COMMUNAUTÉ FRANÇAISE DE BELGIQUE
ACADÉMIE UNIVERSITAIRE WALLONIE-EUROPE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS FOR QUANTIFYING ISOFLAVONES AND EQUOL IN BIOLOGICAL MATRICES

Frédéric DAEMS

Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

Promoteur: Prof. Georges Lognay
Co-promoteur: Dr. Jean-Michel Romnee

2016

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“Il y a dans les chiffres une dimension qui n’est pas quantitative: il est important de toujours maintenir une attitude critique à cet égard, c’est-à-dire de rester conscient des fondements et des limites de la quantification.”

Prof. Jean-Guy Prévost, 2016.

‘‘Que ton alimentation soit ta première médecine.’’

Hippocrate, 5ème siècle av. J.C.

“Tout travail scientifique est une réalisation communautaire et non pas l'œuvre d'un cavalier seul. Qui sait où Darwin se serait égaré en 1837 sans Gould, sans Owen, et sans la vie scientifique très active de Londres et de Cambridge?”

Stephen Jay Gould, 1988.

Daems Frédéric (2016) **Développement et validation de méthodes analytiques pour la quantification des isoflavones et de l'équol dans des matrices biologiques** (Thèse de doctorat). Université de Liège, Gembloux Agro-Bio Tech, Gembloux, Belgique.

Résumé de la thèse:

Les isoflavones sont des composés naturels produits par les plantes et qui suscitent un vaste intérêt en raison de leur association avec une grande variété d'effets sur la santé humaine. Lorsqu'ils sont absorbés par les humains ou les animaux, la grande majorité de ces composés sont métabolisés par des bactéries se trouvant dans le système digestif. Parmi les métabolites microbiens obtenus, l'équol est probablement le composé le plus étudié en raison de ses potentiels effets bénéfiques sur notre santé. Cependant, tous les humains ne sont pas capables de produire de l'équol. L'enrichissement en équol de certains de nos aliments, comme le lait, pourrait donc s'avérer être une stratégie alternative permettant à toute la population de profiter des potentiels bienfaits de ce composé.

L'objectif de cette thèse consiste donc à mettre en place une série d'outils d'analyse permettant de quantifier ces composés dans diverses matrices biologiques et, ainsi, contribuer à l'étude de faisabilité de la production d'un lait naturellement enrichi en équol via une modification de l'alimentation des vaches laitières. Pour cela, quatre procédures analytiques, recourant à l'utilisation d'un système de chromatographie en phase liquide de ultra haute performance couplée à une détection par spectrométrie de masse en tandem (UPLC®-MS/MS), ont été développées et entièrement validées. Les méthodes mises au point ont ensuite pu être utilisées avec succès lors de deux études exploratoires originales qui ont permis; de quantifier la concentration en équol présente dans les laits commercialisés en Belgique et étudier à l'échelle du laboratoire l'évolution de la concentration en isoflavones dans des ensilages de trèfle violet (*Trifolium pratense* L.).

Cette thèse apporte donc de nouveaux outils analytiques fiables qui permettront de poursuivre le développement des connaissances sur la possibilité de produire un lait naturellement enrichi en équol et d'étudier la métabolisation des isoflavones chez la vache laitière.

Mots clés:

Isoflavones – Equol – UPLC®-MS/MS – Procédure analytique – Quantification – Développement – Validation – Vache – Lait – Ensilage – Trèfle violet.

Daems Frédéric (2016) **Development and validation of analytical methods for quantifying isoflavones and equol in biological matrices** (PhD thesis). University of Liège, Gembloux Agro-Bio Tech, Gembloux, Belgium.

Thesis abstract:

Isoflavones are natural plant substances that attract widespread interest because of their association with a large variety of beneficial effects on human health. When they are ingested by animals or humans, most of these compounds are metabolized by bacteria present in the digestive system. Among the isoflavone microbial metabolites, equol is probably the most widely studied because it seems to have numerous health benefits. In the human population, however, not everyone is able to produce equol. Equol enrichment of some food, such as cow's milk, could be an alternative strategy for making equol's health benefits available to everyone.

The main objective of the present thesis was to develop analytical tools for quantifying these compounds in several biological matrices, thus contributing to the feasibility study being conducted on producing milk naturally enriched with equol. For this purpose, four analytical procedures, using ultra performance liquid chromatography coupled with tandem mass spectrometry (UPLC®-MS/MS), were developed and fully validated. The analytical tools were then applied successfully in two original exploratory studies that sought to quantify equol concentrations in commercial cow's milk in Belgium and study the evolution of isoflavone concentration in red clover (*Trifolium pratense* L.) silage in laboratory-scale silos.

This thesis describes new and reliable analytical tools that will help to improve current knowledge about the feasibility of producing cow's milk naturally enriched with equol and to study isoflavone metabolization in dairy cow.

Keywords:

Isoflavones – Equol – UPLC®-MS/MS – Analytical procedure – Quantification – Development – Validation – Cow – Milk – Silage – Red clover.

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nuclear magnetic resonance, ED electrochemical detection, ECD electron capture detection, FID flame ionization detection, TLC thin layer chromatography, CE capillary electrophoresis, ELISA enzyme-linked immunosorbent assay, RIA radioimmunoassay, and TR-FIA time-resolved fluorescence immunoassay…………………………………………….……………………….25

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Chapter I

General introduction

Context

The livestock breeding is regularly criticized for its environmental impact (Hodgson *et al.* 2005; FAO 2006; FAO 2010; Marino *et al.* 2016) and for the effect of its products on human health (Parnaud & Corpet 1997; Chan *et al.* 2011; Superior Health Council Belgium 2013; Michaëlsson *et al.* 2014; Aitken *et al.* 2016). Although livestock breeding helps to recycle industrial by-products, maintain the rural fabric and preserve the landscape and biodiversity, it has become urgent to develop environmentally sustainable production methods and to promote high quality products from animal origin. In recent years, differentiated quality products, regional products or products from organic farming have become very popular among consumers, who have become increasingly aware of the importance of good quality food for their health (Haas *et al.* 2001; Grunert *et al.* 2004; Gracia & de Magistris 2007; Palma *et al.* 2015).

It was in this context that this PhD thesis was undertaken as part of the *PhytoHealth* project. Its main objectives are to develop the knowledge and tools required for enhancing livestock production by conducting research on molecules of interest in the phytoestrogen family, especially the isoflavone microbial metabolite known as equol.

Phytoestrogens constitute a large family of molecules that originate from the plant world, have a chemical structure close to human steroids and are able to bind to estrogenic receptors (Mostrom & Evans 2012; Sirotkin & Harrath 2014). Given the large amount of phytoestrogenic compounds naturally occurring in plants (Fig. 1.1), research has focused on one of the most common categories of phytoestrogens, known as isoflavones. These compounds are found mainly in the *Fabaceae* family (Wu *et al.* 2004; Mostrom & Evans 2012; Vitale *et al.* 2013). They constitute an attractive research topic because of their estrogenic profile and potential impact on human health (Pilsková *et al.* 2010; Mostrom & Evans 2012; Vitale *et al.* 2013; Ko 2014). For more than 20 years, they have attracted considerable interest from the scientific community. As shown in Fig. 1.2, the number of paper published on this type of natural estrogenic compounds began to increase in the early 1990s. All the published papers cover a broad range of scientific topics, including chemistry, bioactivity, isoflavone occurrence in the environment and foods, issues related to metabolism, and pre-clinical and clinical studies on the impact of isoflavones on human and animal health. The sudden growth of interest in the 1990s was linked to an increasing interest in phytoestrogens (Wu *et al.* 2004). When ingested by an

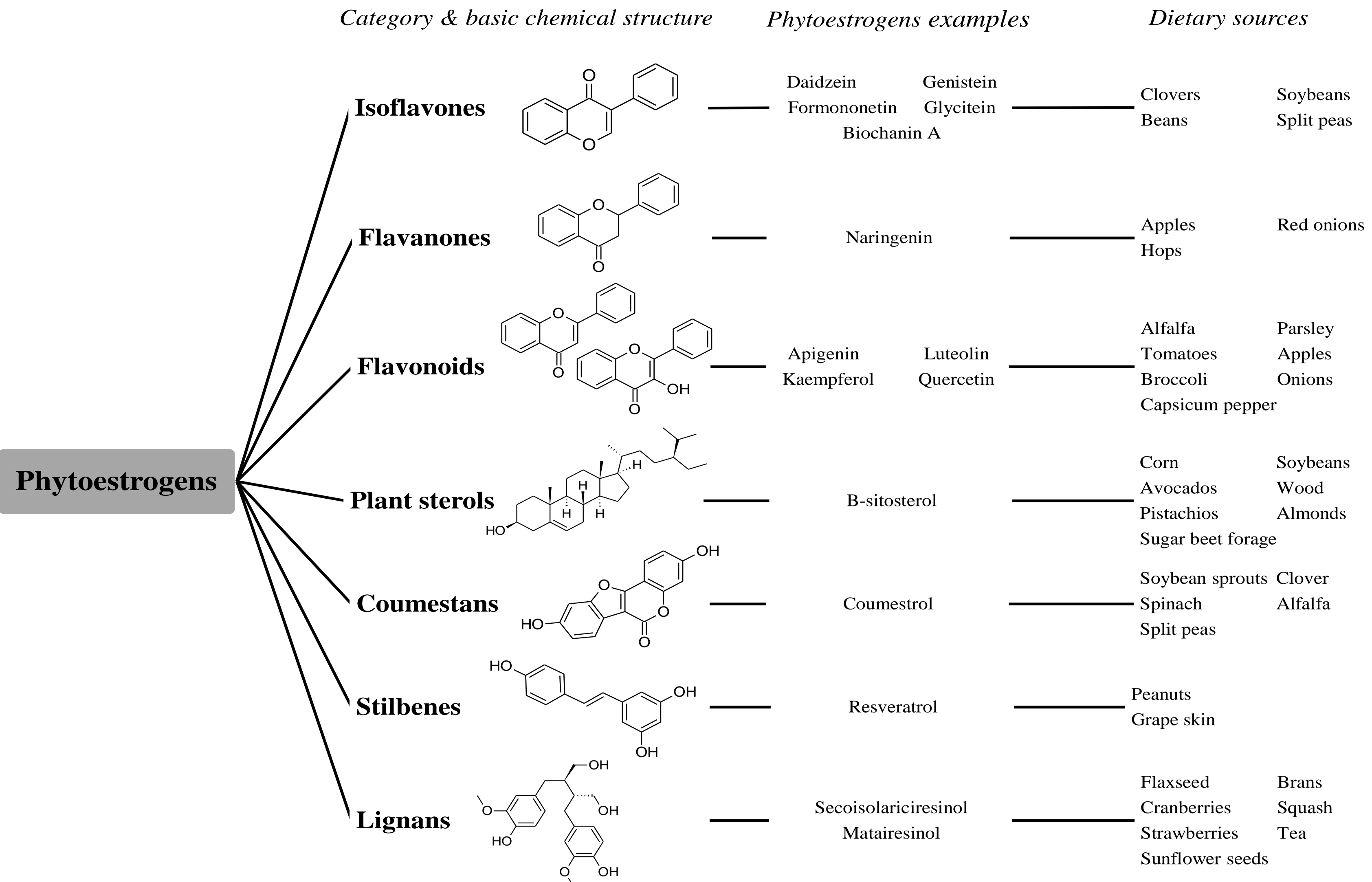

Figure 1.1. Categories of phytoestrogens, their basic chemical structures, compound examples and the dietary sources (adapted from Mostrom & Evans 2012).

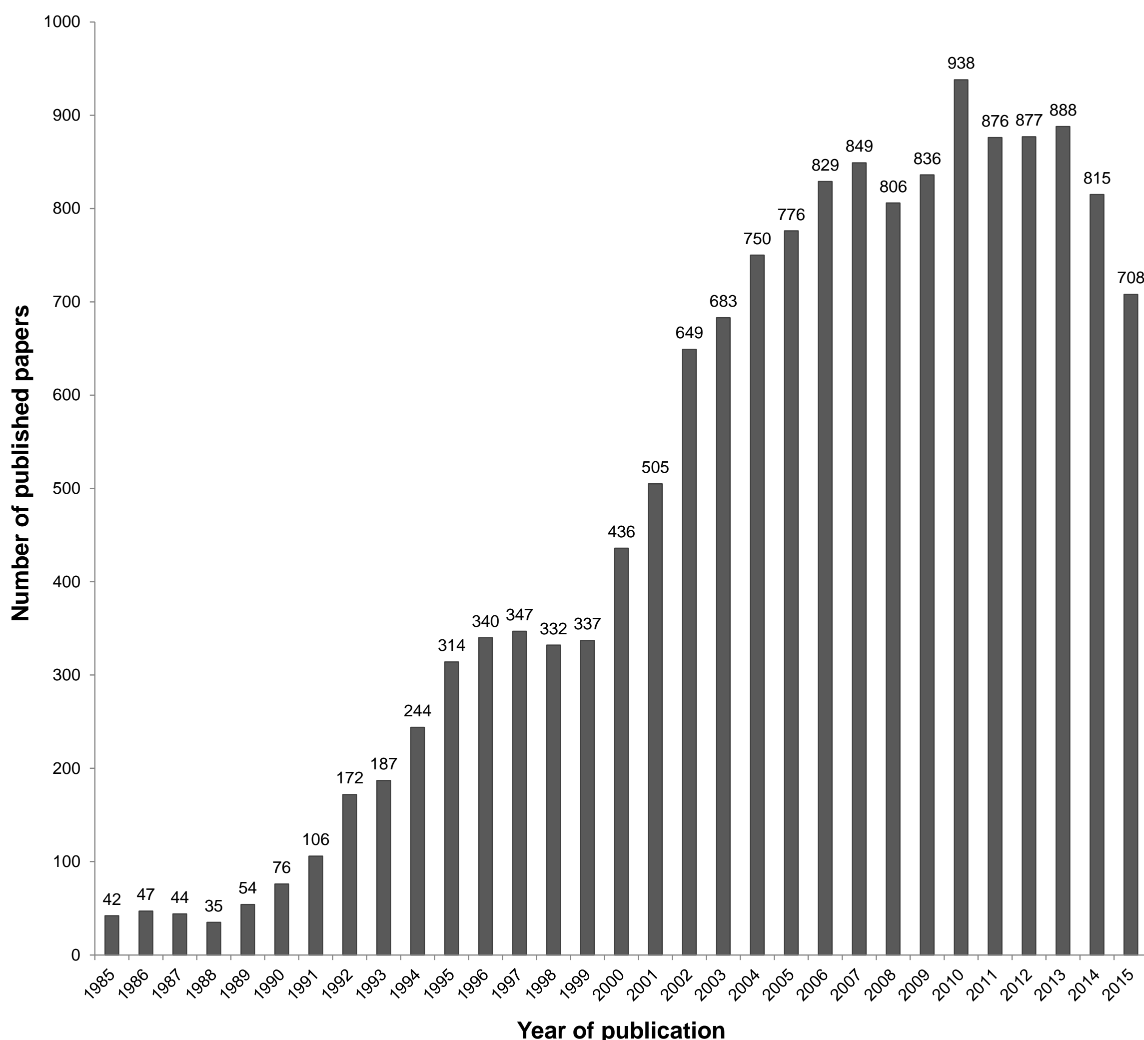

Figure 1.2. Number of papers published per year between 1985 and 2015 on topics related to isoflavones (data from Scopus, April 2015; search keyword 'isoflavone*' and limited to Title/Abstract/Keywords).

animal, isoflavones undergo several transformations by micro-organisms present in the digestive system before being absorbed by the body, and partly excreted in animal products. These microbial metabolites, especially equol, which has a chemical structure similar to 17β-estradiol (Fig. 1.3), could have a beneficial impact on human health. Equol is an isoflavan naturally produced by the metabolization of daidzein by intestinal microflora, with potential chemoprotective and estrogen receptor modulating activities. This non-steroidal estrogen contains a chiral center and occurs as two distinct diastereoisomers with R-(+)equol and S-(-)equol. The first one can be chemically synthesized in laboratory but is not produced naturally by intestinal bacteria. For the second one, various bacteria (lactobacillus, lactococcus, eggerthella and others) have been identified to carry the biotransformation of daidzein to S-(-)equol (Setchell & Clerici 2010a; Jackson *et al.* 2011; Mostrom & Evans 2012). Equol is absent, however, from foodstuffs of plant origin. In addition, most people, especially in the West, lack the

specific microbial populations in their digestive system that are able to transform isoflavones into this potentially beneficial metabolite (Bowey *et al.* 2003; Setchell & Clerici 2010a; Jackson *et al.* 2011; Legette *et al.* 2014). Livestock products are therefore well positioned to provide this microbial metabolite to humans. Among the livestock on farms, dairy cows are of particular interest because they ingest the plants in farm fields and grasslands and appear to have the specific micro-organisms in their digestive system that enable them to produce milk containing equol.

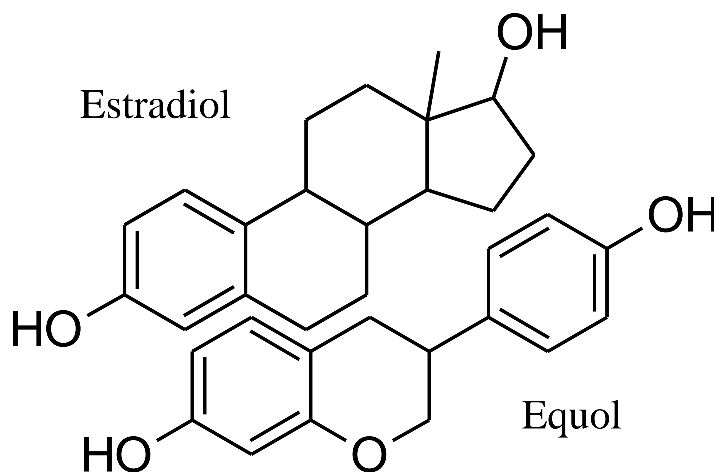

Figure 1.3. Similarity between the chemical structures of 17β-estradiol and equol (adapted from Setchell & Cassidy 1999).

Thesis structure

This PhD thesis represents the first part of the *PhytoHealth* project. Its main objective consists in the development of the analytical tools required for identifying plant varieties with a high isoflavone concentration, for monitoring their metabolism through the digestive system of cows in lactation and for validating the quality of milk produced. The thesis therefore focuses on the development and validation of analytical methods. Some practical applications of these analytical methods have been included in this document to illustrate their usefulness and future use in the project. The thesis consists of eight inter-related chapters. After *Chapter VIII*, lists of bibliographic references and all the relevant scientific works conducted during the thesis are summarized. Each chapter has been published as an independent paper in peer-reviewed journals, except for those presenting the objectives (*Chapter II*), development and validation of analytical procedures for quantifying our target compounds in digestive fluids and feces (*Chapter VII*) and the general discussion (*Chapter VIII*). *Chapter VII* is currently under submission

process in a peer-reviewed journal. A paragraph and diagrammatic illustration were introduced at the start of each chapter in order to provide a link between them and to summarize the main objectives.

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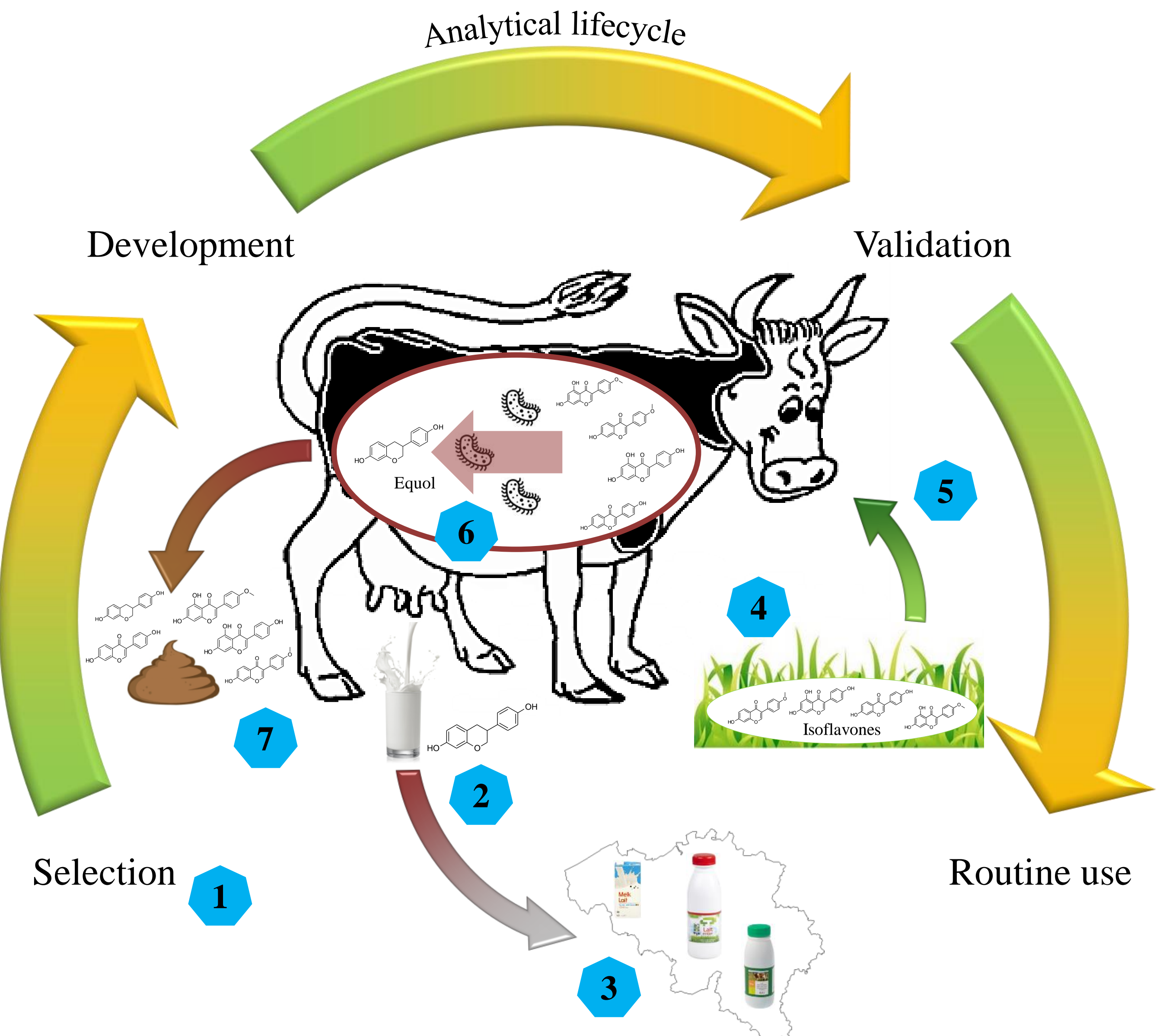
Chapter II

Aims and objectives of the thesis

Objectives

This chapter presents the main objective of this thesis and the selection of target compounds, as well as the underlying objectives that the subsequent chapters seek to address. The research methodology and objectives are presented in Fig.2.1.



1) Which analytical method is most appropriate?
2) How to quantify equol in milk?
3) What is the equol concentration in commercial cow's milk in Belgium?
4) How to quantify isoflavones in forages?
5) What is the impact of ensiling process on isoflavone concentration?
6) How to quantify isoflavones and equol in rumen and duodenum fluids?
7) How to quantify isoflavones and equol in feces?

Figure 2.1. Graphical abstract of *Chapter II.* Research methodology.

Objectives of the study

Phytoestrogens consist in a large family of polyphenolic compounds including several categories of molecules (Mostrom & Evans 2012). The overall objective of this thesis was to develop and validate analytical methods for detecting and quantifying isoflavones and equol in several biological matrices. Equol was selected as the target compound in milk because of its beneficial effects on human health (Setchell and Clerici 2010a; Mostrom and Evans 2012; Jackson *et al.* 2011). In addition, four isoflavones were selected for the development of the analytical methods: formononetin, daidzein, biochanin A and genistein. The first two compounds are the main isoflavones involved in the production of equol (Setchell and Clerici 2010a; Jackson *et al.* 2011; Mostrom and Evans 2012). The other two isoflavones, genistein and biochanin A, were selected because they are well-known isoflavones present in the plant matrix and also have potential health benefits (Mostrom & Evans 2012; Ganai & Farooqi 2015; Srinivas 2015).

