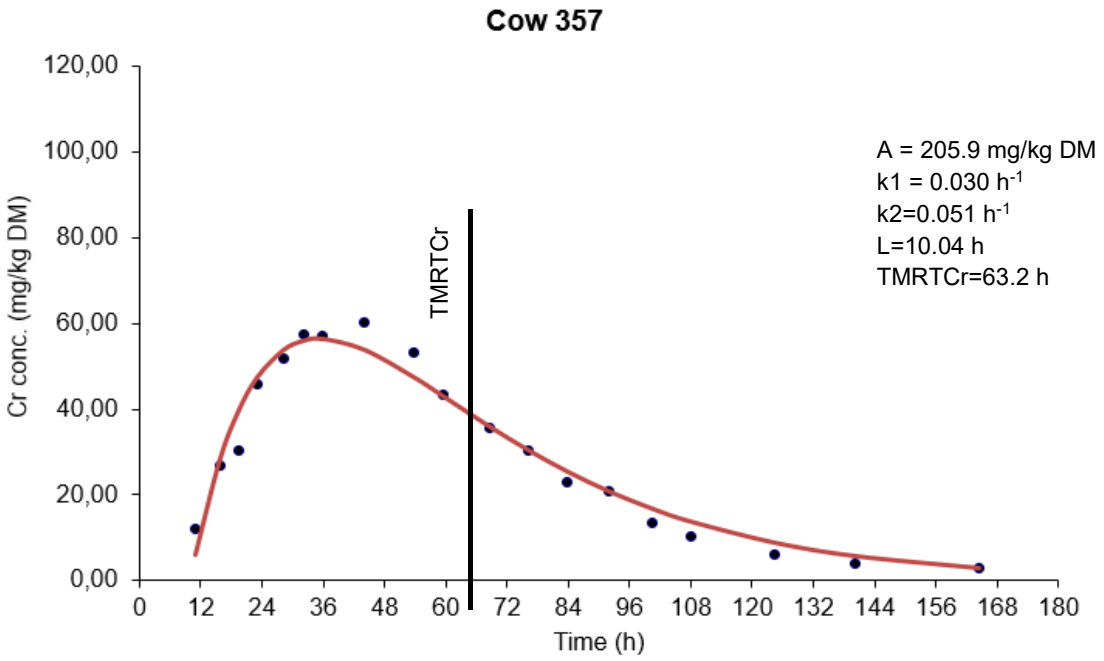

Figure 3 b.

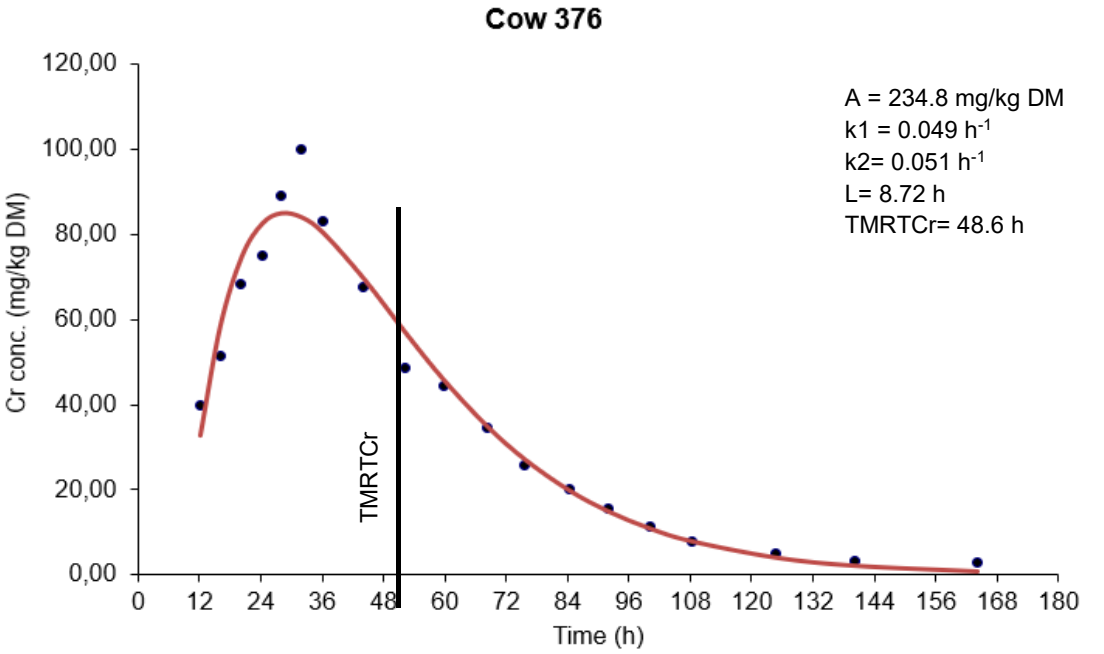

Figure 3 a, b. *The excretion curve of the chemical marker of chromium mordanted fibre measuring the total mean retention time of the solid digesta fraction (TMRTCr) for two randomly chosen cows from the marker method study, cow 357 and 376. The plots were made in Excel® (2016) by making curve fittings with the Excel Solver add in tool and by using the mathematical model described in chapter 3.1.3. The mathematical model solutions of "the pulse dose of chromium" (A), "pool 1" (k1), "pool 2" (k2), "the lag phase" (L) and "the total mean retention time of the solid phase" (TMRTCr) are presented beside each curve.*

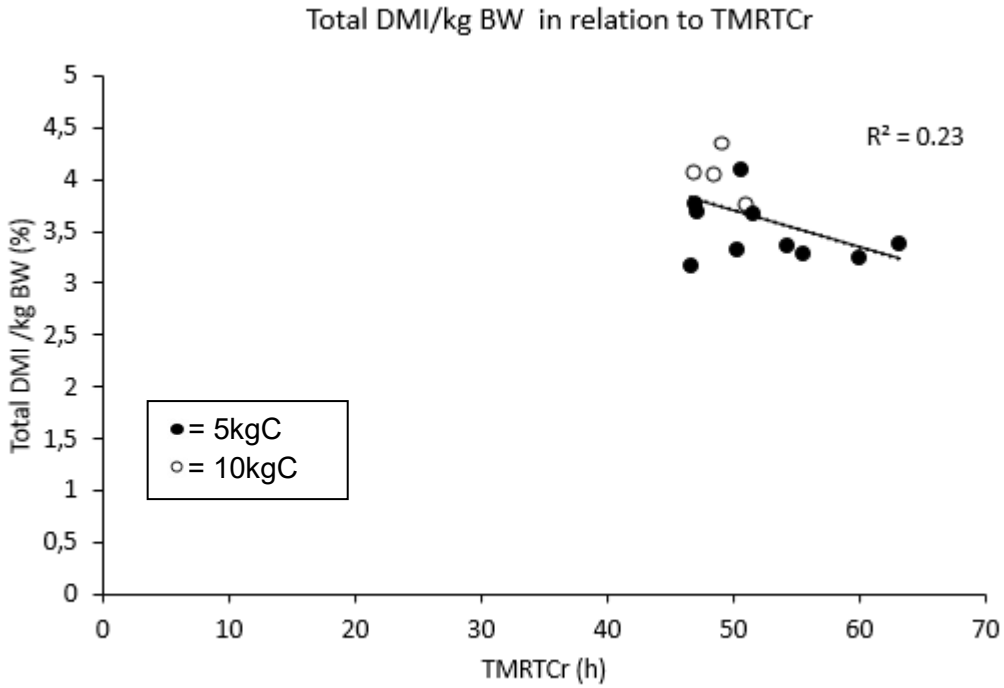

Figure 4. *The dry matter intake per kg of body weight (total DMI/kg BW) compared to total mean retention time of the solid digesta fraction (TMRTCr) measured with an orally inserted pulse dose of chromium mordanted fibre. The figure is a plot containing both diet treatment groups (5kgC and 10kgC). The non-filled marks represent the 10kgC treatment group (n=4) and the black marks represent the 5kgC treatment group (n=10). The plot was made in Excel® (2016). This data was not statistically analysed.*

5.1.2 Liquid phase

Cobalt concentration analyses were not performed on eight out of the 16 cows since the collected faecal samples did not catch the peak excretion of the marker for most of the thitherto analysed cows. The Co analyses and the curve fitting in Excel® (2016) Solver was performed for cows 48, 274, 369, 376, 379, 380, 382 and 1518 where the peak excretion of the Co-EDTA marker was captured for one of the eight analysed cows. According to a control sample, the coefficient of variation for the Co concentration analysis (*ICP-SFMS*) was 19 %. The Co concentration at 84, 92 and 100 h were removed from the final mathematical analysis for all cows, to make a better fit of the curve, since the Co concentration reached 0 mg/kg DM several faecal samples earlier. The faecal samples at h=0 had a mean value of 1.66 mg/kg DM with a min and max of 1.08 and 3.51 mg/kg DM, respectively. Two randomly chosen excretion curves of Co are shown in *Figures 5 a* and *5 b*.

Figure 5 a.

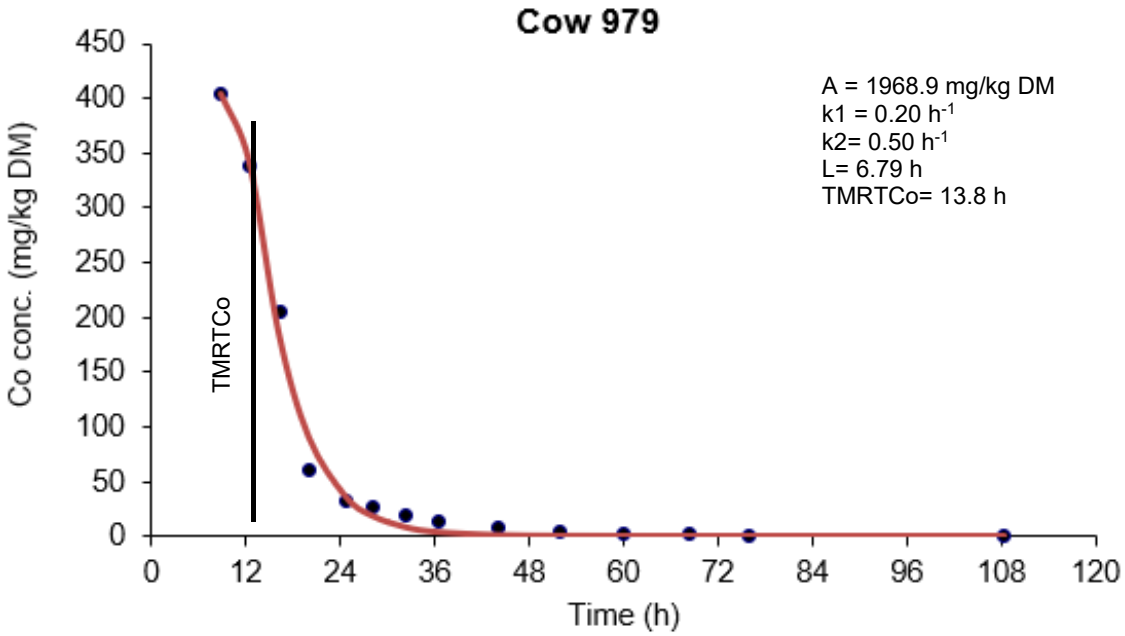

Figure 5 b.