The analytical methods developed were used to gain a better understanding of the production of equol by dairy cows. The first stage involved developing and validating a method for quantifying equol in cow's milk. In the second stage, a method for quantifying the four selected isoflavones in forages was optimized and fully validated. And finally, in the third stage, two analytical methods were optimized and fully validated for simultaneously quantifying the four isoflavones and equol in digestive fluids and feces. Each of these methods followed the analytical lifecycle with an in-house laboratory validation step (Feinberg 2007; EMA 2015).Two of these methods had already been used successfully in two experiments. The first one was used to estimate the equol concentration in commercial cow's milk in Belgium. The second method was used to estimate changes in isoflavone concentration in red clover (*Trifolium pratense* L.) during ensiling in laboratory-scale silos.

The following chapters are structured so that they issues, chronologically, seven specific objectives related to the passage of these estrogenic compounds from forages to cow's milk. These specific objectives are presented as questions in Fig. 2.1. The objectives are:

1) Select the most adequate analytical method for quantifying isoflavones and equol in biological samples.
2) Develop a reliable analytical method for quantifying equol in cow's milk.

3) Assess the equol concentration in commercial cow's milk in Belgium.
4) Develop a reliable analytical method for quantifying daidzein, formononetin, genistein and biochanin A in forages.
5) Assess changes in isoflavone concentration in red clover during the ensiling process.
6) Develop a reliable analytical method that can simultaneously quantify the four selected isoflavones and equol in rumen and duodenum fluids.
7) Develop a reliable analytical method that can simultaneously quantify the five selected compounds in feces.

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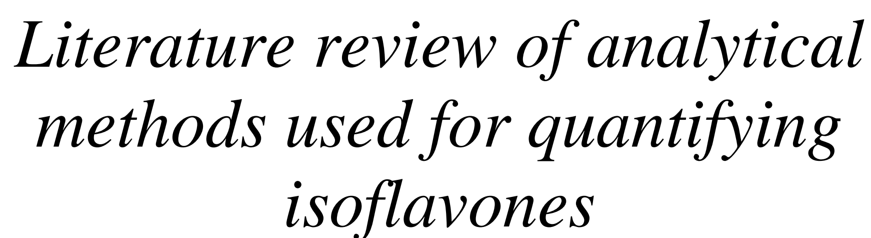
Chapter III



Literature review of analytical methods used for quantifying isoflavones

Objectives

Isoflavones form one of the most common categories of phytoestrogens and constitute an attractive research topic because of their estrogenic profile and potential impact on human health (Pilsková *et al.* 2010; Mostrom & Evans 2012; Vitale *et al.* 2013). In recent years, numerous studies have sought to gain a better understanding of their passage in animal products, especially in cow's milk (Kalač 2013). The main objective of this chapter is to present the results of a comprehensive review of methods used for quantifying isoflavones. In the scientific literature, there are already several reviews of analytical methods for isoflavone quantification (Wang *et al.* 2002; Wu *et al.* 2004; Grynkiewicz *et al.* 2005; Stalikas 2007; Vacek *et al.* 2008; Valls *et al.* 2009; Rostagno *et al.* 2009; Ko 2014; Raju *et al.* 2015), so this review focused specifically on analytical methods for quantifying isoflavones and their metabolites in cow's milk. A structured methodology was used to select recent relevant articles from the Scopus and CAB Abstract databases. This chapter describes the current situation and most recent advances, as well as trends and future prospects, in sample preparation and analysis for the quantification of isoflavones and their metabolites in cow's milk. This literature review allowed selecting the adequate analytical method that will be used throughout as an analytical 'basis' in this thesis. An overview of the methodology used is presented in Fig. 3.1.

Note: [page 23, line 22-24]"... Formononetin (FO) is demethylated into daidzein (DA) and then reduced via hydrogenation and ring scission to equol (EQ) (a microbial metabolite of isoflavone with high estrogenic activity)..." should be replaced by "Formononetin (FO) is demethylated into daidzein (DA), which is then reduced to the intermediate dihydrodaidzein and converted by deoxygenation to equol (EQ) (a microbial metabolite of isoflavone with high estrogenic activity)".

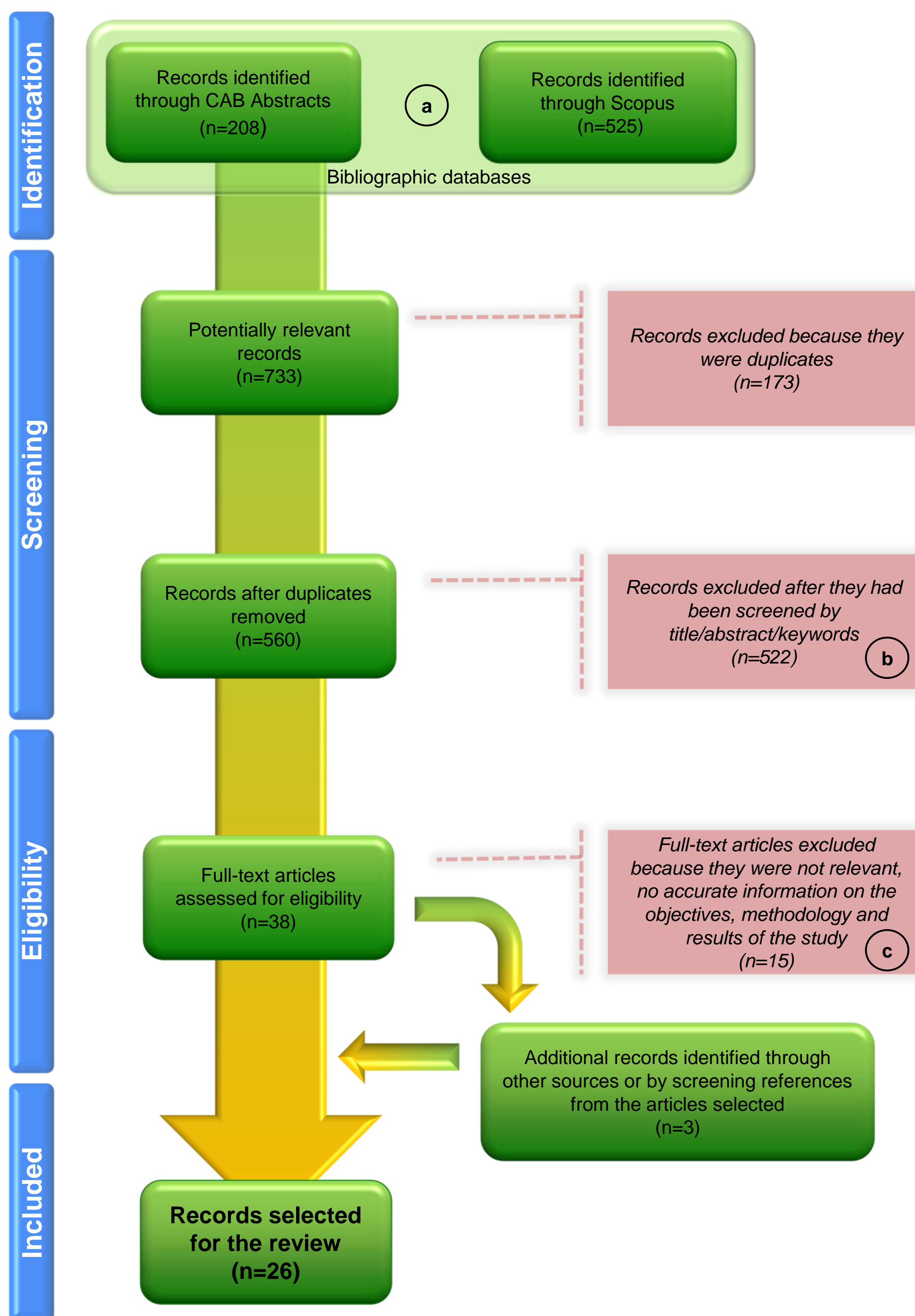


Figure 3.1. Graphical abstract of *Chapter III*. Flow of information through the different steps of the review completed in April 2015. **(a)** Boolean equation: (phytoestrogen* OR "endocrine disruptor*" OR "nonsteroidal* estrogen*" OR "estrogenic metabolite*" OR "plant estrogen*" OR isoflavone* OR isoflavan* OR equol OR daidzein OR formononetin OR genistein OR "biochanin A") AND (milk*) AND (method* OR quantitative OR quantification OR determination OR characterization OR optimization OR validation OR validate OR content* OR concentration* OR level*); applied between 2005 and 2015. **(b)** The following criteria were applied: article type (research article/note/review/book/book section/ thesis were retained, whereas poster/oral presentation/other type of document were removed); text availability; restricted to literature in English; and an analytical method was used for determination of isoflavones or equol in bovine's milk. **(c)** Analytical method described being suitable for the quantitative analysis.

Analytical methods used to quantify isoflavones in cow's milk – A review

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Reference: Daems, F., Romnee, J.-M., Heuskin, S., Froidmont, É., Lognay, G., 2016. Analytical methods used to quantify isoflavones in cow's milk – A review. *Dairy Science & Technology*, 96(3), pp. 261-283.

Abstract

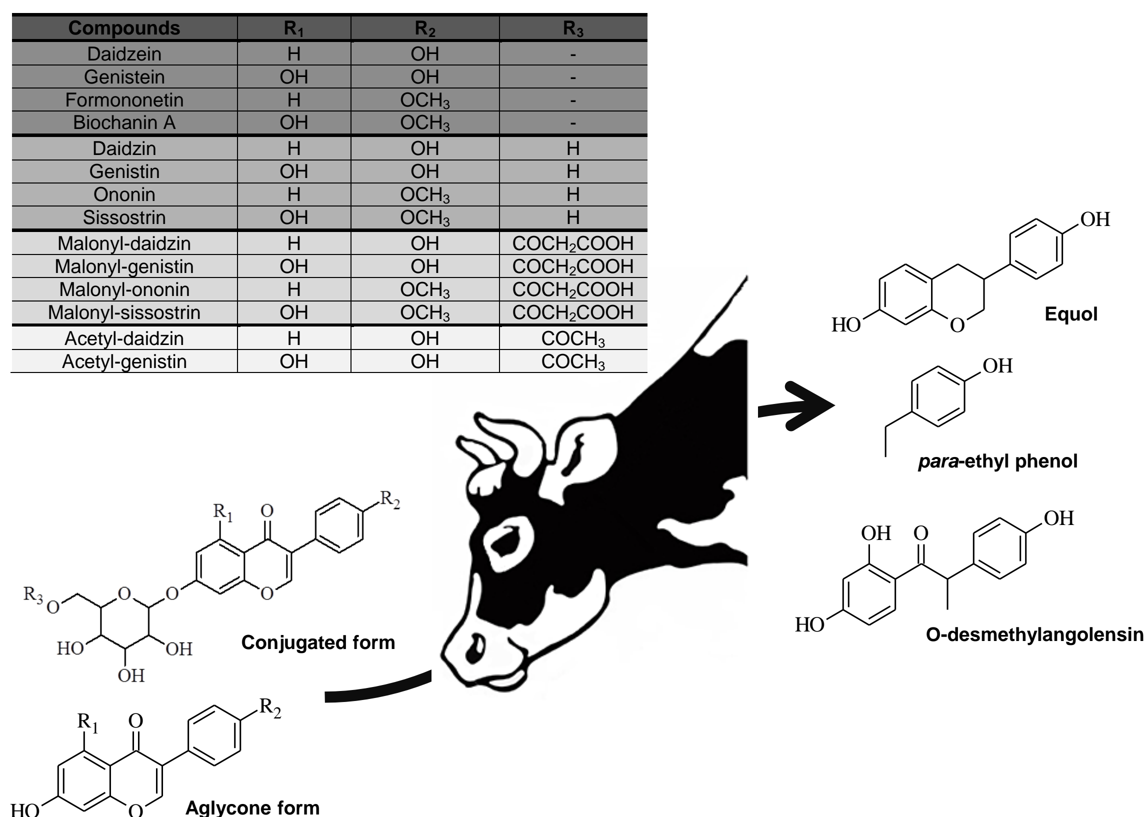
This paper provides an update and comprehensive review of the analytical methods used for quantifying isoflavones and their metabolites in cow's milk. Isoflavones are secondary plant metabolites that are similar to 17 β-estradiol in chemical structure. They form one of the most common categories of phytoestrogens. Numerous health benefits have been attributed to isoflavones, but many of these compounds are also considered to be endocrine disruptors, with adverse effects on health. These contradictory trends offer an attractive prospect for future research, and therefore, sensitive and reliable analytical methods are required to clarify various issues about isoflavones. For this review, a structured methodology was used to select 26 relevant articles published between 2005 and 2015 from the Scopus and CAB Abstract databases. The review discusses individual steps of the analytical procedures described in these articles, including sample preparation, instrumental analysis and validation. The most commonly used analytical procedure is sample preparation involving liquid-liquid extraction and an enzymatic hydrolysis step followed by liquid chromatography with mass spectrometry analysis. Currently, however, there is no standardized procedure for the sample preparation and analysis of isoflavones in milk.

Keywords

Isoflavones – Phytoestrogens – Milk – Quantification – Analysis

Introduction

Isoflavones are classified as phytoestrogens, which are widely distributed in the plant kingdom. These plant secondary metabolites are structurally similar to 17β-estradiol and bind selectively, but weakly, to mammalian estrogen receptors (ER), with a preference for ERβ (Mostrom & Evans 2012; Baber 2013; Vitale *et al.* 2013). These diphenolic compounds appear to have antioxidant activity and could offer alternative therapies for a range of hormone-dependent conditions, including cancer, menopausal symptoms, cardiovascular disease and osteoporosis (Mortensen *et al.* 2009; Mostrom & Evans 2012; Baber 2012; Vitale *et al.* 2013; Ko 2014). They also can be considered as endocrine disruptors, however, with the potential to have adverse health effects (Afssa 2005; Patisaul & Jefferson 2010; Wocławek-Potocka *et al.* 2013; Maggioni *et al.* 2013; Sirotkin & Harrath 2014; Wielogórska *et al.* 2015). The highest concentrations are found in plants of the *Fabaceae* family, such as soybean (*Glycine max* L.) and clover (*Trifolium pratense* L.) (Mostrom & Evans 2012; Ko 2014). In plants, isoflavones are usually found in conjugated forms as glucosides, acetylglucosides and malonylglucosides (Mostrom & Evans 2012; Kalač 2013). When ingested by cows, isoflavones appear to be metabolized mainly in the rumen and the main route of excretion is through feces and urine, with only a small proportion being excreted in milk (Njåstad *et al.* 2014). A diagram of isoflavone metabolization in cows is shown in Fig. 3.2. Biochanin A (BA) is demethylated mainly into genistein (GE) and via a ring cleavage into *para*-ethyl phenol (a compound that apparently has no estrogenic activity) and organic acids (Mostrom & Evans 2012; Kalač 2013; Njåstad *et al.* 2014). Formononetin (FO) is demethylated into daidzein (DA) and then reduced via hydrogenation and ring scission to equol (EQ) (a microbial metabolite of isoflavone with high estrogenic activity). FO can also be metabolized into other metabolites, such as *O*-desmethylangolensin (*O*-DMA) (compounds with low estrogenic activity) (Setchell & Clerici 2010a; Mostrom & Evans 2012; Kalač 2013; Njåstad *et al.* 2014). The aglycones seem to be the most biologically active forms and are quickly absorbed by rumen and gut mucosa (Lundh 1990; Mostrom & Evans 2012; Kalač 2013; Vitale *et al.* 2013). Most of them are conjugated by glucuronic acid (a limited amount is conjugated with sulfate) during this absorption process. A small proportion of aglycones reaches the blood circulatory system, but they are rapidly conjugated in the liver and other tissues (Lundh 1990; King *et al.* 1998; Mostrom & Evans 2012). These



Compounds	R_1	R_2	R_3
Daidzein	H	OH	-
Genistein	OH	OH	-
Formononetin	H	OCH_3	-
Biochanin A	OH	OCH_3	-
Daidzin	H	OH	H
Genistin	OH	OH	H
Ononin	H	OCH_3	H
Sissostrin	OH	OCH_3	H
Malonyl-daidzin	H	OH	$COCH_2COOH$
Malonyl-genistin	OH	OH	$COCH_2COOH$
Malonyl-ononin	H	OCH_3	$COCH_2COOH$
Malonyl-sissostrin	OH	OCH_3	$COCH_2COOH$
Acetyl-daidzin	H	OH	$COCH_3$
Acetyl-genistin	OH	OH	$COCH_3$

Figure 3.2. Chemical structures of some of the main isoflavones occurring in forages and their microbial metabolites found in milk (Saviranta *et al.* 2010; Mostrom & Evans 2012; Vitale *et al.* 2013; Kalač 2013).

isoflavone metabolites then circulate throughout the body and are excreted in feces and urine or transferred to the milk.

In recent decades, due to the growing interest in isoflavones, a diverse range of analytical methods has been developed for isoflavone determination and quantification in plant material, soil, water, food and food supplements, as well as in biological matrices, generating a huge amount of scattered information (Fig. 3.3). The overall analytical method used for isoflavones is highly dependent upon the matrix characteristics, the availability of the techniques, the desired selectivity and the need to obtain information about the chemical structure of these compounds or simply to unambiguously identify previously targeted compounds. Initially, samples are usually freeze-dried or simply frozen. Depending on the matrix analyzed, isoflavones are extracted by classical maceration or liquid-liquid extraction (LLE), but a promising approach that is increasingly being used is to combine extraction/clean-up techniques such as ultrasound-assisted extraction (UAE), solid-phase extraction (SPE), microwave-assisted extraction

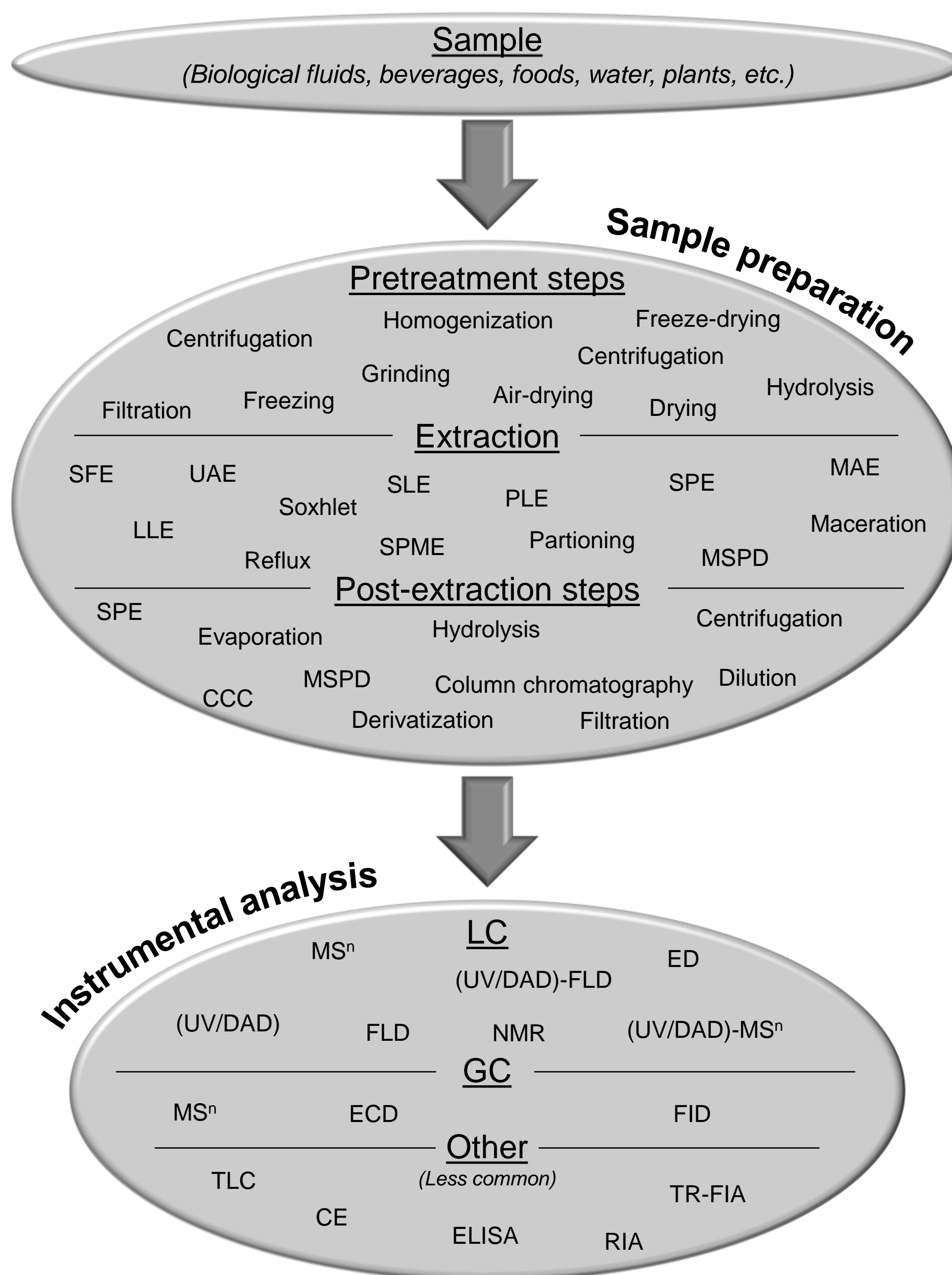


Figure 3.3. Main sample preparation and instrumental analysis methods for the determination of isoflavones and their related compounds. Abbreviations: UAE ultrasound-assisted extraction, SFE supercritical fluid extrac-tion, LLE liquid-liquid extraction, SLE solid-liquid extraction, SPME solid-phase micro-extraction, SPE solid-phase extraction (incorporating the Quechers approach), MAE microwave-assisted extraction, MSPD matrix solid-phase dispersion, CCC counter-current chromatography, LC liquid chromatography (HPLC high-performance liquid chromatography, UPLC ultra-performance liquid chromatography, and UHPLC ultra high-performance liquid chromatography), GC gas chromatography, MS mass spectrometry, UV ultra-violet, DAD diode array detector (also called photodiode array detector, PDA), FLD fluorescence detector, NMR nuclear magnetic resonance, ED electrochemical detection, ECD electron capture detection, FID flame ionization detection, TLC thin layer chromatography, CE capillary electrophoresis, ELISA enzyme-linked immunosorbent assay, RIA radioimmunoassay, and TR-FIA time-resolved fluorescence immunoassay.

(MAE) or matrix solid-phase dispersion (MSPD) (Rostagno *et al.* 2009). Given that isoflavones are often in conjugated forms, a hydrolysis step is sometimes used when the aim is to find aglycones. The number of analytical separation and detection techniques proposed is also numerous. Depending on the matrix and the information required, many of these techniques have been developed using variants of immunoassays (enzyme-linked immunosorbent assay, ELISA; radioimmunoassay, RIA; and time-resolved fluorescence immunoassay, TR-FIA), gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) coupled with various detection modes (mass spectrometry, MS; diode array detection, DAD; fluorescence detection, FLD; and electrochemical detection, ED) (Wu *et al.* 2004; Grynkiewicz *et al.* 2005; de Rijke *et al.* 2006; Stalikas 2007; Vacek *et al.* 2008; Valls *et al.* 2009; Rostagno *et al.* 2009; Ko 2014). Immunoassays are non-chromatographic methods with high sensitivity and specificity for single-component detection, high throughput screening and relatively low cost per sample. For this approach, however, preparing the antibodies for corresponding analytes is a challenging task and it suffers from the likelihood of cross-reactivity between the similar compounds, leading to a possible overestimation of the targeted analyte (Wu *et al.* 2004; Ko 2014). CE is also a promising technique with a high separation resolution and a low amount of sample required, but it is restricted to laboratories that have the necessary technical capacity (Wang *et al.* 2002; Wu *et al.* 2004). The most common analytical method used for quantifying isoflavones remains LC coupled with MS or ultra-violet detection (LC-MS, LC-UV). Thanks to its high sensitivity, MS detection is used preferentially when low amounts of isoflavones are thought to be in the matrix. UV detection is used when isoflavones are thought to be present in larger amounts (Ko 2014).