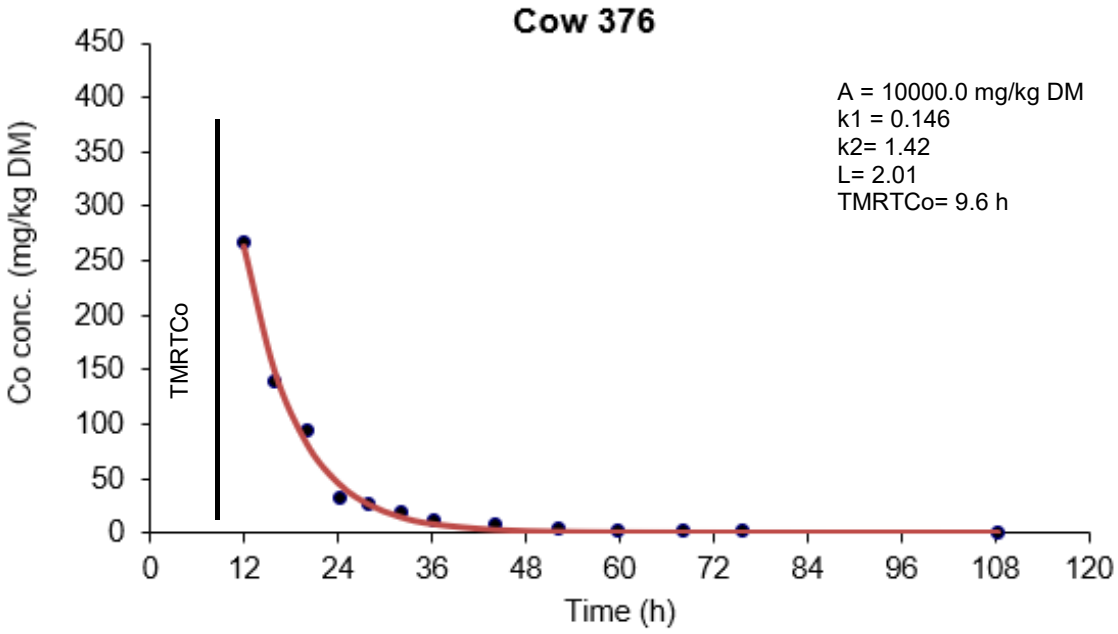

Figure 5 a, b. *The excretion curve of the chemical marker of cobalt EDTA measuring the total mean retention time of the liquid digesta fraction (TMRTCo) is shown for two randomly chosen cows included in the marker method study, cow 979 and 376. The plots were made by using Excel® (2016) Solver add in tool and the mathematical model described in chapter 3.1.3. The mathematical model solutions of "the pulse dose of cobalt" (A), "pool 1" (k1), "pool 2" (k2), "the lag phase" (L) and "the total mean retention time of the liquid phase" (TMRTCo) are presented beside each curve.*

5.1.3 Milk production and feed efficiency

Regression analyses of milk production on TMRTCr was performed for the 5kgC group (n=10). There was no significant relationship between TMRTCr and kg ECM/day (P=0.22 and R^2=0.18) (*Figure 6*). There was neither any significant relationship between TMRTCr and kg milk/day (P=0.87 and R^2=0.00). Milk yield was however significantly related to breed (P=<0.05) where the Holstein had an average milk yield of 37.2 kg/day and the Swedish red had an average milk yield of 31.1 kg. Lactation number (P=<0.05 and R^2=0.50), body weight (P=<0.05 and R^2=0.35), DMI of silage (P=<0.05 and R^2=0.47) and total DMI (P=<0.05 and R^2=0.49), was also significantly and positively related to milk yield (kg). Energy corrected milk was not significantly connected to neither breed (P=0.42), lactation number (P=0.14), body weight (P=0.19), DMI of silage (P=0.31), nor total DMI (P=0.26).

A significant and positive relationship was shown between TMRTCr and feed efficiency (calculated as kg ECM/kg DMI) in the 5kgC (n=10) treatment group. The relationship had an R^2 value of 0.56 and a p-value of <0.05 (*Figure 7*).

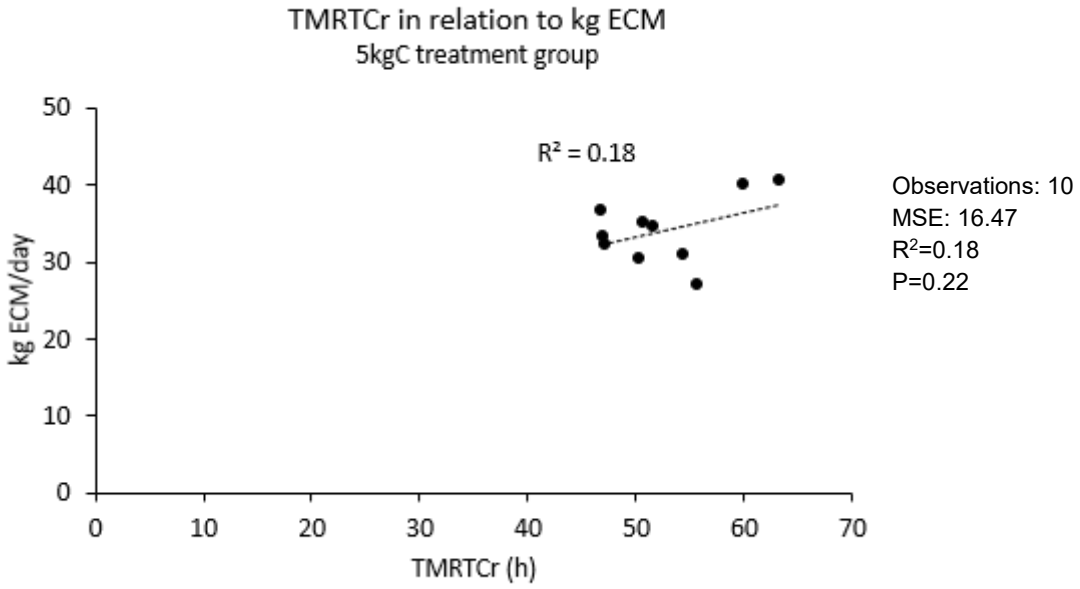

Figure 6. *Kilo gram energy corrected milk/day (kg ECM/day) compared to the total mean retention time of the solid digesta fraction (TMRTCr), measured with an orally inserted pulse dose of chromium mordanted fibre among 10 cows. All included cows had the 5kgC diet. Mean squared error (MSE), R^2 and the p-value are also presented. The plot was made in Excel® (2016).*

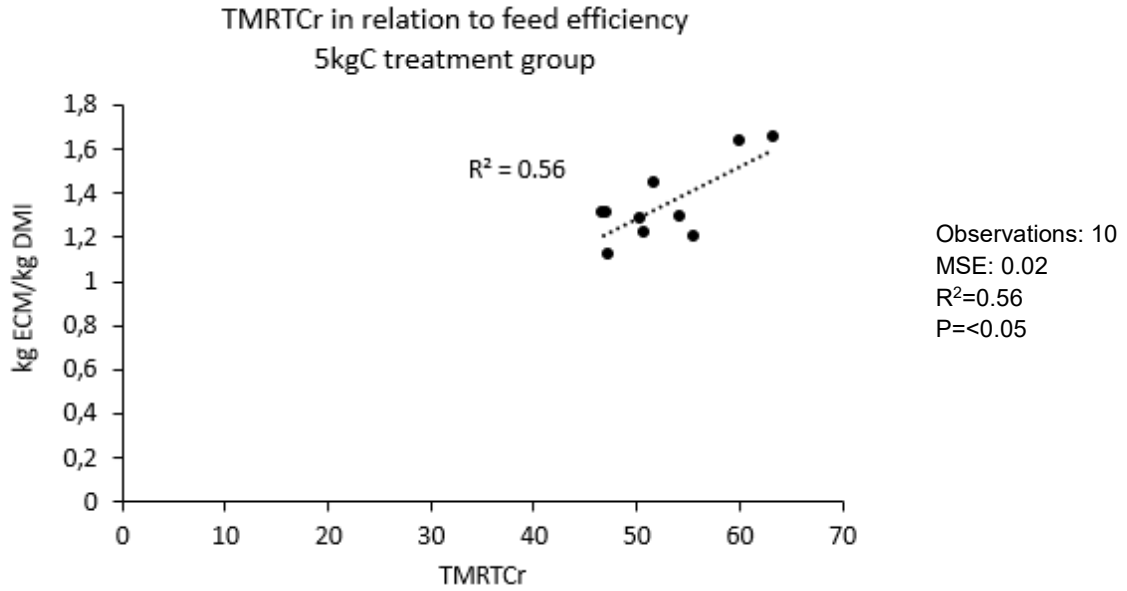

Figure 7. *Feed efficiency (kg ECM/kg DMI) compared to the total mean retention time of the solid digesta fraction (TMRTCr), measured with an orally inserted pulse dose of chromium mordanted fibre among 10 cows. All included cows had the 5kgC diet. Mean squared error (MSE), R^2 and the p-value are also presented. The plot was made in Excel® (2016).*

5.2 Evaluation study results

Since the marker method (amongst other methods to measure passage rate) rarely has been evaluated (Stensig *et al.,* 1998b; Huhtanen *et al.,* 2008), the rumen evacuation technique, which is considered a reliable method (Minde & Rygh, 1997; Volden & Larsen 2011; Huhtanen *et al.,* 2008), was implemented to evaluate the Cr mordanted fibre marker.

The ruminal and faecal average iNDF:NDF ratios were 26 % and 44 %, respectively. The average ruminal fresh weight was 108 kg, and its average DM content was 14.72 kg, with a min of 11.40 kg DM and a max of 16.53 kg DM. The mean rumen fresh weight accounted for 16 % of the total body weight on average. The pooled faecal samples had an average DM value of 15 %, determined by freeze drying. Feed intake data, TMRT-data and data needed for the intake-based calculations of TMRT are presented in *Table 5*.

The SAS proc reg analysis of TMRTin to TMRTCr (*Figure 8*) showed no significant relationship (P=0.16 and R^2=0.71).