Milk is one of the most widely consumed foods in the world and several studies have been conducted in recent years to estimate its isoflavone content and to assess whether or not milk can be considered as a useful or dangerous source of isoflavones for humans. An initial answer was presented in two articles, especially with regard to the impact of cattle feed on the EQ content of milk (Kalač 2011; Kalač 2013). As far as we know, there has not yet been a specific review of the analytical methods used for quantifying isoflavones in cow's milk. This paper therefore reviews the current situation with regard to sample preparation and analysis for the quantification of isoflavones in cow’s milk, including the most recent advances.

The literature overview was conducted online using the Scopus and CAB Abstracts bibliographic databases. Four keywords were identified: 'phytoestrogens', 'isoflavones', 'milk' and 'quantitative analysis'. These keywords and their descriptors were used to create a Boolean equation in the two databases. The most recent studies (2005-2015) were selected. Key data of the selected articles are given in Tables 3.1 and 3.2. Where the authors of a selected article refer to a method published before 2005, the analytical protocol from the reference is summarized and presented in these tables. All the analytical procedures described in these articles were analyzed chronologically: initially, sample preparation procedures were compared and discussed, and then the instrumental analysis and validation parameters were examined.

Sample preparation

Broadly, an analytical method can be split into two distinct parts: sample preparation and sample analysis. Sample preparation is undertaken before instrumental analysis and should ensure that: (1) the target compounds can be extracted from the matrix; (2) interference that could disrupt the detection of the target analyte or damage the chromatographic column can be eliminated or reduced; (3) to allow analysis of samples with low concentrations of the target compound; (4) and the analyte of interest can be transformed into a more suitable form that could be easily separated, detected and quantified. In Table 1, all the information about sample preparation found in the 26 selected articles is detailed and split into four chronological steps. The table also presents the origin of the milk and the isoflavones quantified. Studies using the same sample preparation procedure were grouped together. Articles where the sample preparation procedure was based on a previous study, but with some modifications, were not grouped (Antignac *et al.* 2009; Flachowsky *et al.* 2011; Daems *et al.* 2015). Thirteen sample preparation procedures were identified and are discussed below.

Sample conditioning

As shown in Table 3.1, most milk samples in the experiments reviewed were simply frozen between the sampling and analysis days. In other articles, sample condition was not mentioned. Often, information about the handling of milk samples after

Table 3.1. Summary of isoflavones analyzed, sample storage, and sample preparation methods used between 2005 and 2015 for quantification of isoflavones in milk

Isoflavone *(milk origin)*	Storage	Sample preparation			Reference
		Pre-extraction steps	Extraction	Post-extraction steps	
BA, DA, EQ, FO, GE, and PR *(feeding experiments and health impact experiments)*	Frozen (−20 °C)		- Deproteinized and defatted (2.5 mL milk with 0.25 mL acetate buffer [pH 5–5.2 and 2 mol.L^{-1}], 1 mL heptane and 2 mL acetone) - Centrifugation - Evaporation to dryness (30 °C) - Reconstitution with 0.8 mL water	- Enz. hydrolysis (8 μL of β-glucuronidase/sulfatase from *H. pomatia* type H2, 40 °C, and 3–4 h) - 0.2 mL of MeOH was added - Centrifugation - Supernatant was analyzed	Adler et al. (2015), Adler et al. (2014), Njåstad et al. (2014), Höjer et al. (2012), Nielsen et al. (2012), Andersen et al. (2009a), and Steinshamn et al. (2008)
EQ *(commercial milks)*	Frozen (−18 °C)	- Thawed overnight at 4–6 °C, equilibrated to room T°, and homogenized (2 mL) - Enz. hydrolysis (0.1 mL β-glucuronidase/sulfatase from *H. pomatia* type H2 [≥45,000/3750 units.mL^{-1}], 37 °C, and 2 h) - Cooled - Centrifugation	- Double L/L extraction (n-hexane and ethyl acetate, 3×3 mL) - Evaporation to dryness - Reconstitution MeOH:H_2O (80:20, v/v)	- Filtration (0.2 μm)	Daems et al. (2015)
DA and GE *(commercial milks)*	Frozen (−20 °C) or directly analyzed		- Deproteinized (10 g of milk mixed with 12 mL of 1% (v/v) acetic acid in acetonitrile, 1 g NaCl, and 2.5 g $MgSO_4$) - Centrifugation - Evaporation to dryness - Reconstitution (5 mL water)	- SPE (OASIS-HLB) - Evaporation - Reconstitution in 200 μL of H_2O/MeOH (3:1, v/v) - Filtration (0.2 μm)	Wielogórska et al. (2015)
EQ *(technological processing)*	N.C.		- Deproteinized (10 mL of milk mixed with 3 mL of acetonitrile and centrifuged at 5 °C)	- Supernatant stored at −80 °C - Filtration (0.2 μm)	Tsen et al. (2014)
PU *(technological processing)*	Frozen (≤−10 °C)	- Freeze-drying - Defatted three times with hexane (1:5, w/v)	- Extraction with MeOH (1:3, w/v) - Evaporation to dryness	- Filtration (0.45 μm)	Rastogi et al. (2013)

Table 3.1. (continued)

Isoflavone *(milk origin)*	Storage	Sample preparation			Reference
		Pre-extraction steps	Extraction	Post-extraction steps	
			- Reconstitution in MeOH (30 g.mL^{-1})		
GE and GE-in *(commercial milks)*	N.C.		- Double UAE (10 and 5 mL of acetonitrile, 15 min) - Centrifugation	- Filtration (0.45 μm) - Concentration and dilution - SPE (OASIS-HLB) - Concentration - Dilution	Maggioni et al. (2013)
DA, EQ, FO, and GE *(feeding experiments)*	Frozen (−18 °C)		- L/L extraction (20 mL milk/ 20 mL acetone/acetate buffer pH 5.2 [4:1, v/v]) - Centrifugation - Evaporation (halved)	- Enz. hydrolysis (0.2 mL sulfatase/glucuronidase from *H. pomatia*, 52 °C, and 2 h) - Centrifugation - SPE (C18) - Evaporation to dryness - Reconstitution in MeOH (80%, v/v) - Filtration (0.45 μm)	Flachowsky et al. (2011)
BA, DA, EQ, FO, and GL *(feeding experiments and health impact experiments)*	Frozen (−20 °C)	- Equilibration at room temperature - Enz. hydrolysis (β-glucuronidase, 100 μL.4 mL^{-1} of milk, 37 °C, and 1 h) - Centrifugation (cream and precipitate were discarded)	- Double L/L extraction (hexane and ethyl acetate, 3×3 mL) - Evaporation to dryness - Re-suspension (0.5 mL acetonitrile)	- Filtration (0.2 μm)	Nielsen et al. (2011), Skaanild and Nielsen (2010), Nielsen et al. (2009) and Andersen et al. (2009b)
DA, EQ, GE, and GL *(technological processing and feeding experiments)*	Frozen (-20 °C)	- Enz. hydrolysis (100 μL β-gluconidase [11,400 U.mL^{-1}]/ sulfatase [3200 U.mL^{-1}] from *H. pomatia* type H2 were added at 2 mL of milk and 3 mL of acetate buffer [0.1 M and pH 5], 37 °C, and 12 h)	- L/L extraction (3×2 mL ethylacetate) - Centrifugation - Evaporation to dryness - Reconstitution (0.5 mL methanol:water [1:1, *v/v*])		Křížová et al. (2011a), Křížová et al. (2011b), Krajčová et al. (2010), and Třináctý et al. (2009)
BA, DA, EQ, FO, GE, and GL *(commercial milks)*	N.C.		- L/L extraction (10 mL milk/ 10 mL acetone/acetate buffer [2 M, pH 5.2, 9:1, *v/v*])	- Enz. hydrolysis (0.3 mL *H. pomatia* type H5 [500000 units. 20 mL^{-1} water], 52 °C, and 16 h)	Antignac et al. (2009)

Table 3.1. (continued)

Isoflavone *(milk origin)*	Storage	Sample preparation			Reference
		Pre-extraction steps	Extraction	Post-extraction steps	
				- Reverse-phase SPE (C18) - Normal phase SPE (SiOH)	
BA, DA, EQ, FO, GE, and O-DMA *(commercial milks and feeding experiments)*	Frozen (−80 °C)	- Enz. hydrolysis (100 μL β-glucuronidase [500 units] and 80 μL sulfatase [40 units] were added at 5 mL milk, 37 °C, and 2 h)	- L/L extraction (1.5 mL ammonium acetate buffer 1.5 M+7.5 mL diethyl ether, chilled at −80 °C, centrifugation, and repeated 3 times) - Evaporation to dryness (40 °C) - Reconstitution in 2 mL of sodium acetate buffer (0.2 M and pH 5) containing MeOH 20%	- Centrifugation - SPE (OASIS-HLB 3 cc) - Evaporation to dryness (60 °C) - Reconstitution 200 μL MeOH/sodium acetate buffer (pH 5, 0.2 M, 3:1, v/v) - Centrifugation - Filtration (0.45 μm)	Mustonen et al. (2009) and <u>Hoikkala et al. (2007)</u>
BA, DA, EQ, FO, GE, and GL *(commercial milks)*	Frozen (−20 °C)	- Freeze-dried	- SLE (100 mg/2 mL of 10% MeOH in sodium acetate (0.1%, pH 5), three time)	- Enz. hydrolysis (purified β-glucuronidase from *H. pomatia*, cellulase from Trichoderma reesi, and β-glucosidase from almonds, 37 °C and 16 h) - SPE (Strata C-18E, 50 mg.mL^{-1}) - Dried - Reconstitution (200 μL MeOH 40%)	Kuhnle et al. (2008)
DA, DA-in, GE, GE-in, GL, and GL-in *(technological processing)*	N.C.		- Deproteinized (2 mL of milk mixed with 50 μL IS and 3 mL acetonitrile and stored overnight at 10 °C)	- Equilibrated at room T° - Evaporation to dryness - Reconstitution (2.4 mL 50% acetonitrile) Filtration (0.22 μm)	Uzzan et al. (2007)

The underlined reference corresponds to the referenced method used and cited in the other articles.

Abbreviations: BA biochanin A, DA daidzein, DA-in daidzin (glycoside form of DA), EQ equol, FO formononetin, GE genistein, GE-in genistin (glycoside form of GE), GL glycitein, GL-in glycitin (glycoside form of GL), PR prunetin, PU puerarin, and O-DMA O-desmethylangolensin.

collection and before actual analysis was limited. An exception was Wielogórska *et al.* (2015), who reported that isoflavones (DA and GE) were stable in the matrix for only 2 weeks, indicating that all samples were analyzed immediately after collection or stored at -20°C for no longer than 2 weeks before being thawed for analytical purposes. Poor storage can lead to errors that cannot be corrected later and that will affect the outcome of the final analysis. How should samples be stored, and for how long, without affecting their original isoflavone profile? For matrices such as soybean, some isoflavones (mainly the conjugated forms) have a fairly unstable character (Rostagno *et al.* 2009). In ruminants, glucuronide-conjugated compounds are the major form found in biological fluids (Nielsen *et al.* 2012; Mostrom & Evans 2012). The degradation problem could therefore also arise during milk storage and the information about the conjugated form content could be lost. More attention should be given to the storage and handling of milk sample between the sampling and analysis in future studies.

If freeze-drying is an easy method for milk sample storage, it would be interesting to check isoflavone stability under a range of temperature and storage time conditions. The freezing method could be also compared with other techniques, such as freeze-drying. Isoflavones can be found in powdered milks (Antignac *et al.* 2009; Maggioni *et al.* 2013). Therefore, freeze-drying, which is often used for plant matrices because it does not seem to affect these compounds (Hoerger *et al.* 2011), could be an interesting alternative to the traditional freezing process. These findings need to be taken into account in future studies.

Hydrolysis

Among the 13 sample preparation procedures listed in Table 3.1, only four did not include a hydrolysis step. (Wielogórska *et al.* 2015) proposed a method without a hydrolysis step for the quantification of 19 endocrine disruptors in milk, including DA and GE. Rastogi *et al.* (2013) did not use a hydrolysis step because they sought to quantify puerarin (PU, the 8-C-glucoside of daidzein) from pasteurized toned milk enriched with extracts from *Pueraria tuberosa* Linn (commonly called ‘kudzu’). According to these authors, this glycoside form of isoflavone that is abundant in kudzu might be beneficial in the treatment and prevention of some diseases and cancers. The two other procedures were used by Maggioni *et al.* (2013) and Rastogi *et al.* (2013) to quantify aglycones in infant powdered milk formulas and glycosides in soy fortified milk.

Integrating a hydrolysis step into the analytical protocol depends on the objectives sought. Hydrolysis is often used when the exact nature and composition of isoflavone glycosides in the matrix analyzed are unknown or when target aglycone compounds are sought. It allows the time required for instrumental analysis to be considerably reduced and facilitates the separation of target compounds. It also makes it unnecessary to use standard references that are difficult to obtain, not available or often expensive. Introducing an additional step, however, can increase analytical variability.

As shown in Table 3.1, most of the studies reviewed used a hydrolysis step. Unlike other matrices where three procedures are reported (enzymatic, acidic and, least common, basic hydrolysis) (Schwartz *et al.* 2009; Rostagno *et al.* 2009), for milk only enzymatic hydrolysis is carried out to hydrolyze isoflavones. The authors focused on aglycones because these forms appear to be the most biologically active and can be absorbed by intestinal tract of humans and animals (Mostrom & Evans 2012; Ko 2014). In addition, some isoflavones, such as EQ, seem to be sensitive to acidic conditions (Setchell & Clerici 2010a). Enzymatic hydrolysis involves incubating the sample with enzymes at a fixed temperature (within a range of 37 to 52°C, depending on the study) and over different incubation times (between 1 and ≥ 16 h). A solution buffer (pH about 5) is sometimes used to optimize hydrolysis conditions. Unlike plant matrices (Rostagno *et al.* 2009), only β-glucuronidase/sulfatase from *Helix pomatia* was involved in the hydrolysis of isoflavones in milk. This enzymatic juice contains glucuronidase and sulfatase activities. The former catalyzes the breakdown of complex carbohydrates (glucuronides) and the latter catalyzes the hydrolysis of sulfate esters, the two main forms of isoflavones found in biological fluids (Rostagno *et al.* 2009; Nielsen *et al.* 2012; Ko 2014). Only Kuhnle *et al.* (2008) reported using a mixed solution of three enzymes: β-glucuronidase from *H. pomatia*; cellulase from *Trichoderma reesi*; and β-glucosidase from almonds.

The hydrolysis temperature varied between 37 and 40°C, but it did reach 52°C in some studies (Antignac *et al.* 2009; Flachowsky *et al.* 2011). The temperature generally used by suppliers to evaluate the glucuronidase and sulfatase activities from these enzymes was 37°C. Where a buffer solution was used, the pH of the solution varied between 4.6 and 5.2. The optimal pH values given by suppliers were 5.0 and 6.2 for glucuronidase and sulfatase activities, respectively. Hydrolysis time varied, ranging from 1 h (Andersen *et al.* 2009b) to overnight (Kuhnle *et al.* 2008; Antignac *et al.* 2009).

During an evaluation of the robustness of their hydrolysis protocol based on a one-variable-at-a-time technique, Daems *et al.* (2015) demonstrated that an incubation time of 1 h was sufficient for hydrolyzing conjugated forms of EQ. Another important point, but one on which there is little information in the studies, is the minimum enzyme activity needed to hydrolyze all the conjugated forms of isoflavones present in milk. When enzyme activity values were provided by authors or when estimating them was possible, it was found that they varied greatly across the studies. For example, for their analysis of isoflavones (containing EQ) in commercial milk, Hoikkala *et al.* (2007) used 100 units of glucuronidase activity per ml of sample, whereas Daems *et al.* (2015) used 2,250 units. In the product information data sheets for other biological fluids, Sigma-Aldrich recommends using between 1,000 and 20,000 units per ml of β-glucuronidase type H-2 from *H. pomatia* (EC 3.2.1.31), but no specific information is given on the hydrolysis of milk samples. One disadvantage of using *H. pomatia* juice is that it usually contains appreciable levels of some isoflavones (according to Taylor *et al.* (2005): 13.38 ng $GE.mL^{-1}$ enzyme, 0.25 ng $DA.mL^{-1}$ and 0.58 ng $EQ.mL^{-1}$) which can affect the quantification. Daems *et al.* (2015) and Grace & Teale (2006) did not detect any trace of EQ, and Grace & Teale (2006) found that GE was the major isoflavone in *H. pomatia* juice. These observations show that isoflavone concentrations appear to fluctuate from one enzyme batch to another. The hydrolysis with this enzymatic solution also showed interfering peaks with isoflavone signals in the chromatogram of human milk extracts (Franke & Custer 1996). There are two ways of addressing these problems. One is a pre-clean-up of the enzymatic preparation by SPE (Grace & Teale 2006; Kuhnle *et al.* 2007). The second way, probably the easier of the two, involves working with a blank sample where the milk has been replaced by a buffer solution in order to estimate the isoflavone content in the enzymatic mixture (Taylor *et al.* 2005).

Isolation of target compounds

Solvent extraction is the main step used for the recovery and isolation of bioactive compounds from a matrix before instrumental analysis and it can be performed before or after the hydrolysis step. Additional steps are sometimes necessary to remove lipids or proteins, which can cause interferences during instrumental analysis. Fat is one of the major causes of the matrix effect in LC-MS (Trontelj 2012; Jiang *et al.* 2012) and most of the protocols in Table 3.1 therefore indicate defatting and deproteinizing steps. Lipids can be removed by LLE with an apolar solvent (e.g., n-heptane or n-hexane) and

centrifugation. Proteins are removed by precipitation in acidic conditions and centrifugation. Deproteinization is often used at the same time that the defatting or extraction of target compounds is done because organic solvents can also precipitate the proteins (Trontelj 2012).

LLE is the most widely used technique for extracting isoflavones from milk. The extractions are repeated two to three times and the extracts are pooled. Among the analytical protocols listed in Table 3.1, only three used SLE to recover isoflavones in milk samples (Kuhnle *et al.* 2008; Rastogi *et al.* 2013; Maggioni *et al.* 2013). Only Rastogi *et al.* (2013) deliberately used the freeze-drying process, whereas two other studies used this extraction technique because they were analyzing milk samples in powder form. Optimizing extraction is a challenging task given the wide polarity range of isoflavones. As an example, compared with DA, GE has an additional hydroxyl group and therefore DA and GE are expected to have different solubilities (Harjo *et al.* 2007). In general, conjugated forms are more polar than aglycones and are extracted with polar solvents such as alcohols (MeOH, EtOH) or acetonitrile, often mixed with varying proportions of water. For isoflavone quantification in milk, a hydrolysis step is almost always used in order to remove the polar groups from conjugated forms, leading to the formation of isoflavone aglycones, which are less polar and almost insoluble in water (Harjo *et al.* 2007; Ishii *et al.* 2012; Vitale *et al.* 2013). This is why less polar solvents (diethyl ether or ethyl acetate), with no water added, are used for extraction in milk. The choice of solvent composition should be determined empirically according to the target molecules that the researchers wish to extract.

Looking more closely at the order in which the hydrolysis and isolation steps are done, some authors conducted the hydrolysis step first and then the extraction step, whereas others used the reverse order. This raises the question: is one protocol better than the other? If the less polar solvents are more efficient for extracting aglycone forms, when the hydrolysis is performed after the extraction step the conjugated isoflavones might not be completely extracted, leading to an underestimation of the overall isoflavone content. Some authors (Křížová *et al.* 2011a; Daems *et al.* 2015) reported that isoflavones are distributed more widely in the aqueous fraction than in the lipid fraction. In contrast, Tsen *et al.* (2014) reported that isoflavones are distributed mostly in the lipid fraction and that

Table 3.2. Summary of separation and detection methods used between 2005 and 2015 for quantification of isoflavones in milk

Instrumental analysis		Validation parameters									Reference
Separation	Detection	Quantification mode	LOQ	LOD	Sel.	Lin.	Prec.	Acc.	Stab.	Rob.	
HPLC Column: Zorbax XDB (150× 2.1 mm i.d., 3.5 µm) Mobile phases: (A) 0.5% Acetic acid (B) MeOH Elution: gradient Column T°: N.C. Flow: 0.4 mL.min^{-1} Run time: 14 min Inj. vol.: 50 µL	MS/MS ESI^{+}, MRM mode	Standard addition	N.C.	0.05 - 0.17 ng.mL^{-1}	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Adler et al. (2015), Adler et al. (2014), Njåstad et al. (2014), Höjer et al. (2012), Nielsen et al. (2012), Andersen et al. (2009a), Steinshamn et al. (2008)
UPLC Column: Acquity UPLC® HSS T3 (100×2.1 mm, 1.8 µm) Mobile phases: (A) water/acetonitrile (95/5, v/v) with 0.01% formic acid (B) acetonitrile/water (95/5, v/v) with 0.01% formic acid Elution: gradient Column T°: 40 °C Flow: 0.6 mL.min^{-1} Run time: 5 min Inj. vol.: 10 µL	MS/MS ESI^{+}, MRM mode	External calibration	5 ng.mL^{-1}	0.3 ng.mL^{-1}	V	V	V	V	V	V	Daems et al. (2015)
UHPLC Column: Acquity HSS T3 (100× 2.1 mm, 1.8 µm) Mobile phases: (A) 5 mM ammonium acetate in H_2O/acetonitrile (9:1, v/v) (B) MeOH:acetonitrile (1:1, v/v) Elution: gradient Column T°: 45 °C Flow: 0.4 mL.min^{-1} Run time: 10.5 min Inj. vol.: 10 µL	MS/MS ESI$^{\pm}$, MRM mode	External calibration	0.35–0.36 µg.g^{-1}	0.20–0.21 µg.g^{-1}	V	V	V	V	V	N.C.	Wielogórska et al. (2015)

Table 3.2. (continued)

Instrumental analysis		Validation parameters									Reference
Separation	Detection	Quantification mode	LOQ	LOD	Sel.	Lin.	Prec.	Acc.	Stab.	Rob.	
LC	IT-TOF-MS$^-$	External calibration	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tsen et al. (2014)
HPLC Column: Supelcosil LC-8-DB (250× 4.6 mm i.d., 5 µm)+guard column (40×4.6 mm i.d.) Mobile phases: (A) water (B) MeOH Elution: gradient Column T°: 25 °C Flow: 0.8 mL.min^{-1} Run time: 45 min Inj. vol.: 10 µL	PDA UV: 254 nm	External calibration	500 ng	300 ng	V	V	V	V	N.C.	N.C.	Rastogi et al. (2013)
HPLC Column: LUNA C8 (50×2.0 mm i.d., 5 µm) Mobile phases: (A) 0.05% acetic acid in Milliq water (B) acetonitrile Elution: gradient Column T°: N.C. Flow: 200 µL.min^{-1} Run time: 18 min Inj. vol.: 10 µL	MS/MS ES$^-$, MRM mode	External calibration	N.C.	0.6–0.9 ng.g^{-1}	N.C.	V	V	V	N.C.	N.C.	Maggioni_2013
HPLC Column: Purospher STAR RP-18^e (250× 4.0 mm i.d., 5 µm particle size) Mobile phases: (A) aqueous 1% formic acid (B) acetonitrile 1% formic acid Elution: gradient Column T°: 35 °C Flow: 0.5 mL.min^{-1} Run time: 41 min Inj. vol.: 20 µL	DAD-MS/MS UV: 200–600 nm MS: ESI$^+$, SIM mode	External calibration	0.006–0.7 ng.mL^{-1}	N.C.	N.C.	V	N.C.	V	N.C.	N.C.	Nielsen et al. (2011) and Skaanild and Nielsen (2010), Nielsen et al. (2009) and Andersen et al. (2009b)
HPLC	MS/MS	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Flachowsky et al. (2011)

Table 3.2. (continued)

Instrumental analysis		Validation parameters									Reference
Separation	Detection	Quantification mode	LOQ	LOD	Sel.	Lin.	Prec.	Acc.	Stab.	Rob.	
Column: YMC-Pack ODS-AM 12S05 column (150×4.6 mm i.d., 5 µm) Mobile phases: (A) acetic acid 0.1% (B) acetonitrile/acetic acid [99.9:0.1, v:v] Elution: gradient Column T°: N.C. Flow: 1.0 mL.min^{-1} Run time: 60 min Inj. vol.: N.C.	ESI^{-}, N.C. mode										
HPLC Column: discovery C18 (150×3 mm, 5 µm)+discovery C18 guard column (20×4 mm, 5 µm) Mobile phases: (A) MeOH (B) 0.1% acetic acid water (v/v) Elution: gradient Column T°: 45 °C Flow: 0.7 mL.min^{-1} Run time: 16 min Inj. vol.: 10 µL	MS/MS APCI^{+}, SRM mode	External calibration	2–15 ng.mL^{-1}	0.5–5 ng.mL^{-1}	N.C.	N.C.	V	V	N.C.	N.C.	Křížová et al. (2011a), Křížová et al. (2011b), Krajčová et al. (2010) and Třináctý et al. (2009)
HPLC Column: RP octadecyl grafted silica stationary phase Nucleosil® C_{18}AB (50×2.1 mm, 5 µm+guard column 10×2.1 mm) Mobile phases: (A) methanol (B) water 0.5% acetic acid Elution: gradient Column T°: N.C. Flow: 0.3 mL.min^{-1} Run time: 30 min Inj. vol.: 10 µL	MS/MS ESI^{+}, MRM mode	External calibration	0.05–0.50 ng.g^{-1}	N.C.	V	V	V	N.C.	N.C.	V	Antignac et al. (2009)

Table 3.2. (continued)

Instrumental analysis		Validation parameters									Reference
Separation	Detection	Quantification mode	LOQ	LOD	Sel.	Lin.	Prec.	Acc.	Stab.	Rob.	
HPLC Column: Zorbax Eclipse XDB-C18 (250×4.6 mm+precolumn Zorbax R-P 4×4 mm) Mobile phases: (A) MeOH/10 mM $Na_2HPO_4.2H_2O$ pH 6.5 (B) MeOH 100% Elution: gradient Column T°: 40 °C Flow: 1 $mL.min^{-1}$ Run time: 31 min Inj. vol.: 10 µL	DAD-FLD UV: 260 nm FL: 254 nm (ex) 465 and 310 nm (em)	External calibration	3.3–117.2 $ng.mL^{-1}$	1.3–58.6 $ng.mL^{-1}$	V	V	N.C.	V	N.C.	N.C.	Mustonen et al. (2009) and Hoikkala et al. (2007)
HPLC Column: diphenyl column (Varian Pursuit, 150×2.0 mm i.d., 3 µm) Mobile phases: (A) 40% ammonium acetate (0.1%, pH 4.8) in MeOH (B) 100% MeOH Elution: gradient Column T°: 50 °C Flow: 250 $µL.min^{-1}$ Run time: N.C. Inj. vol.: N.C.	MS/MS ESI^-, N.C. mode	External calibration	N.C.	15 $ng.g^{-1}$ww	V	V	V	V	N.C.	N.C.	Kuhnle et al. (2008)
HPLC Column: C18 YMC-ODS-AM (250×3 mm S5)+C18 guard Column (AJ0-7597) Mobile phases: (A) acetic acid: acetonitrile:water (0.1:9.95:89.95) (B) acetic acid:acetonitrile:water (0.1:89.95:9.95) Elution: gradient Column T°: 40 °C	UV	External calibration	N.C.	N.C.	N.C.	V	N.C.	N.C.	N.C.	N.C.	Uzzan et al. (2007)

Table 3.2. (continued)

Instrumental analysis		Validation parameters									Reference
Separation	Detection	Quantification mode	LOQ	LOD	Sel.	Lin.	Prec.	Acc.	Stab.	Rob.	
Flow: 0.8 mL.min^{-1} Run time: N.C. Inj. vol.: 10 µL											

The underlined reference corresponds to the referenced method used and cited in the other articles.

Abbreviations: LOQ limit of quantification, LOD limit of detection, Sel. selectivity, Lin. linearity, Prec. precision, Acc. accuracy, Stab. stability in extract or sample, Rob. robustness, inj. vol. injection volume, N.C. not communicated, and V communicated.

the defatting process therefore reduces the overall amount of isoflavones in a sample. These are all matters that need to be addressed in future studies.

Purification and concentration extracts

After the extraction of isoflavones in milk, the extract is subjected to a series of post-treatment steps before instrumental analysis. In almost all protocols, centrifugation is performed. As well as eliminating the cream (fat) and the precipitated proteins, it also isolates other small solid impurities. A filtration step is often performed just before the sample extract injection (Uzzan *et al.* 2007; Hoikkala *et al.* 2007; Nielsen *et al.* 2009; Andersen *et al.* 2009b; Flachowsky *et al.* 2011; Rastogi *et al.* 2013; Tsen *et al.* 2014; Daems *et al.* 2015; Wielogórska *et al.* 2015) to prevent damage to the analytical columns.

In milk, isoflavones are usually present in low concentrations (a few ppb or, sometimes, higher for microbial metabolites) (Kalač 2013) and therefore it is necessary to concentrate the target analyte before instrumental analysis. SPE and evaporation were the most commonly used enrichment techniques. SPE using C_{18} bonded silica (Kuhnle *et al.* 2008; Antignac *et al.* 2009; Flachowsky *et al.* 2011) or a universal polymeric reversed-phase sorbent called OASIS-HLB from Waters (Hoikkala *et al.* 2007; Mustonen *et al.* 2009; Maggioni *et al.* 2013; Wielogórska *et al.* 2015) were reported to provide a clean concentrated isoflavone extract. Probably due to its typical chemistry, OASIS-HLB has the advantage of greater retention and good recoveries even if the sorbent runs dry (Rostagno *et al.* 2009). The final extracts are then evaporated until dry and re-suspended with the polar solvent used in the instrumental analysis.

Instrumental analysis

After these preparation steps, the sample extract can be injected into instruments that will allow the target compounds to be separated, detected and quantified. The data in the 26 selected articles are presented in Table 3.2. When different authors used the same analytical methods, the data presented derive from all the information in the articles. Thirteen analytical methods were identified and are discussed below.

Analyte separation

Unlike in other matrices (plants, foods or other biological fluids), there are few separation methods reported in the literature for the quantification of isoflavones in milk.

Over the past 10 years, the only analytical separation technique used has been reverse-phase LC with a binary solvent system that contains acidified water and an acidified polar organic solvent, such as methanol or acetonitrile (Table 3.2).

With the LC technique, a guard column is often placed before the analytical column in order to retain all impurities remaining in the sample extract (Uzzan *et al.* 2007; Hoikkala *et al.* 2007; Trinacty *et al.* 2009; Antignac *et al.* 2009; Křížová *et al.* 2011a; Křížová *et al.* 2011b; Rastogi *et al.* 2013). This also enables the compounds from the extract to start being separated. These small columns are regularly changed and allow the lifetime of the main analytical column to be increased. They often have a stationary phase similar to that of the main column. Columns chosen for isoflavone separation in milk are exclusively packed with reversed-phases, mainly C_{18} with an internal diameter of 2.0-4.6 mm, a length of 50-250 mm and a particle size of 3-5 µm. Two other stationary phase chemistries are sometimes also mentioned in the articles: C_8 (Maggioni *et al.* 2013; Rastogi *et al.* 2013) and diphenyl (Kuhnle *et al.* 2008). Two recent studies report on the use of ultra high-performance LC (UHPLC) columns with a particle size of 1.8 µm, an internal diameter of 2.1 mm and a length of 100 mm (Daems *et al.* 2015; Wielogórska *et al.* 2015). This technology allows separations that have positive effects on both resolution and accuracy. It also increases sample-throughput due to reduced analysis times (Valls *et al.* 2009; Gumustas *et al.* 2013). The reduction of analysis time is illustrated in Table 3.2. For classical LC analysis, the average analysis time is about 35 min, ranging from a minimum of 14 min (Steinshamn *et al.* 2008) to a maximum of 60 min (Flachowsky *et al.* 2011). In the two studies using UHPLC it was between 5 and 10.5 min. These times could be further reduced by manipulating column temperature, flow rate, mobile phase composition and solvent gradients.

The mobile phase flow rate depends upon its composition, column type and temperature. In the selected articles the flow rates varied between 0.2 and 1 $mL.min^{-1}$, the temperature from 25 to 45°C and the injection volume from 10 to 50 µL. The mobile phase is usually a mixture of water and an organic solvent (methanol or acetonitrile) with a small proportion of formic or acetic acids (up to max. 1%). The differing hydrophobicities of isoflavones indicate that the gradient elution mode should be used (Rostagno *et al.* 2009). Since isoflavones exhibit a weak acidic nature, the use of acids in the mobile phase can enhance chromatographic resolution and peak shape (Wu *et al.* 2004; Rostagno *et al.* 2009). Acidification of the mobile phase also significantly increases

the isoflavone limit of detection (LOD) by promoting positive ion formation in the MS source (Wu *et al.* 2004).

Analyte detection

As shown in Table 3.2, the most commonly used detection methods combined with LC are MS and UV detectors. They can be used alone, in combination (Nielsen *et al.* 2009; Andersen *et al.* 2009b) or with other types of detectors (Hoikkala *et al.* 2007; Mustonen *et al.* 2009). Hoikkala *et al.* (2007) used UV-DAD at 262 nm for GE, *O*-DMA and BA and they used FLD for DA and FO (λ_{ex} at 254 nm and λ_{em} at 465 nm), as well as for EQ (λ_{ex} at 254 nm and λ_{em} at 310 nm). Although the LC-FLD method is often more sensitive than the UV absorption methods, the number of isoflavones that are naturally fluorescent is fairly limited (Ko 2014). In addition, the acidification of the mobile phase has a quenching effect that can reduce FLD sensitivity (Vacek *et al.* 2008).

Overall, LC-UV and LC-MS are the two main detection modes used for quantifying isoflavones. In milk, however, MS is preferred to UV detection. UV detection uses the isoflavone characteristic of having at least one aromatic ring that strongly absorbs UV light, with a maximum wavelength ranging from 230 to 280 nm (Wu *et al.* 2004; Stalikas 2007; Rostagno *et al.* 2009; Ko 2014). A range of 300 to 550 nm was sometimes also used as a second maximum wavelength and attributed to the substitution pattern and conjugation of the C-ring (Stalikas 2007). This is not the case with all isoflavones. For example, the microbial metabolites EQ and *O*-DMA exhibit poor UV absorption characteristics, making UV detection unsuitable for their measurement (Setchell & Clerici 2010a; Ko 2014). For isoflavones that exhibit this UV absorption characteristic, the UV-visible spectra of many of them are very similar (Vacek *et al.* 2008). Another disadvantage of this method is its low sensitivity, which restricts its use to quantification in matrices likely to have a large amount of isoflavones, such as plants, plant-derived samples and supplemented foods (Wu *et al.* 2004; Ko 2014). Among the studies presented in Table 3.2, only two used UV as the sole mode of detection (Uzzan *et al.* 2007; Rastogi *et al.* 2013) and both focused on analyzing fortified milk.

In order to overcome the problems of non-specificity and lack of sensitivity, researchers have focused on MS detection. Usually, this method is highly sensitive, with low limits of quantification (LOQ) under or around the ppb level (see Table 3.2). Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are

the two sources most commonly used for quantifying isoflavones in milk. In the selected articles, these sources were used in positive and/or negative mode, except for APCI, which was used exclusively in positive mode with select reaction monitoring (SRM). In the ESI, multiple reaction monitoring (MRM) was used. ESI and APCI interfaces appeared to be suitable for the analysis of isoflavones, but ESI is better suited for the conjugated forms. ESI is the softest and most suitable method for ionization without the in-source fragmentation of the relatively weak glucuronide ether or ester bonds. Thermally labile conjugates form molecular ions in ESI, but with APCI these conjugates decompose because they are not stable enough (Hoikkala *et al.* 2003; Trontelj 2012). In their comparison of ESI and APCI sources for the quantitative determination of phytoestrogens in urine, Rybak *et al.* (2008) showed that, for EQ, ESI was better in terms of measurement precision, sensitivity and LOD.

The benefits of LC-MS include high precision and sensitivity, suitability for determining isoflavones and their metabolites or conjugated forms, less samples manipulation than for GC, and applicability to non-volatile analytes with direct injection of the liquid samples. The higher price of this technology remains its major disadvantage compared with other separation and detection technologies. LC-MS, however, is now a fast-moving field, with many new detection and chromatographic techniques being developed. With their shorter analysis time, lower solvent consumption, better resolution properties and better sensitivity than classical LC, UPLC and UHPLC with MS appear to be the fastest and easiest methods to implement and to produce reliable results.

Validation parameters

So far as we could ascertain, method validations were seldom discussed in the previous review articles on isoflavones and related compounds. Where they were mentioned, the validation parameters are shown in Table 3.2 (when several works used the same analytical procedure, the various data in each study were collected and summarized in this table).

The validation parameters taken into account in this table come from the EMA guidelines (2015), which describe the suitable criteria for the validation of analytical methods used in veterinary drug residue depletion studies. The guidelines could apply to the quantification of isoflavones in milk because these estrogenic compounds can be active in very low amounts. As shown in Table 3.2, most of the studies used external

calibration as the quantification mode for determining the isoflavone content of milk, whereas Steinshamn *et al.* (2008) and related studies used a standard addition protocol. Among these studies, Andersen *et al.* (2009a) reported that the LOD for FO and EQ were 0.047 and 0.170 $ng.mL^{-1}$, respectively. In the other selected articles, the validation parameters most often omitted were sample or extract stability and the robustness of the analytical method proposed. The most comprehensive validation procedures were reported by Wielogórska *et al.* (2015) (for DA, GE and other endocrine disruptors) and Daems *et al.* (2015) (for EQ).

Conclusion

This paper summarizes the current analytical methods used for quantifying isoflavones and their metabolites in cow's milk. Careful examination of methodological aspects showed that most of the studies used LLE to extract isoflavones from milk. Given that aglycones are the most biologically active forms of isoflavones, most of the analytical protocols used a hydrolysis step with β-glucuronidase/sulfatase from *H. pomatia*.

Although non-chromatographic immunoassays and CE with MS or ED detectors are seen as promising techniques for quantifying isoflavones, reverse-phase LC-MS is the only analytical method used in the past 10 years. LC-MS is now a fast-moving field, with many new detection and chromatographic techniques being developed over the past decade that achieve high sensitivity and reduce analysis time. The low isoflavone content (about ppb value) naturally present in milk, except in the case of some isoflavone metabolites, justifies the use of MS as a detection method. The new chromatographic technologies, UPLC and UHPLC, reduce the time needed and achieve good resolution.

Although some analytical protocols have been used frequently by researchers, there is still no standardized procedure available for the sample preparation and determination of these phytoestrogenic compounds in milk. A rigorous validation referring to a guideline would be enough to ensure the reliability of the chromatographic results. For sample preparation, however, a standardized procedure is interesting to compare the results from the different studies.

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Chapter IV

Development and validation of UPLC®-MS/MS method for quantifying equol in cow's milk

Objectives

On the basis of literature overview (*Chapter III*), the ultra-performance liquid chromatography (UPLC®) coupled with tandem mass spectrometry (MS/MS) is the fastest and most sensitive instrumental analysis to produce reliable results for quantifying equol in milk. The quantification of equol in milk was therefore achieved using UPLC®-MS/MS. This chapter describes the development and validation of a reliable analytical method for quantifying this isoflavan in cow's milk. The equol was initially released using enzymatic hydrolysis and was then extracted using a double liquid-liquid extraction. The proposed analytical method was fully validated following European Medicines Agency 'EMA' guidelines (2011). The method was then used for the first time to estimate the equol concentration in commercial cow's milk in Belgium. A graphical abstract of this chapter is presented in Fig. 4.1.

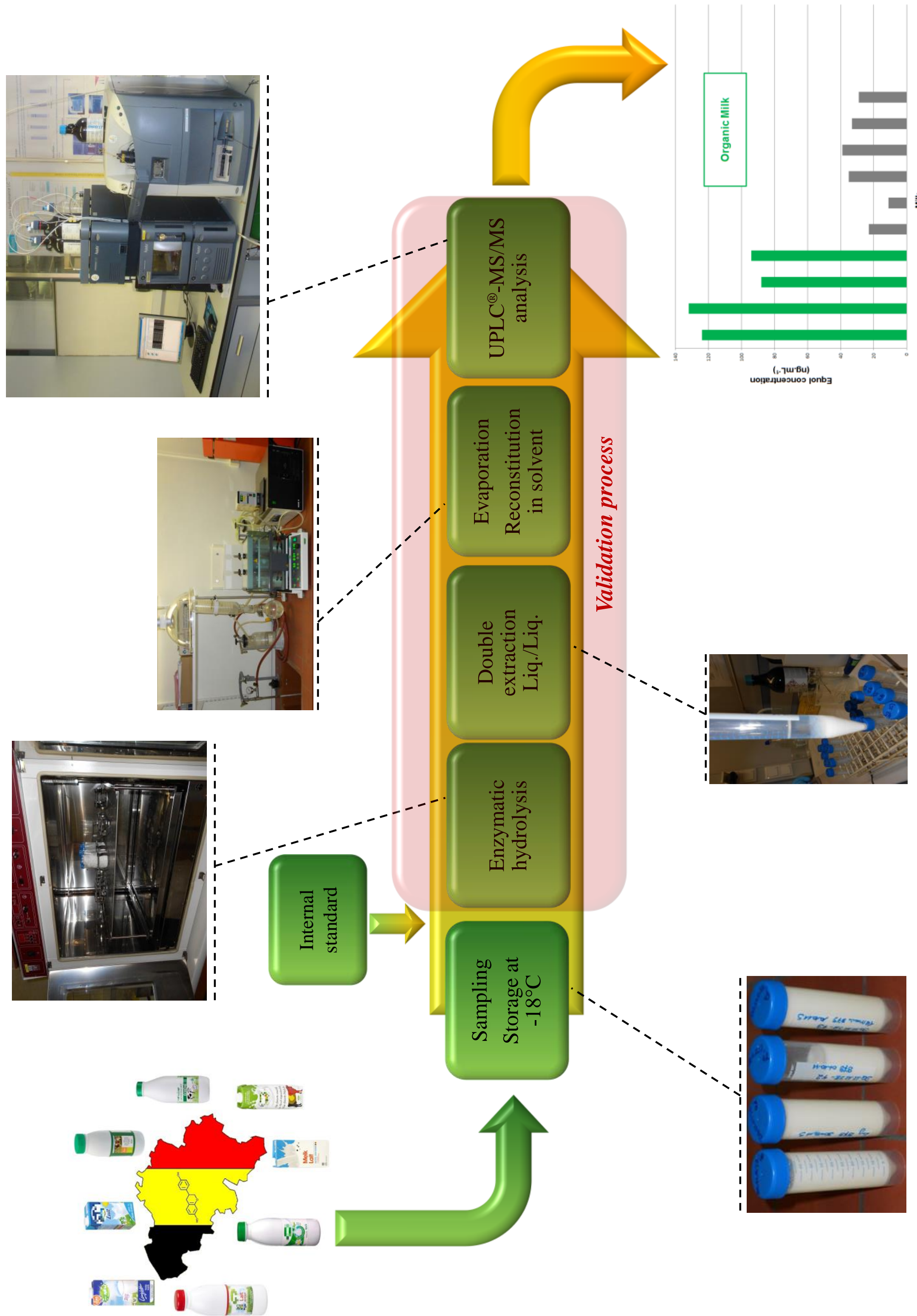

Figure 4.1. Graphical abstract of *Chapter IV*. Analytical procedure developed and validated for quantifying equol in commercial cow's milk in Belgium.

Validating the use of an ultra-performance liquid chromatography with tandem mass spectrometry method to quantify equol in cow's milk

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Abstract

Cow's milk can be used as a potential source of equol in the human diet. In order to study human intake, however, it is necessary to develop a reliable and sensitive analytical method. This paper reports on the validation of an analytical method using ultra-performance liquid chromatography coupled with a tandem mass spectrometry detector to quantify the equol in commercial milks (raw, whole, semi-skimmed, and skimmed milk). The equol was initially released using enzymatic hydrolysis, and it was then extracted using a double liquid/liquid extraction. The analytical method produced a linear calibration curve with a high correlation coefficient ($R^2 \geq 0.996$) between 5 and 1,000 ng.mL^{-1}. Good intra- and inter-day precision (≤5.3% and≤5.2%, respectively) and accuracy (≤8.6%) were achieved. The recovery rate differed slightly among the different types of milk, ranging between 60.6± 1.09% and 82.3±5.21%. Good method repeatability was observed (≤15%). There was neither matrix effect nor carry-over effect, and the sample extracts were stable for at least 7 days of storage at -21 °C and 5 °C. The method proved to be specific, sensitive, precise, and accurate and was used for the first time to quantify the equol content in Belgian commercial cow's milk. In all the samples analyzed, equol was present at a concentration ≥10 ng.mL^{-1} and had a significantly higher content in organic than in conventional milk. The study also found that the mean concentrations of equol were similar for each type of commercial conventional cow's milk.

Keywords

Ultra-performance liquid chromatography (UPLC®) - Tandem mass spectrometry (MS/MS) – Equol – Milk – Validation

Introduction

Equol is a microbial metabolite of daidzein and formononetin, two phytoestrogens present in plants (mainly the Leguminosae family), and is produced by specific bacteria that are present in the digestive system of animals and humans (Setchell & Clerici 2010b; Setchell & Clerici 2010a; Jackson *et al.* 2011). Equol has a strong antioxidant activity (Setchell & Clerici 2010b; Setchell & Clerici 2010a) and could be used as a therapeutic agent against several diseases or cancers (Setchell & Clerici 2010b; Setchell & Clerici 2010a; Jackson *et al.* 2011). In the human population, however, not everyone would be able to benefit from its positive effects because of the lack of the specific microbial populations (Setchell & Clerici 2010b; Setchell & Clerici 2010a; Jackson *et al.* 2011). An alternative strategy for making equol's health benefits available to everyone would be the supply of this molecule in the human diet via food of animal origin. From the range of foods commonly consumed by humans, milk from dairy cows is an interesting potential source of equol (Křížová *et al.* 2011a; Tsen *et al.* 2014). In recent years, many studies have been conducted on how to assess equol in milk and on feeding strategies for increasing the content of this molecule in milk (Steinshamn *et al.* 2008; Mustonen *et al.* 2009; Andersen *et al.* 2009b; Höjer *et al.* 2012; Kalač 2013; Adler *et al.* 2014).