Table 5. *Results and data from the evaluation study performed on the four ruminally fistulated cows. All data, except TMRTCr, is achieved from the rumen evacuation period. Min and max values are presented within brackets*

	Evaluation cows n=4
Body weight, kg	733 (646-780)
Silage aNDFom[1], g/kg DM	420
Silage iNDF[2] content, g/kg NDF	118
Concentrate aNDFom[1], g/kg DM	324
Concentrate iNDF[2] content, g/kg NDF	123
Silage DMI[3]/day	19.05 (14.4-22.6)
Concentrate DMI[3]/day	5.2 (5.1-5.3)
Total DMI[3]/day	24.3 (19.54-27.88)
iNDF[2] intake, g/day	1152 (918-1329)
Rumen iNDF[2]-pool, g	1766 (1365-2049)
Passage rate, intake-based, %/h	2.74 (2.28-2.99)
TMRTin[4], h	36.95 (33.42-43.91)
TMRTCr[5], h	51.0 (47.4-56.3)

[1]aNDFom=amylase neutral detergent fibre method, [2]iNDF=indigestible neutral detergent fibre, [3]DMI=dry matter intake, [4]TMRTin=intake-based total mean retention time of the solid digesta fraction measured by the rumen evacuation method, [5]TMRTCr=total mean retention time of the solid digesta fraction measured by chromium mordanted fibre.

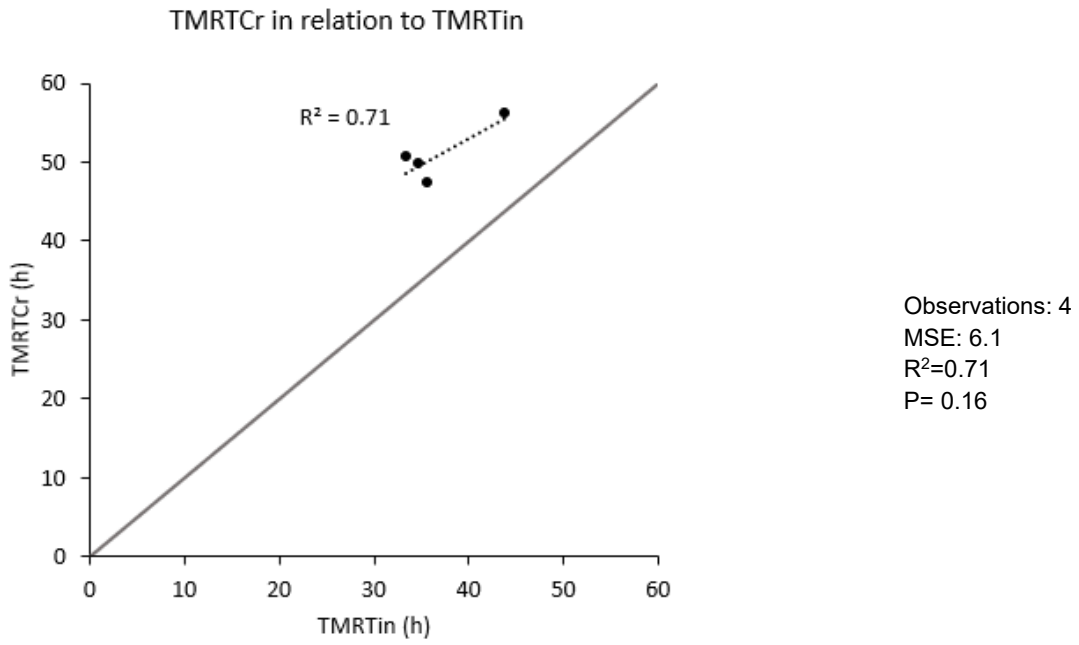

Figure 8. *Results from the evaluation study showing the relationship between the two used methods of measuring total mean retention time (TMRT) of the solid digesta fraction. The TMRT by using the chemical marker of chromium mordanted fibre, h (TMRTCr), is compared to the TMRT achieved by the rumen evacuation method; the intake-based TMRT, h (TMRTin). The fixed line is x=y. Number of observations, error degrees of freedom (error DF), mean square error (MSE) and R^2 is presented separately beside the plot. The plot was made in Excel® (2016).*

6 Discussion

The present study investigated the TMRT of liquid and solid digesta performed by oral administration of Cr mordanted fibre and ruminal addition of Co-EDTA among cows with two different concentrate rations and *ad lib.* access to high-quality silage. The study was performed to investigate the individual differences in TMRT of solid and liquid digesta in relation to forage intake capacities. It was also investigated whether there was a relationship between TMRTCr and milk yield, and TMRTCr and feed efficiency (kg ECM/DMI). Chromium mordanted fibre as a marker for the solid digesta fraction was also evaluated by the performance of a rumen evacuation study on four ruminally fistulated Swedish red cows.

6.1 The marker method study

6.1.1 Total mean retention time of the solid phase

The 10kgC diet had a slightly numerically shorter TMRTCr compared to the 5kgC diet. The individual variation in TMRTCr within the 5kgC treatment group was however larger than within the 10kgC treatment group and overlapped the max as well as min value for the 10kgC treatment group (*Table 4*). This outcome is however not unexpected since the 5kgC treatment group included data from ten cows, while only four cows were included the 10kgC diet. The different concentrate diets had no significant impact of the TMRTCr. The lack of difference in TMRTCr between the treatment groups might be explained by the high-quality forage which had a low NDF content compared to the Swedish average forage of 2017 (*Table 2*). The NDF content in the offered silage was therefore quite similar to the NDF content of the by-product-based concentrate (*Table 2*). The TMRTCr variation of 46.7 h to 63.2 h in the 5kgC diet demonstrates that there is a large individual variation in passage rate among cows fed an *ad lib.* high-quality forage diet. The large variation accounts for the need of further investigations considering individual differences in passage rates among dairy cattle. Parts of the variations in the present study might be described by characteristics such as body weight and breed, even if the present study could not show any significant relationship between these characteristics and TMRTCr when tested by proc Corr or separate means for different breeds with a t-test.