With the growing interest in phytoestrogens and their metabolites, many analytical methods have been developed and reviewed in the literature (Wu *et al.* 2004; Grynkiewicz *et al.* 2005; Stalikas 2007). In ruminants, conjugated equol is the predominant form of equol found in biological fluids (Setchell & Clerici 2010b; Setchell & Clerici 2010a; Mostrom & Evans 2012). A hydrolysis step is therefore required to estimate the overall quantity of equol excreted in milk. The chromatographic technique most frequently used to analyze phytoestrogens and their microbial metabolites in milk samples is liquid chromatography (LC) coupled with a mass spectrometry (MS) detector (Antignac *et al.* 2004; Steinshamn *et al.* 2008; Andersen *et al.* 2009b; Höjer *et al.* 2012; Adler *et al.* 2014). LC is often used because sample preparation is easy and the method can be useful in analyzing more complex forms of phytoestrogens (e.g., with sugar, malonyl, or sulfate fragments) (Wu *et al.* 2004). In recent years, ultra-performance liquid chromatography (UPLC®) has become widely used in this type of analysis (Krajčová *et al.* 2010; Wielogórska *et al.* 2015). This special variant of high-performance liquid chromatography (HPLC) uses a smaller column particle size and can work at much higher pressure than HPLC. In addition, the injection volume is smaller in UPLC than in HPLC

(Churchwell *et al.* 2005; Gumustas *et al.* 2013). These differences improve the speed of analysis as well as the resolution of LC analysis. For equol detection, MS detection techniques are the most widely used because they are very sensitive and can detect very low analyte content in biological fluids (Setchell & Clerici 2010b; Setchell & Clerici 2010a). LC-MS is now a fast-moving field, with many new detection and chromatographic techniques being developed over the past decade aimed at achieving higher sensitivity and reducing analysis time (Wu *et al.* 2004; Prokudina *et al.* 2012; Gumustas *et al.* 2013). Currently UPLC®-MS/MS analysis appears to be the fastest method; it is easiest to implement and produces reliable results.

The aim of this study was to validate a reliable, fast, and easy-to-use analytical method based on UPLC®-MS/MS for the routine quantification of equol in cow's milk. Once validated, this new method was used for the first time to estimate the equol content in a small number of samples of Belgian commercial cow's milk.

Material and methods

Chemicals and reagents

Equol (≥99.0% purity) and β-glucuronidase type H2 from *Helix pomatia* (EC Number: 3.2.1.31, glucuronidase activity, ≥85,000 units per mL and sulfatase activity, ≥7,500 units per mL) were purchased from Sigma-Aldrich (Diegem, Belgium). The internal standard (IS) daidzein-d_4 (98.9% purity) was purchased from C/D/N ISOTOPES (Pointe-Claire, Canada). Methanol (LC-MS reagent) and acetonitrile (LC-MS reagent) came from J.T. Baker (Deventer, The Netherlands); *n*-hexane (HPLC, 99%) from CARLO ERBA reagents (Val de Reuil, France); and ethyl acetate (HPLC, 99.9%) from Biosolve (Valkenswaard, The Netherlands). Formic acid (ULC-MS, 99%) was purchased from Biosolve. The dilution of organic solvents and enzymatic solutions was carried out with deionized water prepared using a Milli-Q® system (Millipore, Overijse, Belgium).

Calibration standard solutions

Separate primary stock standard solutions of equol and IS were prepared in methanol at a concentration of 100 $\mu g.mL^{-1}$. These standard solutions were mixed in the appropriate proportions and serially diluted with deionized water and methanol in order to obtain standard solutions in methanol/H_2O (80:20, *v/v*). Calibration standards, ranging from 5 to 1,000 $ng.mL^{-1}$ for equol with 100 $ng.mL^{-1}$ of IS, were prepared daily for each

level. This calibration range in the UPLC®-MS/MS system corresponds with an equol concentration ranging from 1.25 to 250 ng.mL^{-1} in milk samples. It was chosen in order to take account of the expected levels reported in several studies (Kalač 2011) and dilutions obtained during sample preparation. All the standard solutions were stored at -18 °C and protected from light.

Sampling and storage

In order to apply the proposed method and obtain an initial estimation of equol concentration in Belgian cow's milk, 44 samples (15 whole [fat content 'fat', ≥3.5%], 24 semi-skimmed [fat, 1.5-1.8%] and five skimmed [fat, ≤0.5%]) of various brands were collected from several supermarkets in the country (February 2013). Among these samples, six came from the organic production sector. All samples were ultra-high temperature (UHT) processed, except four of them: three were sterilized and the last one pasteurized and micro-filtered. In addition, five raw milk samples [fat, ≥3.5%] were collected from three Walloon farms, three of them from two conventional farms (farms x and y) and the other two from an experimental farm (farm z). All the milk samples were put in 50-mL Falcon® tubes stored at -18 °C and protected from light for up to 2 months. They were then analyzed in triplicate.

Sample preparation

Sample preparation followed the protocol described by Andersen *et al.* (2009b), with some modifications. Each sample was thawed overnight at 4-6 °C, equilibrated to ambient temperature, and homogenized with a Vortex mixer. An aliquot of 2 mL, 0.1 mL of IS (500 ng.mL^{-1}), and 0.1 mL of β-glucuronidase (glucuronidase activity, ≥42,000 units per mL) was put into a centrifuge tube (15 mL). The mixture was then vortexed, placed in an oven at 37±1 °C, and shaken continuously for 2 h. After hydrolysis, the mixture was cooled in an ice bath for 15 min. It was then centrifuged using an Eppendorf® 5810 centrifuge (Eppendorf, Hamburg, Germany) at 3,220x*g* for 15 min. The creamy layer and the precipitate were discarded, and the liquid phase was recovered and submitted to solvent extraction using *n*-hexane (3x3 mL), followed by ethyl acetate (3x3 mL). In the first extraction, the hexane removed the fat still present in the sample; in the second, the ethyl acetate enabled the compounds of interest to be recovered. Centrifugation (4 min at 3,220xg) was performed between each extraction. The ethyl acetate extract was then evaporated under vacuum with a Büchi Syncore® Polyvap

(BÜCHI Labortechnik AG, Flawil, Switzerland) at 50 °C. The residue was reconstituted in 0.5 mL of methanol: H_2O (80:20, *v/v*) by vortexing for 1 min, filtered through an Uptidisc™ CA 13 mm/0.2 µm (Interchim, Montluçon, France) and analyzed using UPLC®-MS/MS.

Chromatographic conditions

The LC analyses were performed on an ACQUITY UPLC® from Waters (Zellik, Belgium) equipped with a Waters ACQUITY UPLC® HSS T3 column (2.1x 100 mm, 1.8 µm particle size). The column was kept at 40 °C. The vials with samples and standard solutions were stored in an autosampler at 4 °C, and an aliquot of 10 µL was injected using the full loop injection mode. The mobile phase consisted of a water/ acetonitrile mixture (95/5, *v/v*) containing 0.01% formic acid (eluent A) and acetonitrile/water (95/5, *v/v*), as well as 0.01% formic acid (eluent B), with the following gradient profile—90% A/10% B (start), 90% A to 70% A (0.0-1.0 min), 70% Ato 60% A (1.0-2.5 min), 60% Ato 10% A (2.5-3.0 min), 10% A (3.0-4.0 min), and 10% A to 90% A (4.0-4.1 min). The column was then left to re-equilibrate under the initial conditions for 0.9 min, resulting in a total runtime of 5 min. The flow rate was set at 0.6 $mL.min^{-1}$. In order to avoid subsequent contamination, the needle was washed between each injection with 200 µL of eluent B and 600 µL of eluent A.

MS/MS conditions

The eluting compounds were detected using a Quattro Premier XE (Micromass Waters, Zellik, Belgium) with an electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode, and the quantification was performed in multiple-reaction monitoring (MRM). A split was placed at the entrance of the device (split ratio 1/3) to give a mobile phase flow rate of 200 $\mu L.min^{-1}$ inside the mass spectrometer. The ESI source used a standard 125 µm capillary. The optimal source and desolvation temperatures were 140 °C and 350 °C, respectively. Cone gas and desolvation gas flow (both N_2) were set at 50 $L.h^{-1}$ and 700 $L.h^{-1}$, respectively. Argon was used for the collision-induced fragmentation. The optimized capillary and cone voltages were 1.5 kV and 35 V for equol and 1.5 kV and 25 V for IS. As the MRM mode was used, two precursor-product ion transitions were selected: 259>138 (collision energy 'Col. En.', 27 eV) and 259>203 (Col. En., 25 eV) for equol; and 243>133 (Col. En., 13 eV) and 243 > 123 (Col. En., 15 eV) for IS. The first transition was used for

quantification (Q) and the second for confirmation (q). All the data were collected and processed using MassLynx® software with a QuanLynx® program (Micromass Waters, Zellik, Belgium).

Method validation

The UPLC®-MS/MS method used for equol quantification was validated by investigating the following parameters: specificity, linearity range, analytical limits of detection (LOD) and quantification (LOQ), accuracy, precision, carry-over, process efficiency, method repeatability, and sample extract stability. The calibration curve was based on the ratio between the peak areas of equol and IS (A_a/A_{is}) versus the equol concentration (nanograms per milliliter of injected solution). The robustness of the hydrolysis method was also assessed by varying three factors—enzymatic solution concentration, hydrolysis time, and sample volume—and the best conditions were selected. These validation parameters were evaluated according to EMA (2011) guidelines.

Statistical analysis

All the analyses were performed at least in triplicate. Statistical analyses and graphics were performed with Microsoft® Excel software (Microsoft®, Diegem, Belgium) and the JMP® 7 statistical software package (SAS institute, Cary, US). Analysis of variance (ANOVA; α =0.05) and comparison of averages by Tukey's honestly significant difference (HSD) (α=0.05) tests were used to check different validation parameters and to compare the different milk types. A *t*-test (α=0.05) was also conducted to assess the matrix effect.

Results and discussion

Method development

In order to estimate the overall quantity of equol excreted in milk, a hydrolysis step is required. The equol molecule, however, is extremely acid-labile and can easily be destroyed if acidic hydrolytic procedures are used in sample preparation (Setchell & Clerici 2010b; Setchell & Clerici 2010a). For this reason, enzymatic hydrolysis with β-glucuronidase from *H. pomatia* was undertaken to measure the total amount of free equol in the samples.

The choice of enzyme was based on other studies (Wu *et al.* 2004; Antignac *et al.* 2004; Hoikkala *et al.* 2007; Andersen *et al.* 2009b; Adler *et al.* 2014). The sample preparation procedure selected was based on the one reported by Andersen *et al.* (2009b), with slight modifications in the hydrolysis conditions (see Section '*Robustness of hydrolysis process*'). The hydrolyzed sample was then centrifuged and extracted using *n*-hexane in order to carefully remove lipids from the milk (Stalikas 2007). Fat is one of the major causes of the matrix effect in LC-MS analysis (Jiang *et al.* 2012). The second solvent was then used to extract equol (Stalikas 2007).

For chromatographic optimization, a gradient elution with acetonitrile and water that could separate equol and the IS in a reasonably short time was used (Fig. 4.2). A small amount of formic acid was added to the mobile phases to facilitate the dissociation of equol in the solvent system, thus enhancing the chromatographic separation, resolution, and peak shape (Wu *et al.* 2004). The formic acid also significantly increased detection sensitivity by enhancing positive ion formation in the MS detector (Wu *et al.* 2004). The total runtime of the proposed UPLC®-MS/MS method is lower than the other methods described in literature, which range from 16 min (Krajčová *et al.* 2010) to several dozen minutes (Antignac *et al.* 2004; Hoikkala *et al.* 2007; Andersen *et al.* 2009b).

Finally, the optimization of MRM conditions was achieved by infusing standard solutions of equol and IS (1 $\mu g.mL^{-1}$) directly in the MS/MS. The two molecules were detected using a parent ion $[M+H]^+$ and two characteristic daughter ions. After this, the cone voltage, collision energy, temperatures, gas flows, and other parameters were optimized by injecting the individual standard solutions with the mobile phase directly into the MS/MS. For each molecule, the two most abundant fragments were selected as Q candidates. The fragmentation pathways of these two molecules appeared to be similar to those reported by Antignac *et al.* (2003) and Prokudina *et al.* (2012).

Method validation

As the method described by Andersen *et al.* (2009b) had been slightly modified, and because the analytical device and the IS used in this study were not the same, it was mandatory to revalidate the whole procedure.

The occurrence of IS in the milk samples and enzymatic solution was checked, and no trace of IS was found nor was any trace of equol found in the enzymatic solution.

Specificity

Under the optimized UPLC®-MS/MS conditions, equol and IS were well separated on the chromatogram, and no interference in the integrate window was observed around the retention times (Fig. 4.2). The IS and equol retention times were 1.252±0.0070 and 1.823±0.0073 min (*n*=587), respectively. This implied that the relative retention times (RRT) between equol and IS were always included in an RRT tolerance range of ±2.5% (Antignac *et al.* 2003). In addition, for each compound, the ratio between the signals obtained for the confirmation transition and the one used for quantification (q/Q) was calculated, and it remained stable during the validation and routine analysis: 0.74 (relative standard deviation [RSD] of 6.9%) and 1.00 (RSD of 2.4%) for IS and equol (*n*=595), respectively. These RSD values were significantly lower than the tolerance ion ratio of 20% reported by Antignac *et al.* (2003).

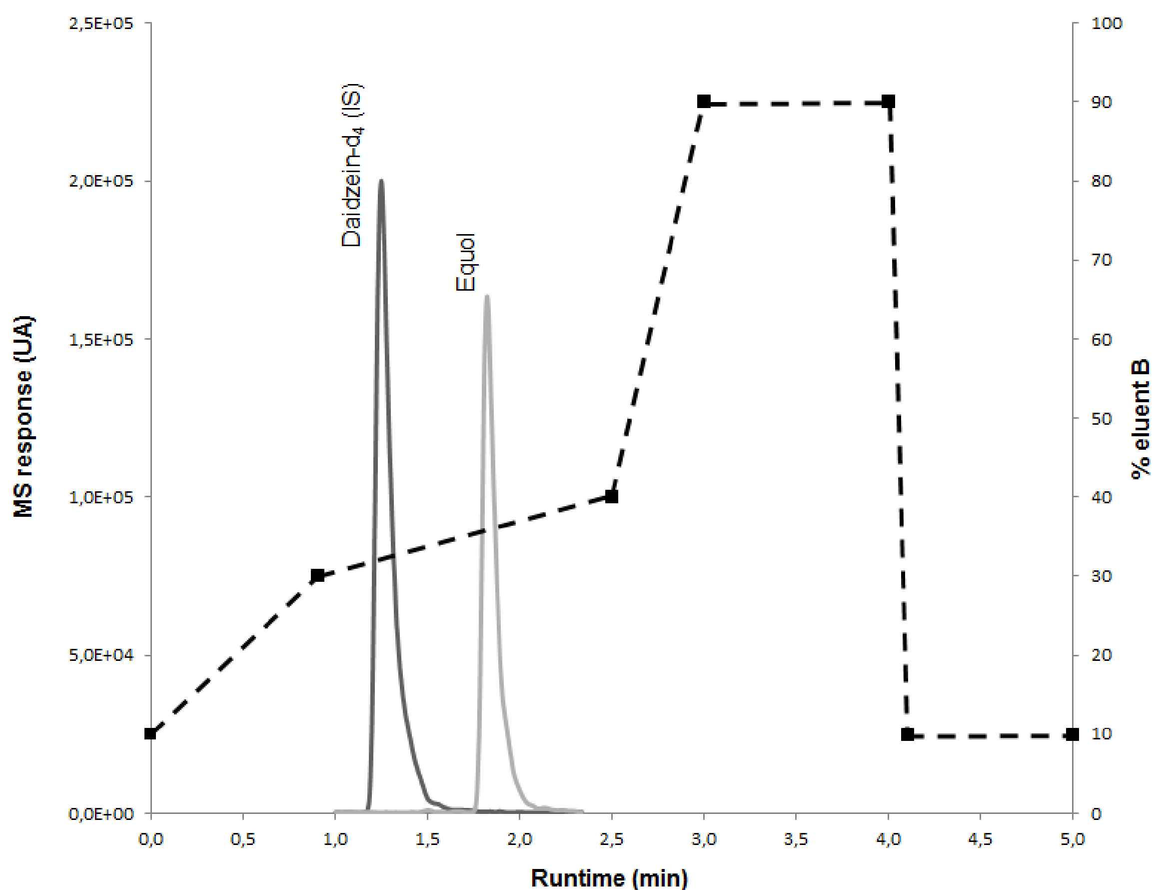

Figure 4.2. Optimized elution gradient (*dotted line*) and TIC of equol and daidzein-d_4 (IS) set at 50 ng.mL^{-1} and 100 ng.mL^{-1}, respectively.

Calibration curve

The calibration curve was constructed by injecting six replicates of each equol standard solution with IS (at 100 ng.mL^{-1}) at concentration levels ranging from 5 to 1,000 ng.mL^{-1}. In order to validate the calibration curve, several linearity criteria needed to be met: minimum of six concentration points per curve; RSD for each point not to exceed 20% for low points or 15% for high points (EMA 2011); and correlation coefficient (R^2) to be more than 0.99 (Fiechter *et al.* 2013). The representative regression equation for the calibration curve was (Eq. 1):

$$y = 0.0066(\pm 0.00012)x + 0.0077(\pm 0.00335) \quad (4.1)$$

where y was the analyte-to-IS ratio and x the equol concentration (n=6 and R^2 ≥0.99). In addition, an ANOVA with lack-of-fit test was used to confirm if the least squares linear regression model between the A_a/A_{is} ratio and concentration was adequate for describing the observed data (α=O.05) (De Bock *et al.* 2012). The ANOVA *P* value and the lack-of-fit test were <0.0001* and 0.5960, respectively. These results show that the use of linear calibration curves between 5 and 1,000 ng.mL^{-1} is satisfactory for quantifying the equol content of milk samples. It is the same order of magnitude as the range proposed by (Krajčová *et al.* 2010).

In this study, the LOQ was arbitrarily set at the lower calibration level (5 ng.mL^{-1}) because this limit value was judged satisfactory. The LOD was determined as the concentration at a signal-to-noise (S/N) of 3 (Fiechter *et al.* 2013). The S/N of equol was calculated in methanol/H_2O (80:20, v/v) with a concentration close to the fixed lower calibration level (n=5), and it was estimated at 0.3 ng.ml^{-1}. The LOD was not estimated in blank matrix because there was no milk without equol in the samples. The limits of the proposed method are substantially the same as those cited in other studies using an MS detector (Antignac *et al.* 2004; Andersen *et al.* 2009b; Krajčová *et al.* 2010), but far lower than those obtained using ultra-violet detectors (Hoikkala *et al.* 2007; Mustonen *et al.* 2009).

Instrumentation accuracy and precision

Intra-day (six injections within 1 day) and inter-day (six injections on 3 days) precision (expressed as RSD%) at four standard concentration points included in the calibration range (25, 50, 200, and 750 ng.mL^{-1}) were determined. Accuracy (expressed

as bias%) was also evaluated each day for the same standard concentrations. Standard solutions were prepared daily by different analysts. Table 4.1 summarizes the precision and accuracy of the UPLC®-MS/MS method. The intra- and inter-day RSD values did not exceed 5.3 and 5.2, respectively, nor did they exceed the maximum allowed value of 15% (Ellis 2008; EMA 2011). For accuracy, the maximum bias between the measured and assigned values was observed for the standard concentration at 200 $ng.mL^{-1}$ during the second and third day of analysis. The two values (7.6% and 8.6%) were far lower than the allowed value of 15% (Ellis 2008; EMA 2011). These observations indicate that the UPLC®-MS/MS method used had an acceptable level of precision and accuracy.

Carry-over

Carry-over is one of the most commonly encountered problems in the quantification of metabolites in biological samples using LC-MS/MS (Prasain *et al.* 2010). In order to check this parameter, 10 µL methanol/H_2O (80:20, *v/v*) solution was injected after the injection of three high-standard concentration solutions containing the two molecules (2,000 and 200 $ng.mL^{-1}$ of equol and IS, respectively). No traces of equol or IS were found in the methanol/water solution.

Robustness of hydrolysis process

An evaluation of the robustness of the hydrolysis procedure was performed in three raw milk samples from an experimental farm. A one-variable-at-a-time technique was used to identify the conditions that provided the best hydrolysis efficiency.

For the first experiment, four enzymatic concentrations between ≥85,000 and ≥10,600 units per mL of β-glucuronidase were tested in 4 mL of raw milk samples for 1 h. All the analyses were carried out four times. A Tukey's HSD test showed that there was a significant relationship between the enzymatic concentration and the amount of equol obtained (Fig. 4.3). A decrease in enzymatic concentration led to a decline in the equol content found at the end of the process. This indicated that, for low β-glucuronidase activities, enzymatic hydrolysis was incomplete in the milk samples. This statistical test also demonstrated that there was no significant difference between the undiluted and twice-diluted enzyme solutions, ≥85,000 and ≥42,500 units per mL. In the second experiment, four hydrolysis times were tested in triplicate (between 1 and 20 h), with 4 mL of raw milk samples and an enzymatic concentration set at ≥42,500 units per mL^{-1}. In

Table 4.1. Estimation of precision and accuracy of the UPLC®-MS/MS method, process efficiency, and assessment of sample extract stability after a 7-day storage at 5±1 °C and -21± 1°C

Instrumentation precision and accuracy					
Injected concentration ($ng.mL^{-1}$)	Day	Concentration found ($ng.mL^{-1}$)[a]	Intra-day[a] (RSD, %)	Inter-day[b] (RSD, %)	Accuracy[a,c] (Bias, %)
25	1	23.5±1.24	5.3	5.2	6.3±4.76
	2	24.8±1.09	4.4		2.8±3.17
	3	23.5±1.00	4.3		6.1±3.53
50	1	48.5±2.08	4.3	3.8	3.9±3.16
	2	47.9±1.92	4.0		5.3±1.71
	3	49.0±1.53	3.1		3.1±1.64
200	1	200.4±7.26	3.6	5.2	2.8±1.95
	2	215.2±7.30	3.4		7.6±3.65
	3	215.1±11.33	5.3		8.6±3.45
750	1	744.1±27.26	3.7	3.0	2.8±2.10
	2	752.0± 16.71	2.2		1.8±1.11
	3	743.3±25.34	3.4		2.4±2.36

Process efficiency	Recovery				ME%[e]
	80 $ng.mL^{-1}$	200 $ng.mL^{-1}$	400 $ng.mL^{-1}$	800 $ng.mL^{-1}$	
Raw milk	64.9±7.58 (11.7%)[f]	63.4±1.10 (1.7%)	66.6±1.59 (2.4%)	60.6±1.09 (1.8%)	-11.6
Whole milk	64.9±5.26 (8.1%)	64.8±4.64 (7.2%)	60.8±5.50 (9.1%)	67.8±1.92 (2.8%)	-7.8
Semi-skimmed milk	82.3±5.21 (6.3%)	78.7±4.26 (5.4%)	67.3±2.60 (3.9%)	64.4±6.36 (9.9%)	0.5
Skimmed milk	80.0±2.99 (3.7%)	75.7±4.30 (5.7%)	73.9±2.55 (3.4%)	74.0±1.81 (2.4%)	10.9

Sample extract stability milk	First analysis ($ng.mL^{-1}$)[g]	After a storage at -21 °C ($ng.mL^{-1}$)[h]	After 7 a 7-d storage at 5 °C ($ng.mL^{-1}$)[h]
Raw milk	52.6±4.20[i]	53.3±0.42[i]	54.7±8.64[i]
Whole milk	33.7±2.70[i]	36.5±1.19[i]	35.6±2.77[i]
Semi-skimmed milk	44.6± 1.68[i]	45.6±0.92[i]	47.0±0.80[i]
Skimmed milk	49.0±0.47[i]	47.6±1.52[i]	50.1±0.30[i]

[a] n=6; [b] n=18; [c] Bias%=Abs((Conc.$_{calc}$.-Conc.$_{Nom}$)/Conc.$_{Nom}$)*100; [d] Recovery(%)=(Conc.$_{Calc}$./Conc.th.)*100; [e] MatrixEffect(%)=((Slope$_{Matrix}$-Slope$_{MeOH}$)/Slope$_{MeoH}$)*100; [f] RSD% in brackets (n=4); [g] n=4; [h] n=2; and [I] For each type of milk in the verification of sample extract stability, values indicated are not significantly different (P value>0.05).

this experiment, no significant effect was observed among the four hydrolysis times (Fig. 4.3). These observations are interesting because they show that it was possible to start the enzymatic hydrolysis on the eve or day of analysis, depending on the time available. In the third experiment, five sample volumes were tested in triplicate, from 1 to 5 mL, with an enzymatic concentration of ≥42,500 units per mL^{-1} and for a period of 2 h. In this last experiment, it was shown that sample volume variations had a significant impact on the amount of equol obtained (Fig. 4.3). The concentrations found for the three sample volumes between 1 and 3 mL were not significantly different, but they were slightly higher than those found when 4 and 5 mL were used. The small difference (only 5 $ng.mL^{-1}$) between these two volume ranges was enough to indicate a significant impact of volume samples. There was a more opaque and consistent hexane phase (first liquid/liquid extraction) for high-volume samples, making sample preparation a little more complicated than for low sample volumes. Therefore, with high sample volumes, this first extraction could result in the involuntary loss of equol in the upper fatty phase.

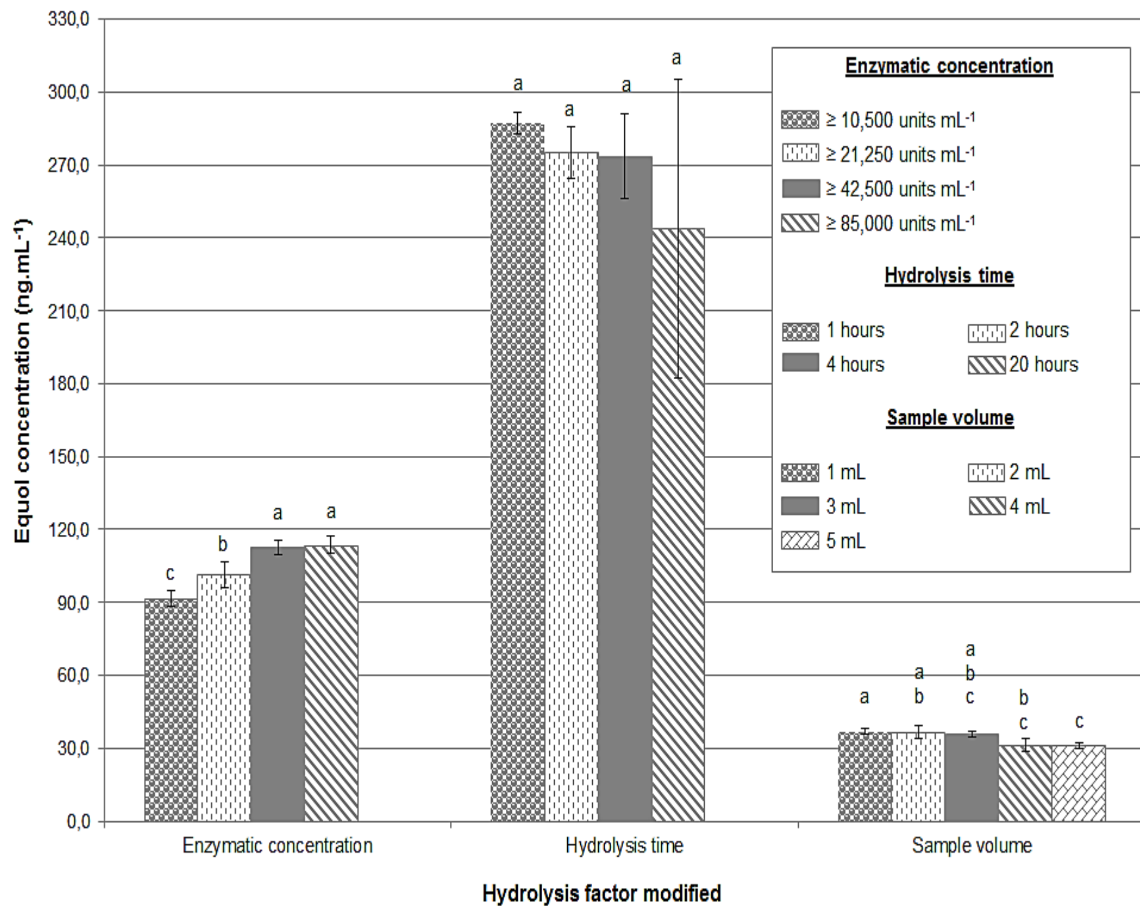

Figure 4.3. Robustness evaluation of the enzymatic hydrolysis process (n≥3). Bars with the same letters (*a*, *b*, and *c*) are not significantly different (*P* value > 0.05).

After these three experiments, the hydrolysis conditions were fixed at 2 h of hydrolysis, with a sample volume of 2 mL and a concentration of ≥42,500 units per mL^{-1} β-glucuronidase type H2 from the *H. pomatia* solution. The reliability of the hydrolysis procedure was therefore enhanced for several reasons: with a 2 mL sample volume, fewer problems occur during skimming and hexane extraction; there is a significant financial saving with the double dilution of the enzymatic solution; and, with 2 h of hydrolysis time, the conjugated forms of equol appear to be thoroughly hydrolyzed after 2 h. The length of time taken by the hydrolysis process (with β-glucuronidase from *H. pomatia*) used in this study was far shorter than that reported by Krajčová *et al.* (2010), but it is substantially the same as the times reported by Antignac *et al.* (2004), Hoikkala *et al.* (2007), and Andersen *et al.* (2009b) in their studies of the equol content of milk.

Process efficiency

Process efficiency was evaluated by determining the extraction recovery and matrix effect for each type of milk (Kruve *et al.* 2008). The main difficulty was the absence of a true blank milk sample without equol. For these two validation parameters, a blank milk sample had to be prepared each time in order to estimate the equol content already in the sample.

Extraction recovery focusing on the efficiency of separating analyte from the samples was studied in the four types of milk at four concentration levels. The concentration found in milk samples spiked at the start of the sample preparation process was compared with the expected concentration. Each milk sample was spiked with 100 µL of standard solution at 400, 1,000, 2,000, and 4,000 $ng.mL^{-1}$ in order to obtain a final equol concentration of 80, 200, 400, and 800 $ng.mL^{-1}$. The analysis was carried out four times. As shown in Table 4.1, in all cases, the recovery rates ranged between 60 and 82%. This range straddled the lower limit value, fixed at 70% for this range of concentration (Ellis 2008; EMA 2011). The mean recovery rate was 69.4± 8.07%. These results accord with those reported by Andersen *et al.* (2009b), but are lower than those reported in other studies (Hoikkala *et al.* 2007; Mustonen *et al.* 2009). In these latter studies, however, the recovery rate was not calculated with spiked samples of the target compound but with the IS used. When all the recovery rates were statistically analyzed, no difference was found between each concentration spiked, even when the two lowest concentrations appeared to produce better recovery rates for skimmed and semi-skimmed milk. There was a

significant difference, however, between each type of milk (*P* value<0.001*). The Tukey's HSD test showed that recovery was similar (about 64%) for raw and whole milk, but higher for the two other types of milk (about 74%). This increase appeared to be correlated with the milk fat content, indicating that fat removal with *n*-hexane extraction would reduce equol recovery rates. It is important to note that the RSD value for extraction recovery repeatability was 11.6% for all types of milk and concentration levels analyzed. This value was acceptable and derived mainly from the variability among the types of milk. During routine analysis, therefore, the correction factor calculation was carried out for each type of milk on each day of analysis (*n*=3, final concentration spiked, 200 $ng.mL^{-1}$).

The matrix effect was used to describe equol and IS ionization efficiencies. Ion-enhancement or ion-suppression was observed occasionally when target analytes and matrix components co-eluted. It was also studied for each type of milk by comparing the slopes of the calibration curves obtained after adding appropriate calibration standard solutions in dry residues with the slopes arising from the same standard solutions in methanol/H_2O (80:20, *v/v*). For this comparison, eight samples of the same milk were extracted, of which seven residue extracts were reconstituted with the appropriate seven standard calibration solutions, and one was reconstituted with meth-anol/H_2O (80:20, *v/v*) (blank), with its response being subtracted from the other seven samples. The percentage difference between the slope of the calibration curve obtained by adding standard solutions in dry residues and the one arising from methanol/H_2O (80:20, *v/v*), taking the latter as the reference, was less than 12% (in absolute terms) for all types of milk (Table 4.1). A slightly negative effect (signal suppression) was observed for the raw milk from farms and for the whole milk. For the other two types, however, the opposite effect was observed. For the semi-skimmed milk, the signal enhancement was very weak. These values were within a tolerance range (-20 and 20%) for which there was no matrix effect (Ferrer *et al.* 2011). In addition, a *t*-test (α=0.05) was used to compare each couple of slopes (external calibration in methanol/H_2O (80:20, v/v) vs. standard addition in dry residues) and to establish if the slope differences were statistically significant (Salomone *et al.* 2011). For each couple of calibration curves, the statistical treatments were not significant, indicating that there was no significant matrix effect for any of the four types of milk. The preparation of matrix-matched standards could therefore be omitted, and

quantification was carried out through external calibration curves in methanol/H_2O (80:20, v/v).

Method repeatability

Intrinsic variability in a complete sample work-up (hydrolysis, extraction, and UPLC®-MS/MS analysis) was assessed throughout the validation process and sample analysis. During the estimation of recovery rates, robustness of hydrolysis protocol and sample analysis, at least three repetitions were conducted. Thus, the RSDs were derived for each situation and were always lower than the maximal tolerance limit fixed at 15% (Ellis 2008). The repeatability of all analytical methods was therefore judged to be completely satisfactory.

Sample stability

The stability of sample extracts was also investigated. Initially, four samples of each type of milk were prepared and analyzed. Then, for each type of milk, two samples were stored in a freezer (-21 ±1 °C) and the other two in a fridge (5±1 °C) for 7 days. After this period, sample extracts were analyzed a second time. As shown in Table 4.1, no significant difference was found with the Tukey's HSD test between the equol content found in the first analysis and after a 7-days storage in the fridge or freezer. There was therefore no equol degradation in the methanol sample extracts stored at -21 °C and 5 °C for at least 7 days.

Equol content of commercial milk in Belgium

The validated method was then successfully used to determine the equol content in Belgian milk. In order to assign a correction factor to the results, a recovery rate was calculated daily. A higher number of semi-skimmed milk samples was taken into account because this type of milk is the one most heavily consumed by the Belgian population. As shown in Fig. 4.4, equol was present in all the milk samples. Within each type of milk, the equol content varied widely between samples. When comparing mean values, no significant difference was observed among the four types of milk (ANOVA *P* value, 0.7256) randomly selected from the available milk types on the Belgian market in February 2013. The average values were estimated at 35.7± 12.05, 37.2±6.96, 38.1± 5.50, and 23.1 ±12.01 ng.mL^{-1} for raw, whole, semi-skimmed, and skimmed milk, respectively.

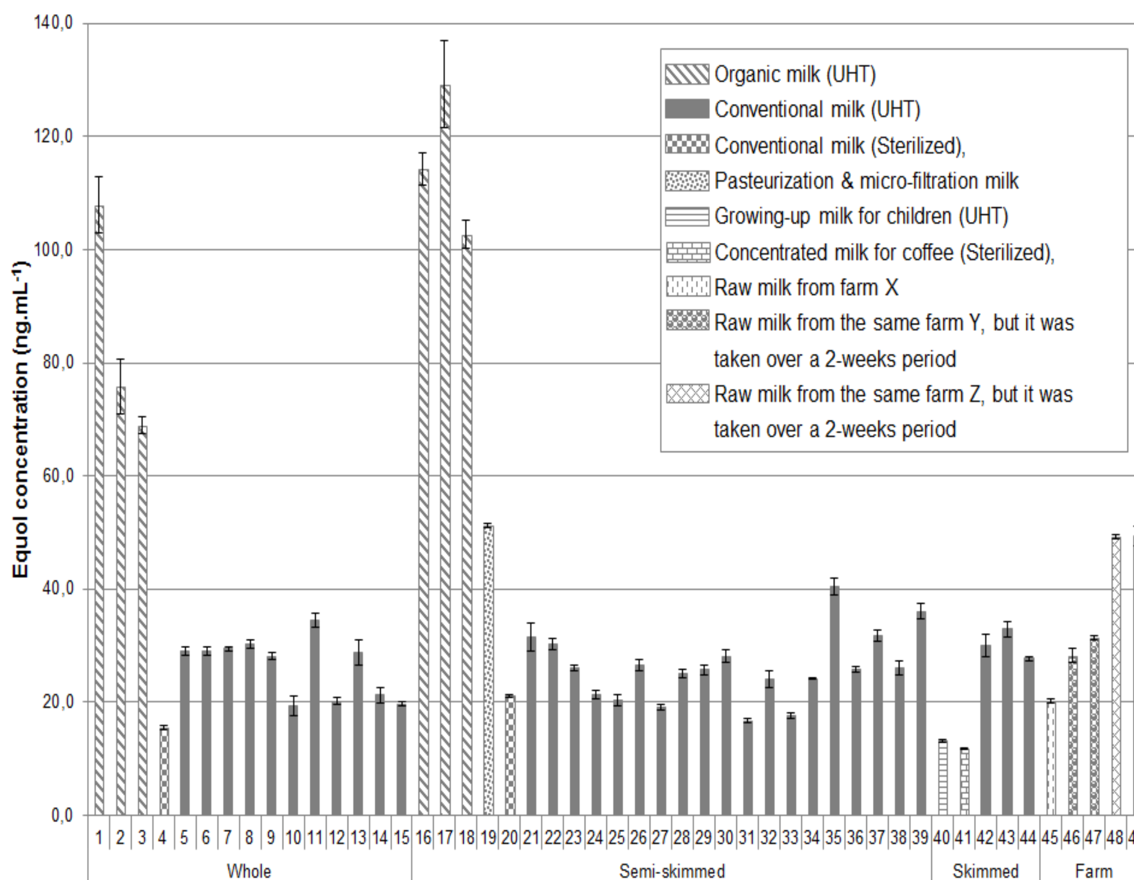

Figure 4.4. Equol content found in Belgian commercial cow's milk and raw milk analyzed by UPLC®-MS/MS. "*UHT*" is the abbreviation of ultra-heat treatment. Each sample was analyzed in triplicate (*n=3*), and the standard deviation is represented by the *error bars*.

These results accorded with those reported by other studies, which showed that there was no difference between skimmed and full cream milk samples (Antignac *et al.* 2004) and that there were lower concentrations in cream than in milk (Křížová *et al.* 2011a). This means that equol is not a particularly lipophilic compound and therefore has little affinity with the lipid milk fraction. This interpretation goes against a recent study, however, where the authors reported that equol is distributed to a larger extent in the lipid fraction than in the aqueous fraction and therefore that the skimming process reduces the amount of equol in cow's milk (Tsen *et al.* 2014). More research is needed to clarify this situation, but this initial screening indicated that equol did not seem to be affected by the lipid fraction in milk.

There was a highly significant difference, however, between organic milk and the milk from the agricultural sector classed as 'conventional' (ANOVA P value<0.0001). Equol concentration in organic milk varied from 70 to 130 ng.mL, whereas in conventional milk it varied from 10 to 50 ng.mL^{-1}. This difference between organic and

conventional milk has also been reported from other countries (Antignac *et al.* 2004; Hoikkala *et al.* 2007). The equol concentration found in conventional milk in Belgium (10-50 µg.L^{-1}) was higher than the mean value reported in the United Kingdom (about 10 µg.L^{-1}) (Kuhnle *et al.* 2008), equal to that reported in France (about 36 µg.L^{-1}) (Antignac *et al.* 2004) and slightly lower than that reported in Finland (about 61 µg.L^{-1}) (Hoikkala *et al.* 2007). The concentration found in organic Belgian milk (70-130 µg.L^{-1}), however, was lower than the mean values reported for France and Finland (about 191 and 411 µg.L^{-1}, respectively) (Antignac *et al.* 2004; Hoikkala *et al.* 2007). This variability in equol content between countries could be linked partly to the use of different analytical methods. It could also be due to many other factors, such as sampling period, animal geographical origin, dairy cow species, or the type of feedstuff used (red clover content in the diet, for example). In this study, sampling was done during the second half of the winter period. The milk was therefore assumed to have been produced in late autumn or at the beginning of winter. The study was therefore limited to milk available for Belgian consumers at this period, and no more information on sample history or cow feeding regimes was available. One factor that might explain the striking difference between the equol content of organic and conventional milk samples could be the type of feed used in these two cattle management systems. Organic agricultural practices are supposed to use the high part of grass products to feed animals, often rich in Leguminosae species that are a great source of equol precursors, especially red clover (Křížová *et al.* 2011a; Höjer *et al.* 2012). It was also found that milk from different farms had significantly different equol concentrations (ANOVA *P* value, 0.0075), whereas the concentrations in milk from the same farm remained stable over the 2-week period. This accords with the finding reported in an Australian study where, for a fixed period, equol concentration varied solely according to milk geographical origin (King *et al.* 1998). There also appeared to be no difference in equol concentration between UHT and sterilized milk. This observation is in line with other studies where no degradation in isoflavones with an increase in temperature during milk processing was observed (King *et al.* 1998; Křížová *et al.* 2011a). In our study, however, not enough analysis was done on sterilized milk to confirm this observation statistically. The only sample of pasteurized milk analyzed seems to have had more equol content than UHT milk, but, as previously noted, not enough analysis was done during this first screening. Finally, analyses of milk for growing children and concentrated milk for coffee were also done. Equol was found in

both these types of milk samples (13.2±0.16 and 11.8±0.19, respectively) but at lower concentrations than in other samples.

Conclusions

This paper describes the validation of a reliable, fast, and sensitive UPLC®-MS/MS method for quantifying equol concentration, over a range of 5-1,000 $ng.mL^{-1}$, in raw and processed milk sold in supermarkets. A major advantage of this analytical method is the short sample analysis runtime required, which is important for high sample throughput. The extraction procedure is easy to perform and suitable for routine work. The application of this validated method gave an initial indication of the equol content in commercial cow's milk in Belgium. Equol was found in all the samples analyzed, with a concentration ranging from 10 to 50 $ng.mL^{-1}$ for conventional milk and from 70 to 130 $ng.mL^{-1}$ for organic milk. The results obtained show the suitability of this method for analyzing estrogenic metabolite-equol in cow's milk and its potential for use in further investigations.

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Conflict of interest/Compliance with ethics requirement statements

Frédéric Daems, Christophe Jasselette, Jean-Michel Romnee, Viviane Planchon, Georges Lognay, and Eric Froidmont declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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Chapter V

Optimization and validation of an analytical method for quantifying isoflavones in forages

Objectives

The previous chapters showed that equol concentration was higher in organic milk than milk from the conventional agricultural sector. This striking difference could be linked to the type of feed used in these two management systems, and specifically grass forages. In order to check this hypothesis, the objective of this chapter was to develop an analytical method for the simultaneous quantification of four selected isoflavones in forages. It is based on aglycones released by methanolic ultrasound-assisted extraction (UAE), followed by enzymatic hydrolysis and UPLC®-MS/MS analysis. It was developed following a clear and structured methodological approach that involved the use of a response surface methodology in order to optimize sample preparation. A complete validation was then performed following international recommendations (EMA 2015). A preliminary study on silages was also conducted to illustrate the method's usefulness. An overview of this chapter is presented in Fig. 5.1.

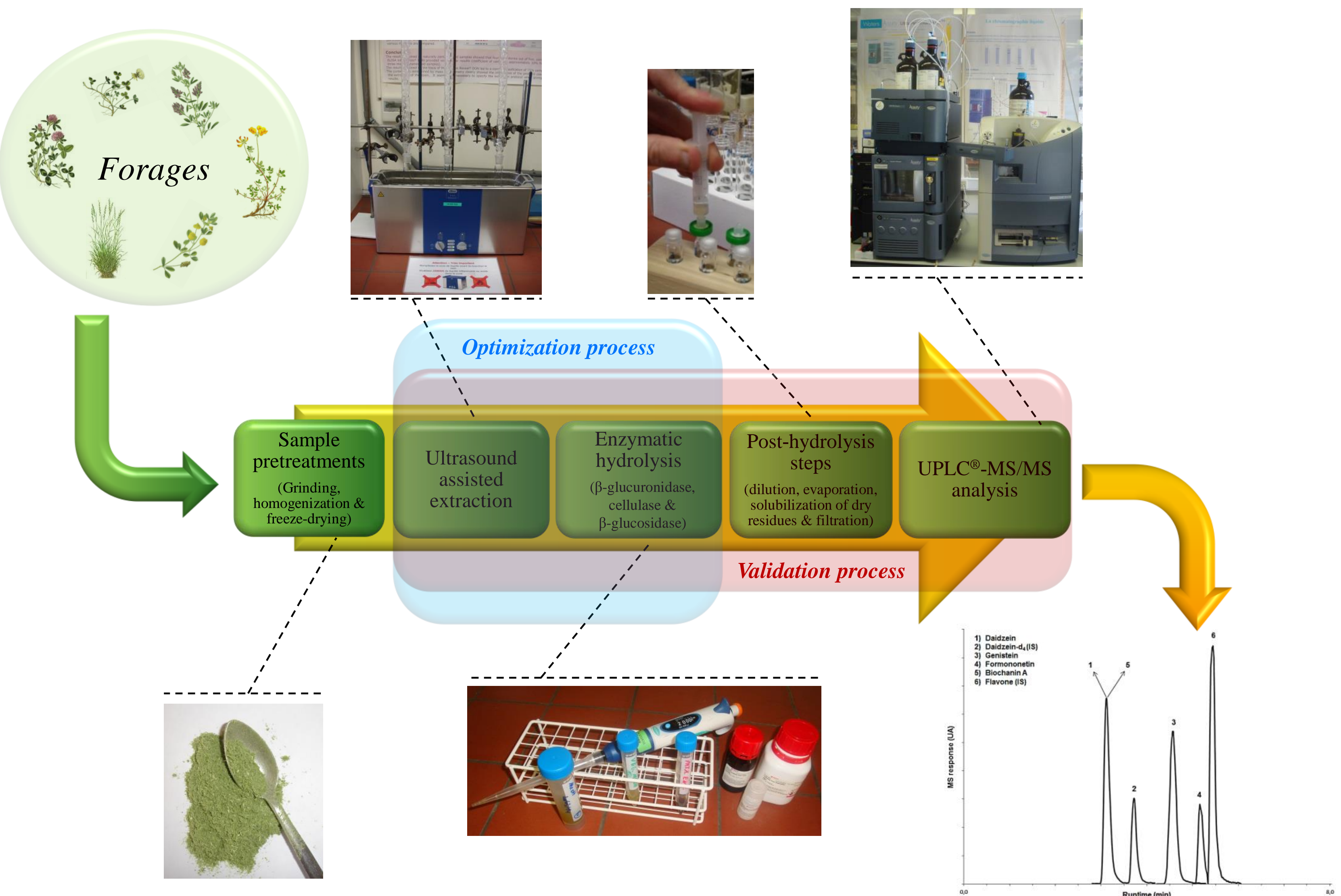

Figure 5.1. Graphical abstract of *Chapter V*. Analytical procedure developed and validated for quantifying isoflavones in forages.