No significant relationships between DMI and TMRTCr were seen within the 5kgC treatment group, neither when considering forage DMI per kg BW and TMRTCr performed on both treatment groups together. The variation in TMRTCr could thus not prove the hypothesis that TMRT(Cr) is negatively related to the cows' individual forage intake capacity. To make further statistical analyses, accounting for differences in breed, parity, BW *et cetera* could potentially explain the eventual relationships between forage intake and passage rate. This is theorised since Huhtanen & Kukkonen (1995) has shown that the passage rate increases with increased feed intake, and because Paloheimo & Mäkelä (1959) showed a curvilinear negative correlation between DMI and the reticulorumen MRT of DM. However, Robinson *et al.* (1987) who measured the rumen digesta passage rate with Cr mordanted fibre and rumen evacuations found that the Cr mordanted fibre had the highest passage rate at intermediate feed intakes, while the rumen evacuation method, in accordance with Huhtanen & Kukkonen (1995) resulted in a decreased passage rate out of the rumen when feed intake decreased.

6.1.1.1 Feed efficiency and milk production

A significant and positive relationship was shown between TMRTCr and feed efficiency (ECM/DMI) among the cows in the 5kgC treatment group. This means that a long retention time is associated with a higher feed efficiency. Since Meng *et al.* (1999) observed a reduced digestibility and an altered microbial activity when the dilution rate (passage rate) of liquid digesta was increased, the relationship between feed efficiency and TMRTCr in the present study was thought to be due to the same function. The anticipated reduced fibre degradation would thus potentially reduce the degradability of other nutrients as well, since these might get trapped inside the less digested feed particles. The cows individual NDF digestibility, and organic matter digestibility, was however not investigated in the present study. It would be motivated to further analyse this, to better understand and motivate what part of the digestibility that is most affected by the reduced feed efficiency.

Regarding the milk production, no significant relationship was shown for neither kg ECM nor milk yield (kg) when subjected to TMRTCr in the 5kgC treatment group. Since milk production is known to be affected by several characteristics on individual (Edwards & Tozer, 2004; Phillips, 2010; Cattle Statistics, 2018), as well as environmental level (Phillips, 2010; Wingren, 2018) it may be suggested that the impact of other characteristics singly or collectively affect the milk yield more than TMRTCr does on its own. In the present study, breed, lactation number, body weight and DMI of silage had a significant impact on the milk production (kg), which demonstrates that these characteristics outcompetes the potential impact of TMRTCr. No significant relationships were however shown between kg ECM and breed, parity, body weight, DMI of silage or total DMI. The present study did not select for animals of the same breed, parity, *et cetera*. To find potential relationships considering milk yield and TMRT, a statistical model accounting for differences in breed and body weight *et cetera* would probably be preferred but was not used due to a low number of cows. To select animals that has similar characteristics could be another potential solution. Data from more cows might also be needed.

The previous discussed significant, positive relationship between TMRTCr and feed efficiency may also open for the discussion whether a potential future selection for cows with fast passage rate is a way of counterworking the rumen chamber and the microbial degradation potential, even though it is associated with DMI (Okine and Mathison, 1991) which in turn is linked to milk production (Hristov *et al.,* 2000, Nielsen *et al.,* 2007). The first sentence of this master thesis was that ”Ruminants has the unique ability to digest and utilize energy from cell wall materials”, an ability which thus become reduced due to a shorter retention time in the reticulorumen. The ability to digest and utilize energy from cell wall materials at different TMRT would be interesting to further investigate in relation to productivity. An optimal balance between passage rate and digestibility is central, since these two parameters are both competing and affecting each other (Van Soest 1994; Fox *et al.,* 2004; McDonald *et al.,* 2011).

6.1.2 Total mean retention time of the liquid phase

The TMRTCo was shorter comparted to TMRTCr, which was expected since it is consistent to comparisons between other studies measuring the liquid and/or solid fraction of digesta (Cherney *et al.,* 1991; Minde & Rygh, 1997; Volden & Larsen, 2011; Lee & Hristov, 2014; Ahvenjärvi *et al.,* 2018). The results of the liquid phase in the present study does however have a large uncertainty level since the curve-fitting was not trustworthy. Statistical analyses of TMRTCo were

therefore not performed in the present master thesis. The curve-fitting will be further discussed later in this discussion. Additional uncertainty factors considering TMRTCo, except the upcoming curve fitting issues, are that the faecal samplings did not catch the peak excretion of the marker for seven out of the eight cows (see example in *Figure 5a* and *5b*), which reduces the ability to make an accurate curve fit. The calculated TMRTCo was at some occasions shown to be passed before the first post marker-dosing faecal sampling was performed (see *Figure 5b*). However, the curves tended to be flattened out around h=8 for two cows (see example in *Figure 5a*), which indicate that the peak excretion may have been close in time to the first faecal sample, but earlier than h=8. Additional faecal samplings, earlier than h=8, and collection of faecal samplings with shorter intervals than 4 h during the first 24 hours, could easier catch the excretion curve of the Co-EDTA marker. Another study measuring the passage rate of the liquid fraction made the first post-dose faecal samplings at h=4 (Ahvenjärvi *et al.,* 2018). This was followed by further faecal samplings with tighter intervals compared to the present study: every second hour until h=24, followed by greater intervals until the last collection at h=96 (Ahvenjärvi, *et al.,* 2018). This, or something similar, could potentially be put into practice in a future study.

6.1.3 Curve fitting

The curve fitting which was performed on the data from all analysed cows was performed in a pre-programmed Excel sheet, using the Excel® (2016) Solver add in. The solid digesta data was also evaluated using a separate curve fit program, Table curve2D®.