Quantification of four isoflavones in forages with UPLC®-MS/MS, using the Box-Behnken experimental design to optimize sample preparation

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Abstract

A performant method for the simultaneous quantification of daidzein, genistein, formononetin, and biochanin A in forages using an UPLC®-MS/MS was developed and fully validated. The ultrasound-assisted extraction and enzymatic hydrolysis used in the sample preparation step were optimized using the Box-Behnken experimental design. The optimal extraction conditions used for a representative mix of forage plants were 80 °C, 10 min, and 55 % methanol, and for hydrolysis, they were 20 °C, 18 h, and pH = 6. The chromatographic separation was achieved using an Acquity UPLC® HSS T3 column, with a water/ methanol linear gradient containing 0.01 % of formic acid at a 0.55 $mL.min^{-1}$ flow rate. The four isoflavones were detected by ESI mass spectrometry in positive ion MRM mode. The method allows high throughput analyses of samples and showed an adequate linear regression model for all isoflavones over a range from 5 to 125 $ng.mL^{-1}$. There were good intra- and inter-day precisions (≤8.2 and ≤7.6 %) and accuracy (≤11.4 and ≤7.1 %). The recovery rates were in an acceptable range of 70-120 %, except for biochanin A, where the rate was about 50 %. Good method repeatability was also observed, and there was no matrix effect or carryover problem. The sample extracts were stable for at least 6 days of storage at -21 and 6 °C. The method proved to be sensitive, precise, and accurate for discriminating a wide variety of forages likely to be grazed by ruminants according to their isoflavone contents and to observe the impact of storage process on isoflavone content in forages.

Keywords

UPLC®-MS/MS - Optimization - Experimental design - Validation - Isoflavones- Forages

Introduction

Isoflavones are secondary plant metabolites and form one of the most common categories of phytoestrogens (Mostrom & Evans 2012; Vitale *et al.* 2013). Isoflavones are polyphenolic compounds that are structurally similar to 17β-estradiol. In addition to their involvement in plant normal growth and development, numerous health benefits have been attributed to isoflavones (Mostrom & Evans 2012; Vitale *et al.* 2013; Ko 2014), but many of them are also considered to be endocrine disruptors, with the potential to cause adverse health effects (Patisaul & Jefferson 2010; Sirotkin & Harrath 2014). They are found mainly in the *Fabaceae* family, such as soy (mainly daidzein and genistein) (Wu *et al.* 2004; Rostagno *et al.* 2009; Mostrom & Evans 2012; Vitale *et al.* 2013) or red clover (mainly formononetin and biochanin A) (Wu *et al.* 2004; Tsao *et al.* 2006; Saviranta *et al.* 2010), usually conjugated with a glucosyl group, which is often esterified with acetyl or malonyl groups (Rostagno *et al.* 2009; Saviranta *et al.* 2010; Mostrom & Evans 2012; Vitale *et al.* 2013). These conjugated forms are hydrolyzed in the human or animal gut into their aglycone forms before being metabolized (Mostrom & Evans 2012; Ko 2014).

Many different sample preparations (including extraction, hydrolysis, and purification steps) and analytical methods for quantifying plant phytoestrogens (including isoflavones) have been reported in the literature (Wang *et al.* 2002; Wu *et al.* 2004; Grynkiewicz *et al.* 2005; Stalikas 2007; Vacek *et al.* 2008; Rostagno *et al.* 2009; Ko 2014). The development of an optimized extraction procedure that would suit all of them is a challenging task. Ultrasound-assisted extraction (UAE) is now the most commonly used technique to extract isoflavones from plants matrix. This technique is cheap, has low instrumental requirements, and enhances extraction efficiency (Rostagno *et al.* 2003; Rostagno *et al.* 2009). The quantification of isoflavones is usually performed using reversed phase liquid chromatography (RP-LC) with gradients of a binary solvent system that contains acidified water and an acidified polar organic solvent, such as methanol or acetonitrile, and ultraviolet (UV) or mass spectrometry (MS) detection (Wu *et al.* 2004; Stalikas 2007; Ko 2014).

Considering the multiple sample preparation methods found in scientific literature, the wide variety of forage plants likely to be grazed by ruminants, and the wide variation in isoflavone concentrations, the objective of this study was to develop a reliable and performant analytical method for quantifying four isoflavones in one of the most important sources of isoflavones in cow feed. Response surface methodology (RSM) was

used to optimize sample preparation. The whole analytical method was validated and used to measure daidzein, genistein, formononetin, and biochanin A in forages silages originating from three different meadows. This development is part of a larger study on the metabolization of the biomolecules of interest and the potential accumulation of metabolites such equol in milk (Daems *et al.* 2015; Daems *et al.* 2016a).

Materials and Methods

Chemicals and Reagents

A freeze dried forage sample containing six plants *(Trifolium pratense* L., *Trifolium repens* L., *Medicago sativa* L., *Lotus corniculatus* L., *Medicago lupulina* L, and *Lolium perenne* L.; 1:1:1:1:1:1, w/w/w/w/w/w) was mixed with flaxseed meal [4:1, w/w; 93.7 ± 0.1 % dry matter). Formononetin 'FO' (CAS number: 485-72-3), biochanin A 'BA' (491-80-5), genistein 'GE' (446-72-0), and flavone 'IS' (525-82-6) were bought from Sigma-Aldrich (Diegem, Belgium). Daidzein 'DA' (486-66-8) was acquired from Cayman Europe (Tallinn, Estonia). Daidzein-d_4 (1219803-57-2) was purchased from C/D/N ISOTOPES (Pointe-Claire, Canada). Individual molecule stock solutions (100 $\mu g.mL^{-1}$) were prepared in methanol and stored at -20 °C in the dark.

β-Glucosidase (from almonds, ≥6 $U.mg^{-1}$, 9001-22-3), *β*-glucuronidase (type H-2 from *Helix pomatia,* ≥ 85000 $units.mL^{-1}$, 9001-45-0), and cellulase (from *Aspergillus niger,* ≥0.3 $units.mg^{-1}$, 9012-54-8) were bought from Sigma-Adlrich (Diegem, Belgium). Sodium acetate (127-09-3) was obtained from Merck KGaA (Darmstadt, Germany). Throughout the study, the powder enzymes and *Helix pomatia* juice were dissolved in different proportions of 0.2-M sodium acetate buffer solution set at pH values between 4 and 6 (depending on the experimental design).

Methanol 'MeOH' (LC-MS and HPLC grade) and acetonitrile 'ACN' (HPLC grade) were obtained from J.T Baker (Devender, Netherlands). Ethanol (96 %, AnalaR NORMAPUR) 'EtOH' was obtained from VWR (Fonte-nay-sous-bois, France), and formic acid (ULC-MS, 99 %) was obtained from Biosolve (Valkenswaard, the Netherlands). Deionized water has been prepared using a Milli-Q® Gradient system (Millipore, Overijse, Belgium).

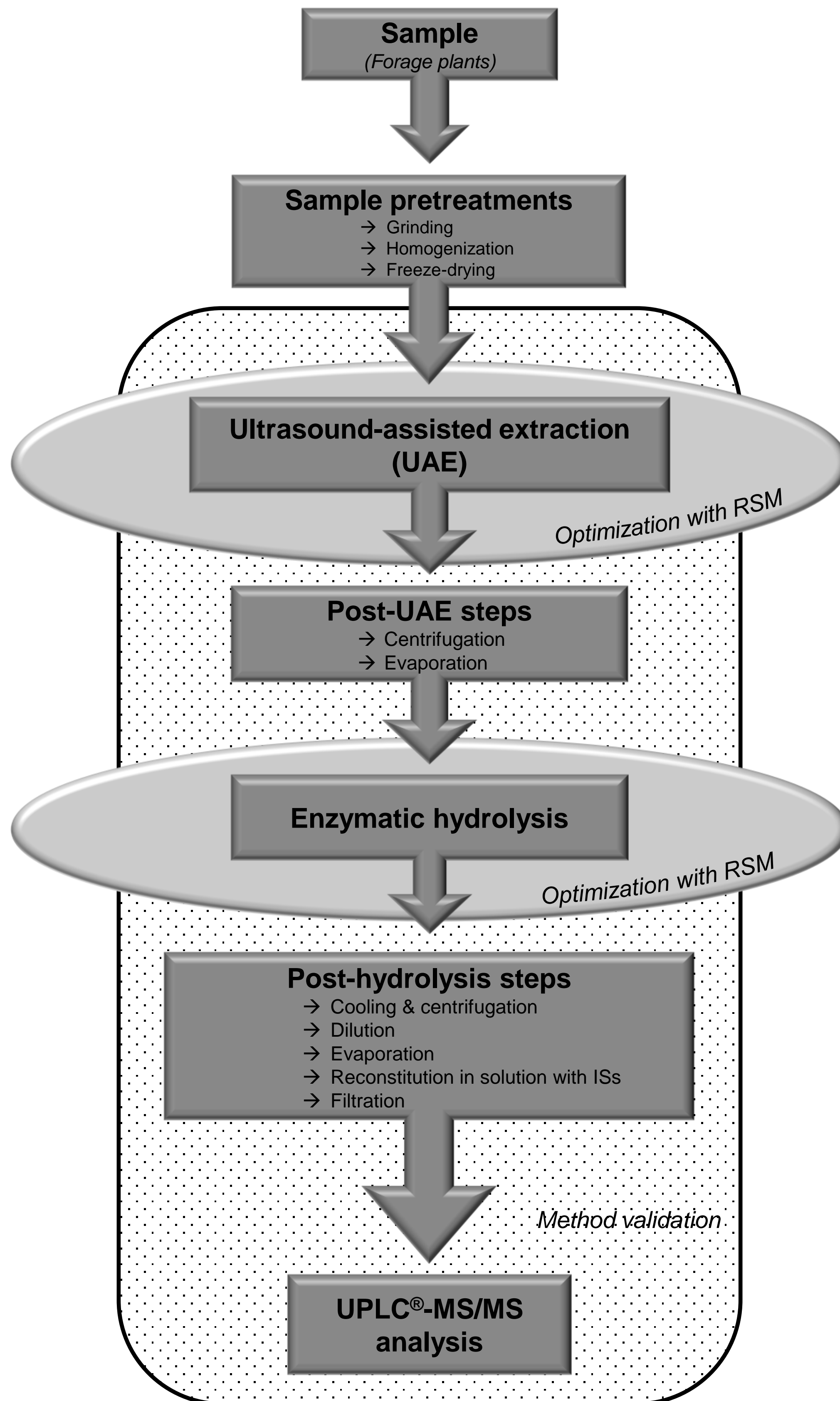

Figure 5.2. Overview of the sample preparation optimization and validation described in this study.

LC-MS/MS Analysis

The LC analyses were performed on an ACQUITY UPLC® from Waters (Zellik, Belgium) equipped with a Waters ACQUITY UPLC® HSS T3 column (2.1 x 100 mm, 1.8-µm particle size) with a preconnected in-line filter (0.20 µm). The column was kept at 65 °C. The vials were at 4 °C, and an aliquot of 10 µL was injected. The mobile phase consisted of ultrapure water with 0.01 % of formic acid (eluent A) and methanol with the same percentage of acid (eluent B). The gradient elution was operated at a flow rate of 0.55 $mL.min^{-1}$ according to the following optimized gradient profile allowing good chromatographic resolution: 30 % B (0.0-1.0 min), 30-70 % B (1.0-6.0 min), 70-90 % B (6.0-6.1 min), 90 % B (6.1-6.5 min), and 90-30 % B (6.5-6.6 min). The column was then left to reequilibrate under the initial conditions for 1.4 min, resulting in a total runtime of 8 min.

The eluting compounds were detected using a Waters Quattro Premier XE (Micromass® Waters, Zellik, Belgium) with an electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode, and the quantification was performed using multiple reactions monitoring (MRM). A splitter was placed at the entrance of the device (split ratio 9/20) to give a mobile phase flow rate of 250 $\mu L.min^{-1}$ inside the mass spectrometer. The optimal source and desolvation temperatures were 140 and 350 °C, respectively. Cone gas and desolvation gas flow (both N_2) were set at 50 and 700 $L.h^{-1}$, respectively. Argon was used for the collision-induced fragmentation. All the data were collected and processed using the MassLynx® software with a Quanlynx® program (Micromass® Waters, Zellik, Belgium).

Sample Preparation

An overview of the sample preparation procedure is shown in Fig. 5.2. Extraction was undertaken in an ultrasonic bath (S80H, Elmasonic, Singen, Germany). 500 ± 5 mg of freeze-dried sample were mixed with 25 mL of solvent into a glass test tube with NS joints (Lenz Laborglas, Wertheim, Germany), with a Vigreux column (Lenz Laborglas, Wertheim, Germany). The best extraction conditions were determined using the experimental design described hereafter.

The extract was then poured into a centrifuge tube (50 mL) and centrifuged (5 min at 3200g). The 1 mL of the supernatant was then evaporated to dryness at 65 °C, and with a gentle stream of nitrogen. The dry residues were solubilized with 1 mL of sodium

acetate buffer. The solution was sonicated (5 min at 750 W and room temperature) and vortexed, and then 2 mL of the enzymatic solution was added. The mixture was then placed in an oven and shaken continuously (conditions: see experimental design). The mixture was centrifuged (5 min at 3200 g). An aliquot of supernatant was diluted with several H_2O:methanol (40:60,v/v) solutions, depending on the suspected isoflavone content and to prevent possible matrix effects (Ferrer *et al.* 2011; Hoerger *et al.* 2011). After this, 0.5 mL of each diluted extract was transferred into a glass tube (15 mL) and evaporated to dryness. The dry residues were then reconstituted in 1 mL of H_2O:methanol (40:60, v/v) with $IS_{Flavone}$ at 20 ng.mL^{-1} and $IS_{Daidzein\text{-}d4}$ at 50 ng.mL^{-1}. The reconstituted solution was filtered through an Uptidisc™ CA 13 mm/0.2 µm (Interchim, Montluçon, France) and analyzed by UPLC®-MS/MS.

Experimental Designs

The Box-Behnken experimental design, involving three factors and three levels, was used to optimize both the extraction and hydrolytic conditions. The levels chosen for each factor in this study were based on the single-factor experiments and literature. The complete design consisted of 16 combinations, including four replicates of the center point.

The experimental results were analyzed by quadratic stepwise regression to fit the second-order equation (Eq. 5.1):

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{3} B_{ij} X_i X_j \qquad (5.1)$$

where Y stands for isoflavone yield, X_1 - X_2 - X_3 for independent variables, β_0 for the model intercept, and B_i - B_{ii} - B_{ij} for regression coefficients of variables for linear, quadratic, and interaction terms, respectively. The JMP®7 statistical software package (SAS institute, Cary, USA) was used to obtain the coefficients of the quadratic polynomial model and to determine the best conditions for the ultrasound-assisted extraction 'UAE' and hydrolysis steps. Y was expressed in µg.g^{-1} of dry matter 'DM', which was determined at 103 ± 2 °C until constant weight (Reynaud *et al.* 2010).

For UAE optimization, the dried resuspended residues were mixed with 2 mL of enzyme mix solution (pH = 5), which contained ≥0.06, ≥4.2 and ≥3400 units.mL^{-1} of cellulase, β-glucosidase, and β-glucuronidase, respectively. In line with two other studies (Kuhnle *et al.* 2007; Konar *et al.* 2012), the pH of buffer solution, temperature, and

Table 5.1. Box-Behnken design and the response for isoflavone yield extracted from the forage mix

Assay	X_1	X_2	X_3	Isoflavone yield (µg.g^{-1}DM)		
				MeOH	EtOH	ACN
	Temperature (°C)	Duration (min)	Solvent proportion (%)	Observed value	Observed value	Observed value
1	50	50	50	741.9	744.5	687.4
2	50	50	50	751.2	776.9	664.0
3	80	50	90	692.6	611.1	679.0
4	50	90	90	657.6	517.6	617.2
5	20	50	90	677.3	424.5	588.6
6	20	90	50	769.4	740.5	691.6
7	80	90	50	720.1	515.0	648.5
8	50	50	50	726.0	558.8	744.5
9	20	10	50	776.6	660.4	715.5
10	80	10	50	830.2	631.1	708.9
11	50	90	10	286.2	341.6	560.1
12	50	50	50	654.2	671.0	730.3
13	50	10	90	646.0	478.4	571.6
14	20	50	10	238.6	264.7	297.1
15	50	10	10	352.0	273.5	517.6
16	80	50	10	587.5	563.8	697.2

Table 5.2. Box-Behnken design and the response for isoflavone yield hydrolyzed from the methanolic extract

Assay	X_1	X_2	X_3	Isoflavone yield (µg.g^{-1} DM)
	Temperature (°C)	Duration (h)	pH (U pH)	Observed value
1	60	21	6	421.7
2	40	21	5	691.2
3	20	18	5	758.0
4	40	21	5	690.8
5	40	24	6	903.6
6	40	18	6	907.1
7	20	24	5	742.7
8	20	21	4	611.1
9	60	24	5	404.3
10	40	21	5	613.6
11	20	21	6	765.7
12	60	18	5	608.7
13	60	21	4	581.4
14	40	18	4	736.5
15	40	21	5	642.9
16	40	24	4	727.3

duration of the hydrolysis process was fixed at 5, 37 °C, and 18 h, respectively. Three extraction solvents (ACN, EtOH, and MeOH) were evaluated. Extraction temperature (X_1), duration (X_2), and solvent proportions (X_3) were chosen as independent variables. The three levels for each factor were: X_1 (20, 50, and 80 °C); X_2 (10, 50, and 90 min); and X_3 (10, 50, and 90 % organic solvent/water). The 16 combinations are summarized in Table 5.1. After the statistical treatments, the better extraction conditions were validated by performing seven extractions. In addition, the extraction efficiency was also checked by performing three consecutive extractions.

The enzymatic hydrolysis step was optimized using a second Box-Behnken experimental design. The three factors and levels selected were: hydrolysis temperature (X_1: 20, 40, and 60 °C); hydrolysis time (X_2: 18, 21, and 24 h); and pH (X_3: 4, 5, and 6). The 16 combinations are shown in Table 5.2. The experimental results were analyzed according to the aforementioned method, and the model was validated.

Method Validation

The validation was performed according to the EMA guidelines (2015). The calibration curves were based on the ratio between the peak areas and that of IS (A_a/A_{is}, daidzein-d$_4$ (50 ng.mL^{-1}) was used for daidzein and flavone (20 ng.mL^{-1}) for the other three compounds) versus the standard concentration. A stock solution containing the four analyte (1 µg.mL^{-1}) was prepared in H_2O:methanol (40:60, v/v). The working standard solutions were then prepared by diluting this stock solution with different volumes of H_2O:methanol (40:60, v/v).

Silage Samples Analysis

Silage samples originating from three experimental meadows located in the Walloon Agricultural Research Center (Gembloux, Belgium) were analyzed. The first one 'P.Wiame' was predominantly composed of a mix of red clover (*Trifolium pratense* L.), white clover (*Trifolium repens* L.), and cocksfoot (*Dactylis glomerata* L.). The second one 'P.Class' was a classical permanent pasture constituted with a great part of ryegrass (*Lolium perenne* L.) and other local grasses (*Poaceae* sp.). Other plants were also present, such as: rumex (*Rumex* sp.), dandelion (*Taraxacum* sp.), or ribwort (*Plantago* sp.). The last one 'P.Dact' was constituted largely of cocksfoot. There was also the presence in low proportion of alfalfa (*Medicago sativa* L.), white and red clover. The meadows were mowed during summer 2013. Forages were prewilted on the field before wrapping in bale

and stored until winter period. After opening, each bale was sampled, ground, freeze-dried, and stored in opaque vacuum bags at -21 °C until analysis.

Statistical Analysis

Most of the analyses were performed at least in triplicate. The Box-Behnken assays were performed only once, except for the center point and validation of the models, which were performed four and seven times, respectively. Silage samples were analyzed in duplicate. Statistical analysis and graphics were conducted using Microsoft® Excel software (Microsoft®, Diegem, Belgium) and the JMP® 7 statistical software package (SAS institute, Cary, US). Analysis of variance (ANOVA; $\alpha = 0.05$) and the comparison of averages by Tukey's HSD ($\alpha = 0.05$) test were used to compare process and validation parameters.

Results and Discussion

Matrix and Isoflavones Choices to Optimize Extraction Process

The proposed method was intended to quantify, as accurately as possible and in a single analytical run, four isoflavones in forage plants. All analyses were performed on one homogeneous sample obtained by blending several ground and lyophilized plants with flaxseed meal to give a representative sample of cows feed.

Development of the LC-MS/MS Method

Figure 5.3 shows the optimized elution gradient next to the total ion chromatogram (TIC) of the standard solution of the four isoflavones and the two ISs. The four isoflavones were eluted and reported by Valls *et al.* (2009) for this type of stationary phase. As this figure shows, the peaks of DA and $IS_{Daidzein\text{-}d4}$ had the same retention times, but with the use of MRM, there was no difficulty in discriminating these two compounds. In addition, no interference was observed when plant extracts were injected.

The optimization of MRM conditions was achieved by infusing standard solutions of isoflavones and ISs (1 $\mu g.mL^{-1}$ in methanol) directly in the MS/MS. The positive mode was selected for each molecule, which was detected using the precursor ion and two characteristic product ions (BA 285.2 > 213, 269.1; DA 255 > 199, 137; GE 271.1 > 153, 215; FO 269.2 > 197, 237.1). The most abundant fragments were selected for quantification *(Q),* and the second most abundant were used for confirmation (*q*). The optimized parameters and ions selected are shown in Table S5.1.

Isoflavones are generally solubilized in methanol containing water. To optimize the dissolution, a mixture of the four isoflavone and the two ISs at 50 ng.mL^{-1} was prepared in methanol/water ratios ranging from 10 to 100 % and injected in triplicate (Fig. S5.1). A 60 % aqueous methanol was chosen, because this proportion led to the best solubilization and analytical repeatability. As shown in Fig. S5.2, this solvent proportion was also chosen for the solubilization of isoflavone from dry residues, because this proportion led to a low and stable residual of isoflavones after reconstitution step.

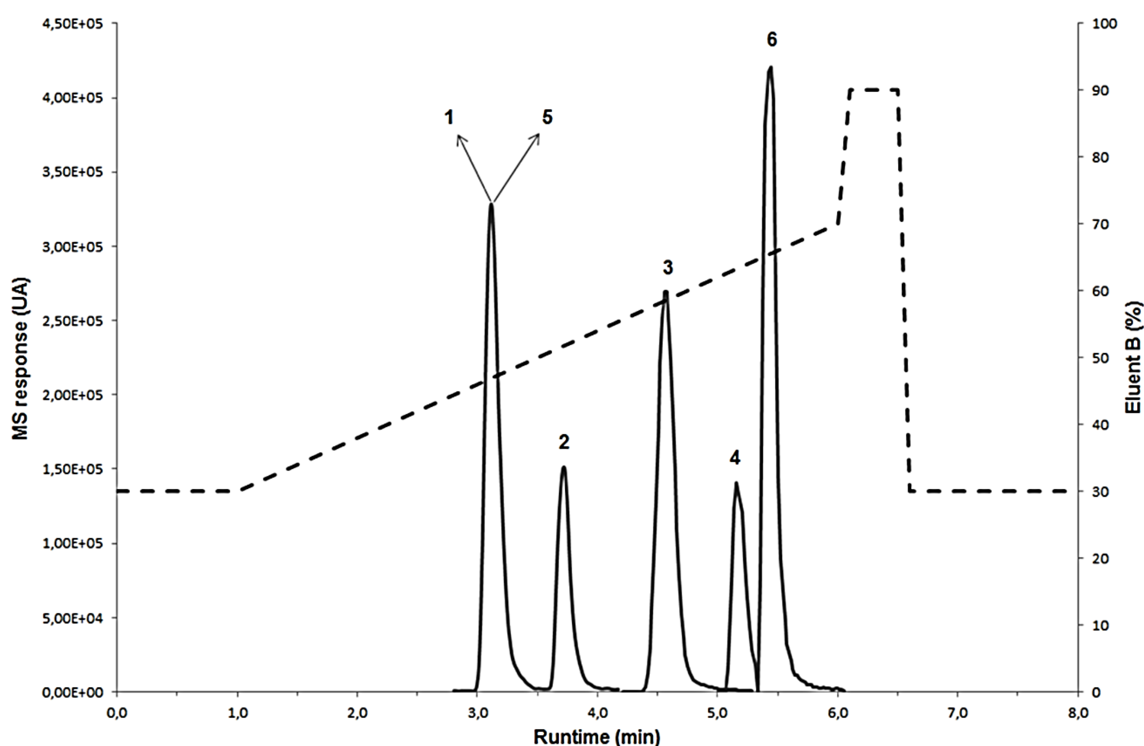

Figure 5.3. Optimized elution gradient and overlay graphs of the TIC of standard solutions of daidzein (1), genistein (2), formononetin (3), biochanin A (4), daidzein-d4 'IS' (5), and flavone 'IS' (6). Compounds 1-5 and 6 are set at 50 ng.mL^{-1} and 20 ng.mL^{-1}, respectively.