Minor differences in the numerical outcomes of A, k1, k2, L, and TMRTCr were shown depending on curve fit occasion. These differences were considered as negligible, but no statistical comparisons were performed to compare these different outcomes. No visual differences on the curves could be seen on the occasions with different numerical outcomes, and the curve fit R^2 was >0.95 for all cows at all occasions. However, if considering TMRTCo, the solution sometimes differed greatly from time to time when observing data from the same cow. For example, cow 376 (*Figure 5b*) had a first TMRTCo of 9.570 h while a later curve fit occasion gave a TMRTCo of *13.692 h* (data written in italics was not presented in results). The different outcomes in TMRTCo was, just like TMRTCr, caused by different results of A, k1, k2 and L. However, despite the big differences in curve fittings for TMRTCo, there were also cases with either small differences or no differences in the numerical outcomes for A, k1, k2, L and TMRTCo. Even in all the case of TMRTCo curve fittings, no visual differences on the predicted curves could be seen between curve-fitting occasions, and the curve fit R^2 was >0.95 for all cows at all occasions. The big variation in numerical outcomes depending on curve fit occasion for TMRTCo indicates the need of a more reliable approach of making the prediction.

One potential reason behind the different curve-fit outcomes could be the limitations in the Solver-program and its GRG engine since the "Excel® Solver add-in program" cannot ensure it gives the best results for non-linear problems due to the algorithm used by the GRG engine (Frontline Systems Inc, personal message 2018-08-23). Different starting values, which are affected by the analysis order of the data, affect the program's work through the algorithm (Frontline Systems Inc, personal message 2018-08-23; Lasdon *et al.,* 1974). This may lead to different results for the same mathematical problem depending on when in the order of data the problem

is solved (Frontline Systems Inc personal message 2018-08-23; Lasdon *et al.,* 1974). These programme limitations clearly correspond to the observed different outcomes in the present study.

If data from the present study happens to be further analysed, the solutions from the Solver are more likely to be similar if the "Multistart"-option is used in the program (Frontline Solvers, 2018) and could therefore be recommended to use in the future. However, even if the program then gives the same solution at different occasions, it cannot guarantee it gives the best solution (Frontline Solvers, personal message, 2018-08-23), which indicates the weaknesses of the programme to solve the mathematical model used in the present study. To predict the curves by using a different computer program may be another option. The Solver and the GRG engine can however not be the only reason behind the different outcomes in TMRT, since the different outcomes in TMRTCr were negligible, while TMRTCo could differ with several hours between different tries even if both TMRTCr and TMRTCo were analysed in the same programme. One contributing factor to the large differences in TMRTCo within single cows may be due to the large allowed minimum and maximum values for A ($100.00 \geq A \leq 10000.00$) in the curve-fit formula, which was needed to be able to make a proper curve-fit. Faecal samplings before h=8 could potentially enable a reduced allowed interval for "A". Additionally, the curve might make a better fit if additional samples around Co=0 mg/kg would be removed (the faecal samples taken in the end of the sampling session), since these values affect the shape of the curve, and pull it down closer to zero. The discussed difficulties of getting a trustworthy TMRTCo might also indicate the need of a totally different mathematical model.

6.2 The evaluation study

There has been limited focus on validation of different digestion kinetic models in research (Huhtanen *et al.,* 2008). Focus has generally been on fitting markers to different mathematical models without checking its certainty (Huhtanen *et al.,* 2008). Evaluations of the marker method have been requested (Huhtanen *et al.,* 2008), and the present study performed an evaluation study by using the rumen evacuation technique to evaluate the Cr mordanted fibre marker.

The present study showed no significant relationship between TMRTCr and TMRTin. The TMRTCr was (numerically) longer compared to the rumen evacuation technique (*Figure 8*). This differs from other studies, which found that the rumen evacuations had a slower passage rate than the marker method (Robinson *et al.,* 1987; Minde & Rygh, 1997). Parts of the reason for the different outcome in the present study may be due to the fact that the marker method intended to measure the TMRT of the cell wall fraction (NDF) of the forage, while the rumen evacuation technique measured the iNDF fraction, and not the whole NDF fraction. The rumen evacuation technique did additionally measure TMRT on all ingested feed, including the concentrate, which differs from the marker method which specifically measured the passage rate of the marked forage. However, ruminal evacuations can be performed in different ways, and Robinson *et al.* (1987) based the rumen evacuations on ruminal decline of NDF which was not the case in the present study. These types of differences may contribute to difficulties comparing results between studies.

Lund *et al.* (2007) showed that the ruminal MRT of iNDF differ from dNDF. The study also concluded that the rumen evacuation technique, based on a one compartment model, overestimated the ruminal MRT of dNDF. That conclusion does not add any further understanding to the outcome in the present study's data, since the iNDF value was 118 g/kg NDF for the silage, and 123 g/kg NDF for the concentrate in the present study; this shows that most of the NDF fraction is pdNDF that thus rather would be overestimated considering TMRT instead of underestimated. Nevertheless, the study by Lund *et al.* (2007) also showed that the iNDF fraction generally had a shorter ruminal MRT, compared to dNDF (Lund *et al.,* 2007). This may support the outcome in the present study, since the NDF fraction contained a greater part of pdNDF than iNDF. That would thus lead to a longer overall TMRTCr, compared to the iNDF-based rumen evacuations. This potential explanation for the differences between TMRT does however not explain the difference between this study and studies that rather showed a shorter TMRT of the marker method compared to the rumen evacuations since the same analytical fractions were marked in the studies. Potential differences in iNDF/NDF-values between the studies may however explain some of the differences. Furthermore, the shape and structure of the feed particles where most of the iNDF or NDF is located, does most likely influence the particles' FSG, and thus their tendency of leaving the reticulorumen (Hristov *et al.,* 2003) and accordingly the MRT. These physical structures may differ between feeds in different studies and may therefore explain why TMRTCr in the present study was (numerically) longer compared to TMRTin even though previous mentioned studies rather showed the opposite. The fact that the cows in the present study had a large forage intake may be another potential explanation.

Above discussed potential reasons for the outcome in the present study are only theories. The outcome and the absence of safe proof for the outcomes underlines the need of further investigations to evaluate the marker method study in the present research project. The insignificant relationship between TMRTin and TMRTCr does show that the present study cannot verify the marker method as a reliable way of estimating TMRT.

6.2.1 The need for further evaluation

Even though the present marker method needs further evaluations, the orally administered markers are less invasive compared to for example the rumen evacuation technique (Owens & Hanson, 1992) and the practical procedure of the rumen evacuations probably disturb diurnal behaviours of the cow to a larger extent compared to the marker method. Considering these aspects, the marker method may be a better way to estimate passage rate when considering animal welfare and labour requirement. Due to economics, the marker method probably also enables more animals to participate in the studies (if needed). The marker method may therefore be positive for many reasons, but only if it is a reliable method.

TMRTin was the only parameter that could be used for comparison with the marker method in the present study. An output-based TMRT could not be calculated. This was because the study did not estimate the faecal output by total faecal collection (Huhtanen et *al.,* 2007), by the steady state method achieved by chemical markers (Owens & Hanson, 1992; Ferret *et al.,* 1999), or by the use of the internal marker AIA (Morris *et al.,* 2018). The pulse dose method (Susmel *et al.,*

1996), by estimating the faecal output from the pulse dose of Cr mordanted fibre marker, was neither implemented in the present thesis, since that kind of estimation could not verify the Cr mordanted fibre marker. By for example performing AIA analyses on feed samples and multiple faecal samples (Morris *et al.,* 2018), the faecal output could be estimated. The estimated faecal output could then be used to calculate the output based TMRT, which in turn could be compared to the achieved values of TMRTin, as well as TMRTCr.

More research, and more data are probably needed to further evaluate the marker method. The fact that two out of four cows were throwing silage out of the feeding troughs in the present study reduced the reliability of the outcome.

7 Conclusion

The present study investigated the TMRT among dairy cows with different abilities to consume large quantities of roughage and its impact on the milk production. The study also performed a rumen evacuation session among four ruminally fistulated cows to examine whether the solid digesta marker of Cr mordanted fibre was a reliable way of estimating the TMRT of the solid digesta fraction.

A positive and significant relationship was shown considering feed efficiency and TMRTCr. This means that the energy utilization is greater among cows with a slower passage rate of the solid digesta fraction which was hypothesised in the present thesis. No significant relationship was however observed between TMRTCr and kg milk, and neither between TMRTCr and kg ECM. The present study could therefore not prove the hypothesised negative relationship between TMRT(Cr) and milk production. No relationship between the cows' individual TMRTCr and their individual forage intake capacities was shown and the hypothesised negative relationship between forage intake and TMRT(Cr) could thus not be proved. Further studies are required to investigate the TMRT's relation to milk production and feed efficiency considering fibre digestibility, as well as digestibility of other feed fractions, to give a better understanding of TMRT in relation to productivity among cows with different abilities to consume large quantities of roughage. No statistical analyses were performed on the liquid digesta fraction in the present study since the collected data was not considered reliable. TMRTCo could thus not be used to investigate the hypotheses in the present master thesis.

The evaluation study, where rumen evacuations, as well as a marker method session was performed, could not verify the Cr mordanted fibre marker as a reliable method to measure TMRT of the solid phase of digesta. No significant relationship was shown between TMRTCr and TMRTin. TMRTCr was shown to overestimate the TMRT compared to the intake-based rumen evacuation calculations. This outcome was different compared to other studies on this subject. Further research to evaluate the marker method is needed to get a better understanding of the reliability of this non-invasive method. It is also important to have full control of the cows' feed intake to get reliable results. The usage of more cows might also be required.

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10 Appendix

I.

The 24 parameters subjected to proc CORR, Pearsons correlation coefficient, among the cows in the 5kgC treatment group. All parameters are presented in alphabetical order, where division calculations are listed in the end:

Body weight (kg)
Breed
DMI of concentrate (kg/day)
DMI of silage (kg/day)
DMI, total (kg/day)
Drinking water (kg/day)
ECM (kg/day)
Parity
Lactation week
Milk yield (kg/day)
Sampling group
TMRTCr
TMRTCo
water from concentrate (kg/day)
water from silage (kg/day)
water intake, total (kg/day)
wet concentrate intake (kg/day)
wet silage intake (kg/day)

$$\frac{DMI\ of\ silage\ (kg/day)}{BW\ (kg)}$$

$$\frac{DMI\ (\text{kg/day})}{BW\ (kg)}$$

$$\frac{ECM\ (kg/day)}{DMI\ of\ silage\ (kg/day)}$$

$$\frac{ECM\ (kg/day)}{DMI\ (kg/day)}$$

$$\frac{Milk\ yield\ (kg/day)}{DMI\ \ (kg/day)}$$

$$\frac{Milk\ yield\ (kg/day)}{DMI\ of\ silage\ (kg/day)}$$