Optimization of UAE

Response Surface Methodology

MeOH, EtOH, and ACN were evaluated for their efficiency in extracting isoflavones in a forage plant mix according to the conditions of Table 5.1. As shown in this table, the isoflavone concentrations found in the assays from the center point (four analyses for each solvent) were close to each other. Moreover, as shown in Table 5.3, it was found that extraction yield was influenced mainly by solvent proportion (X_3).

$$Y_{MeOH-Total} = 718.325 + 46.0625X_1 - 21.4375X_2 + 151.15X_3 - 25.725X_1X_2 - 83.4X_1X_3 + 19.35X_2X_3 + 59.65X_1^2 - 3.9X_2^2 - 228.975X_3^2 \quad (5.2)$$

$$Y_{EtOH-Total} = 690.85 + 28.8625X_1 + 8.9125X_2 + 73.5X_3 - 49.05X_1X_2 - 57.15X_2^2 - 227.875X_3^2 \quad (5.3)$$

$$Y_{ACN-Total} = 698.8375 + 55.1X_1 + 0.475X_2 + 48.05X_3 - 9.125X_1X_2 - 77.425X_1X_3 - 132.7875X_3^2 \quad (5.4)$$

The second-order polynomial models describing the correlation between isoflavone extraction yield and the three variables for each solvent are presented in Eqs. 5.2-4. The statistical significance of these equations was determined using the ANOVA results shown in Table 5.3. The ANOVA p-values indicated that the models were significant (p value <0.05) for the three solvents. The lack-of-fit tests were not significant (p values >0.05), and therefore, no lack of matching between the data and the model can be shown. The goodness of fit was checked by the coefficient of determination (R^2) (Zou *et al.* 2010). There is no specific rule relating to the minimum value accepted for this coefficient, but 80 % is a commonly accepted limit (Joglekar & May 1987). Lower values can be tolerated, providing that the quality of the model has been proved (Malcolmson *et al.* 1993). For UAE with MeOH, R^2 was 95 %, implying that only 5 % of the total variation could not be explained by the model. The R^2 values were lower for EtOH and ACN, but remained within an acceptable range. These results suggested that the models adequately represented a large part of the real relationship between the chosen factors in the range of selected values.

In Table 5.3, the best R^2 value was found for MeOH, indicating that the model for extraction with MeOH provided a better explanation of the variations observed than the two other models. In addition, with these equations, the best extraction yield was predicted for MeOH, which was ultimately chosen for the UAE optimization.

Among the four target compounds present in the forage mix, FO and BA were the most abundant. With regard to DA and GE, only several dozen micrograms per gram dry matter were quantified. If UAE had been optimized, considering the overall isoflavones, the compounds present at the highest levels would probably have influenced the choice of extraction conditions. This situation was considered in developing the optimization protocol, and a second-order polynomial model was fitted for each compound (see Eqs. 5.5-8). As shown in Table 5.3, all ANOVA p-values were lower than 0.05, and all lack-

of-fit p-values were higher than 0.05, indicating that the quality of the models has been also proved for each analyte.

$$Y_{MeOH-FO} = 412.75 + 27.625X_1 - 13.4125X_2 + 81.1125X_3 - 59.15X_1X_3 + 21.725X_2X_3 + 30.6125X_1^2 - 8.6125X_2^2 - 97.0125X_3^2 \quad (5.5)$$

$$Y_{MeOH-BA} = 31.025 + 15.85X_1 - 7.65X_2 + 51.975X_3 - 16.475X_1X_2 - 19.725X_1X_3 - 6.475X_2X_3 + 25.125X_1^2 + 5.675X_2^2 - 112.325X_3^2 \quad (5.6)$$

$$Y_{MeOH-DA} = 16.875 + 0.0675X_1 - 0.3375X_2 + 2.4875X_3 - 1.05X_1X_2 - 0.15X_1X_3 + 0.725X_2X_3 + 0.95X_1^2 - 1.175X_2^2 - 3.625X_3^2 \quad (5.7)$$

$$Y_{MeOH-GE} = 57.675 + 1.925X_1 - 0.025X_2 + 15.6X_3 - 0.05X_1X_2 - 4.35X_1X_3 + 3.4X_2X_3 + 2.9625X_1^2 + 0.2125X_2^2 - 16.0375X_3^2 \quad (5.8)$$

As shown in Table 5.3, the MeOH proportion had significant linear and quadratic effects on all isoflavone extractions. The combination of these two observations allowed an optimum level for each isoflavone to be obtained for a solvent mixture containing between 50 and 70 % of MeOH. Temperature interacted significantly with MeOH concentration for GE extraction, reflecting the situation with overall isoflavone extraction. For duration, however, no effect on isoflavone extraction was found.

The lack of a significant impact of duration and temperature is likely the cause of the numerous extraction conditions found in scientific literature. The concentration range found, however, was slightly lower than that cited in the literature for forage plants (≥70 % of organic solvent) (Wu *et al.* 2004; Tsao *et al.* 2006; Zgórka 2009; Reynaud *et al.* 2010; Hoerger *et al.* 2011). One possible explanation could be the presence of numerous conjugated forms in plant matrices, which are more polar than the aglycone forms. For other plant matrices (Kiss *et al.* 2010; Niranjan *et al.* 2011), this solvent composition and proportion is not uncommon and has even been recommended (Rostagno *et al.* 2003). Increasing the water proportion in the extraction solvent helps the effective swelling of the plant matrix and increases the propagation of ultrasonic waves (Biesaga 2011). Nevertheless, according to Rostagno *et al.* (2003), there should be no more than 60 % of water to prevent oxidative reactions that could reduce extraction efficiency.

Choice of Conditions and Verification of the Model

Equations 5.2 and 5.5-8 generated earlier allowed the best extraction conditions for total and individual isoflavone to be extracted (Table 5.3). The optimal temperature

Table 5.3. Model-fitting results for UAE optimization with the three solvents and each isoflavone for MeOH are presented in the upper part of the table. Isoflavone content predicted and obtained under optimal and selected UAE conditions are presented in the lower part of the table

Model-fitting

Statistical parameters	$EtOH_{Total}$	ACN_{Total}	$MeOH_{Total}$	FO_{MeOH}	BA_{MeOH}	GE_{MeOH}	DA_{MeOH}
R^2	0.725	0.741	0.950	0.830	0.967	0.983	0.884
R^2 Adj	0.542	0.570	0.874	0.637	0.917	0.958	0.709
ANOVA *p* value	0.0322*	0.0256*	0.0030***	0.0353*	0.0009***	0.0001***	0.0308*
Lack-of-fit *p* value	0.4226	0.1007	0.1830	0.1313	0.8872	0.1747	0.2764
Factors with significant effect	X_3X_3***	X_3X_3***	X_3X_3*** X_3*** X_1X_3*	X_3** X_3X_3*	X_3X_3*** X_3***	X_3*** X_3X_3*** X_1X_3*	X_3X_3*** X_3**

Verification of the prediction model

Compound	Condition	UAE variables X_1 (°c)	X_2 (min)	X_3 (%)	Y (µg.g^{-1} DM) Predicted[d]	Pred. Inter.[6]	Obtained (n = 7)[f]
Total	Optimal	80	10	54.4	869.8 ± 135.4	1036.6 - 703.1	892.3 ± 57.4
	Selected	80[a]	10[b]	55[c]	869.7 ± 135.6	1036.6 - 703.0	
FO	Optimal	80	19.96	50.73	476.3 ± 85.9	619.1 - 333.4	517.2 ± 50.1
	Selected	a	b	c	474.2 ± 98.6	620.4 - 328.2	
BA	Optimal	80	10	57.13	305.1 ± 45.6	374.5 - 235.8	291.6 ± 26.8
	Selected	a	b	c	304.9 ± 45.4	360.8 - 249.0	
GE	Optimal	80	90	68.35	66.0 ± 6.7	73.9 - 58.1	62.9 ± 6.2
	Selected	a	b	c	63.6 ± 6.4	71.4 - 55.7	
DA	Optimal	80	29.73	60.67	19.2 ± 2.7	23.3 - 15.1	20.7 ± 2.3
	Selected	a	b	c	18.9 ± 3.5	23.2 - 14.6	

* Significant at 0.05, ** Significant at 0.01, *** Significant at 0.005. X_1, X_2, X_3 and Y correspond to temperature, duration, solvent concentration and isoflavone yield, respectively
[a] Selected temperature
[b] Selected duration
[c] Selected solvent proportion
[d] Predicted content ± half confidence interval (α = 0.05)
[e] Prediction interval of 95 %
[f] Mean value ± standard deviation

was the same for each compound. The situation, however, differed for the other two variables, and a compromise was needed. The optimal duration was very different for each individual compound. This variable, however, showed no significant impact on extraction yield (Table 5.3). To reduce the sample preparation time, therefore, this variable was arbitrarily set at 10 min. For the last one, the optimum concentration for each compound was obtained for solvent proportions between 50.7 and 68.3 % of MeOH. The selected extraction conditions finally fixed were: temperature 80 °C; duration 10 min; and MeOH concentration 55 %. In Table 5.3, the predicted yield for each compound

with the selected conditions is within the standard deviation of the predicted value with the optimal conditions. This finding shows that these selected conditions represent a good compromise.

To verify the predictive capacity of the model, seven extractions with the selected conditions were performed. The total isoflavone yield was 892.3 ± 57.4 $\mu g.g^{-1}$ DM, which was within the prediction interval (1036.6-703.0 $\mu g.g^{-1}$ DM). This finding was the same for each individual compound (Table 5.3). These data proved the validity of the models designed in this study.

The extraction efficiency was also checked by performing three consecutive extractions under the selected conditions (Fig. S5.3). This investigation demonstrated that according to the molecules, 85-90 % have been recovered after one extraction. This value and the good reproducibility of the measurements were judged acceptable for a single extraction.

Optimization of Hydrolysis Conditions

Choice of Enzymes

Enzymatic hydrolysis was preferred to acidic hydrolysis, because some authors have cited the problem of incomplete cleavage of conjugated forms, as well as stability problems for some compounds (Rostagno *et al.* 2009; Schwartz *et al.* 2009). *β*-Glucosidase, cellulase, and *β*-glucuronidase/arylsulfatase from *Helix pomatia* digestive juice are the enzymes commonly used to perform isoflavone hydrolysis. The combination of these three enzymes has been reported to be a more effective way for determining total isoflavone content in vegetables (Konar *et al.* 2012).

Before optimization with RSM, the impact of each enzyme and their concentrations on hydrolysis yield were investigated. Hydrolysis with each enzyme alone, as well as with the enzyme mix, was tested in triplicate. Three blank assays using only a buffer solution without enzymes were also undertaken. It was found that very low isoflavone content was found in the extracts without hydrolysis. These findings confirm that isoflavones are present mainly in conjugated forms in plants (Rostagno *et al.* 2009; Saviranta *et al.* 2010; Mostrom & Evans 2012; Vitale *et al.* 2013). In any case, each enzyme seems to play a role in the hydrolysis of conjugated isoflavones, and using the three enzymes in solution is, therefore, probably the best approach (Fig. S5.4). The

concentration of the enzymatic suspension was also investigated. An enzymatic solution that contains ≥12, ≥24, and ≥10,200 units.mL^{-1} of cellulase, β-glucosidase, and β-glucuronidase, respectively, was prepared. This enzymatic solution was then diluted 2, 4, and 8 times with sodium acetate buffer solution (pH = 5). All tests were carried out in triplicate. It was found that enzyme mix concentration had no impact on the hydrolysis yield of DA and BA. For GE and FO, however, the stock solution and the one diluted twice had a better hydrolysis yield than the other two dilute solutions (Fig. S5.5). In this study, the enzymatic solution with ≥6, ≥12, and ≥5100 units.mL^{-1} of cellulase, *β*-glucosidase, and *β*-glucuronidase, respectively, was finally selected.

One disadvantage of using *Helix pomatia* digestive juice is that it usually contains appreciable levels of some isoflavones that can affect quantification (Kuhnle *et al.* 2007; Alves *et al.* 2010). The isoflavone concentrations found in crude solution, however, appear to fluctuate over time. For this reason, blank samples were made for each experiment to correct the concentration found by subtracting the isoflavone amount in the hydrolysis reagent from the amount quantified in each sample. When the extract was diluted by a factor of minimum 10 in the post-hydrolysis steps, however, the three isoflavones were present as traces, and there was no difficulty in quantification.

Response Surface Methodology

The hydrolysis conditions, including temperature, duration, and pH as independent variables, were also analyzed using RSM. The results of the experimental design are given in Table 5.4. The second-order polynomial models for the total amount of isoflavones and for each individual compound are given in Eqs. 5.9-13, and their statistical significances are given in Table 5.4.

$$Y_{Hyd.-Total} = 659.625 - 107.675X_1 - 29.05X_2 + 42.725X_3 - 47.275X_1X_2 - 78.575X_1X_3 + 1.425X_2X_3 - 127.425X_1^2 + 96.225X_2^2 + 62.775X_3^2 \quad (5.9)$$

$$Y_{Hyd.-FO} = 451.45 - 53.7X_1 - 23.6125X_2 + 22.1875X_3 - 32.75X_1X_2 - 47.25X_1X_3 - 5.475X_2X_3 - 85.7875X_1^2 + 54.7875X_2^2 + 42.7875X_3^2 \quad (5.10)$$

$$Y_{Hyd.-BA} = 128.95 - 38.9375X_1 + 0.725X_2 + 14.2625X_3 - 7.55X_1X_2 - 23.325X_1X_3 + 10.6X_2X_3 - 31.0875X_1^2 + 34.6875X_2^2 + 18.8125X_3^2 \quad (5.11)$$

$$Y_{Hyd.-GE} = 62.825 - 13.8375X_1 - 5.3875X_2 + 6.25X_3 - 6.925X_1X_2 - 6.95X_1X_3 - 3.35X_2X_3 - 9.475X_1^2 + 6.975X_2^2 + 0.8X_3^2 \quad (5.12)$$

$$Y_{Hyd.-DA} = 16.28125 - 1.1875X_1 - 0.75X_2 + 0.0375X_3 - 1.1X_1X_3 - 0.325X_2X_3 - 1.0625X_1^2 + 0.3875X_3^2 \quad (5.13)$$

All the ANOVA p-values indicated that the models were significant (p value <0.05). The lack-of-fit tests were not significant (p values >0.05), and therefore, no lack of matching between the data and the model can be shown. All the R^2 values were higher than 80 %, except for DA, which had R^2 of 78 %. All the models, therefore, proved to be suitable for an adequate representation of the relationship between the selected variables and isoflavone yield after hydrolysis. As shown in Table 5.4, all the variables interacted significantly, but in different way, depending on the compound. Nevertheless, temperature (X_1) was the most significant parameter and showed significant linear and/or quadratic effects (p value <0.05) on the hydrolysis of all isoflavones. An optimum below the middle of the selected temperature range was found for each analyte. Temperature also interacted significantly with pH (X_3) for DA. Duration (X_2) had a significant quadratic effect only on BA hydrolysis, which reflects overall isoflavone hydrolysis, and a linear effect on GE hydrolysis. The last variable, pH (X_3), had a significant linear impact only on GE hydrolysis.

The optimal temperatures were quite far below the temperature of 37 °C cited in other studies (Kuhnle *et al.* 2007; Andersen *et al.* 2009b; Konar *et al.* 2012) and used by the suppliers for estimating the activity of their enzymes. This finding is not unusual, though Steinshamn *et al.* (2008) performed hydrolysis with a cellulase at ambient temperature. In other studies, hydrolysis duration has usually been reported to be a minimum of 16 h (Kuhnle *et al.* 2007; Steinshamn *et al.* 2008; Konar *et al.* 2012), with the exception of Andersen's *et al.* (2009b) work, whose hydrolysis protocol with *β*-glucuronidase lasted only 1 h. The results suggest that overnight (16-20 h) hydrolysis is appropriate. Even if the last variable seems to have lower impact than the other two, pH = 6 was defined as the optimal value for all tested compounds. This optimum pH was one unit above the value usually used in other studies (Kuhnle *et al.* 2007; Steinshamn *et al.* 2008; Konar *et al.* 2012) and by enzyme suppliers.

Choice of Conditions and Verification of the Model

The best hydrolysis conditions for total and individual isoflavones were determined in the same way as before. As shown in Table 5.4, pH was the same for each compound. For the two other variables, the best conditions differed among individual

Table 5.4. Model-fitting results for enzymatic hydrolysis optimization are presented in the upper part of the table. Isoflavone content predicted and obtained under optimal and selected hydrolysis conditions are presented in the lower part of the table

Model-fitting

Statistical parameters	Isoflavone total	FO	BA	GE	DA
R^2	0.908	0.885	0.892	0.938	0.785
R^2Adj	0.770	0.714	0.729	0.845	0.598
ANOVA *p* value	0.0162*	0.0295*	0.0254*	0.0054**	0.0311*
Lack-of-fit *p* value	0.1058	0.1636	0.1724	0.5330	0.0851
Factors with significant effect	X_1X_1**	X_1X_1**	X_1***	X_1***	X_1**
	X_1**	X_1*	X_2X_2*	X_1X_1*	X_1X_3*
	X_2X_2*		X_1X_1*	X_3* X_2*	

Verification of the prediction model

Compound	Condition	Enzymatic hydrolysis variables			Y (µg.g^{-1} DM)		
		X_1 (°c)	X_2 (h)	X_3 (U pH)	Predicted[d]	Pred. Inter.[e]	Obtained (*n* = 7)[f]
Total	*Optimal*	*21.6*	*24*	*6*	*940.7 ± 182.6*	*1123.3 - 758.1*	827.8 ± 25.3
	Selected	20[a]	18[b]	6[c]	900.5 ± 191.9	1084.5 - 716.5	
FO	*Optimal*	*32.1*	*18*	*6*	*613.8 ± 99.2*	*732.2 - 495.6*	523.1 ± 32.6
	Selected	a	b	c	582.7 ± 128.9	706.3 - 459.1	
BA	*Optimal*	*20.0*	*24*	*6*	*246.8 ± 66.7*	*310.7 - 182.8*	217.8 ± 11.1
	Selected	a	b	c	209.0 ± 66.7	272.9 - 145.1	
GE	*Optimal*	*25.3*	*18*	*6*	*90.6 ± 14.4*	*106.2 - 75.0*	70.7 ± 3.8
	Selected	a	b	c	90.0 ± 16.7	106.0 - 73.9	
DA	*Optimal*	*20*	*18*	*6*	*19.0 ± 2.2*	*21.5 - 16.5*	16.2 ± 0.8
	Selected	a	b	c	19.0 ± 2.2	21.5 - 16.5	

* Significant at 0.05, ** Significant at 0.01, *** Significant at 0.005. X_1, X_2, X_3 and Y correspond to temperature, duration, pH and isoflavone yield, respectively
[a] Selected temperature
[b] Selected duration
[c] Selected pH
[d] Predicted content ± half confidence interval (α = 0.05)
[e] Prediction interval of 95 %
[f] Mean value ± standard deviation

compounds, and a compromise, therefore, had to be found. The optimal working conditions were finally fixed as follows: temperature of 20 °C for 18h at pH = 6. The predicted yield for each compound with the selected conditions was within the standard deviation of the predicted value with the optimal conditions. This finding shows that these selected conditions represent a good compromise.

To verify the predictive capacity of the model, seven extractions under the selected conditions were also performed. The total isoflavone yield was 827.8 ± 25.3 µg.g^{-1} DM, which was within the prediction interval (1084.5-716.5 µg.g^{-1} DM). This was

also true for each individual compound (Table 5.4). These data proved that the models designed for this hydrolysis optimization were valid.

Method Validation

Two internal standards: daidzein-d_4 for daidzein and fla-vone for the other three analytes, were used for quantification. No trace of flavone was found neither in the plant mix nor in the enzymatic solution. The validation parameters of the analytical method were first checked to ensure reliable responses from UPLC®-MS/MS, after which the validation of the complete method was undertaken by checking recoveries, ME, repeatability, robustness, and stability of hydrolyzed methanol extracts under two storage conditions.

The specificity of the detection of each compound was characterized by its retention time (RT) and by two precursor-product ion transitions. The RT and *Q/q* ratios are shown in Table 5.5. These two parameters were observed throughout the development and validation process. The relative retention time (RRT) for each analyte was always included in the RRT tolerance range of ±2.5 %, and the *q/Q* ratio was also always included in a tolerance range of ±20 % (Antignac *et al.* 2003; Moreno-González *et al.* 2013).

The calibration curves were established by injecting six replicates of each isoflavone standard solution with ISs (50 ng.mL^{-1} for daidzein-d_4 and 20 ng.mL^{-1} for flavone) at concentrations ranging from 5 to 125 ng.mL^{-1} for all target isoflavones. This range was judged satisfactory given the isoflavones that can be found in forages. To validate these calibration curves, several linearity criteria needed to be met: a minimum of five concentration points per curve; the relative standard deviation (RSD) for each point not to exceed 20 % for points near LOQ or 15 % for others (EMA 2015); and a correlation coefficient (R^2) equal to or higher than 0.99 (Fiechter *et al.* 2013). In addition, an ANOVA with lack-of-fit test was used to confirm if the least squares linear regression model between the A_a/A_{is} ratio and concentration was adequate for describing the observed data (α = 0.05) (De Bock *et al.* 2012).

The LOD and LOQ were determined as the concentration at a signal to noise (S/N) of 3 and 10, respectively (Fiechter *et al.* 2013). The S/N of each compound was calculated in a blank matrix spiked with isoflavones at concentrations near the lower calibration concentration (LCC) (n = 5). None of the forage samples, however, was free

of the four target analytes. Flaxseed was, therefore, used to evaluate limits of these four isoflavones. The results are shown in Table 5.5. The LOQ values were 0.50, 0.50, 0.53, and 4.46 ng.mL^{-1} for DA, BA, FO, and GE, respectively. These values were experimentally confirmed by analyzing other flaxseed samples spiked with the target analytes at concentrations equal to or slightly higher (< 20 %) than calculated limits (n = 6) (Salomone *et al.* 2011). It was found that the variation in the peak area did not exceed 20 %. The LOQs calculated were lower than the LCCs. Therefore, even if these LOQs were estimated only in flaxseed samples, LCCs set at 5 ng.ml^{-1} would be considered satisfactory.

Intra-day (five injections within 1 day) precision (expressed as RSD %) and accuracy (expressed as bias %) at four concentrations (15, 30, 50, and 80 ng.mL^{-1}) were determined. Inter-day (five injections over 3 days) precision and accuracy were also evaluated for the same standard concentrations (Table S5.2). The intra- and inter-day RSD values did not exceed the maximum allowed value of 20 % for the points close to LOQ or 15 % for the other ones (Ellis 2008; EMA 2015). The maximum values for BA were at 15 ng.mL^{-1} with 8.2 and 7.6 % for intra- and inter-day, respectively. For accuracy, the maximum bias between the measured and assigned values for intra- and inter-assay was observed for BA at 30 ng.mL^{-1}. The two values (11.4 and 7.1 %) were lower than the allowed value of 15 % (Ellis 2008; EMA 2015). These observations indicated that the UPLC®-MS/MS method used had an acceptable level of precision and accuracy.

To check the carryover, 10 µL of H_2O:methanol (40:60, v/v) was injected after the injection of three high-standard concentration solutions containing the four analytes (250 ng.mL^{-1}) and ISs (50 and 100 ng.mL^{-1} for flavone and daidzein-d_4 respectively). No signals with S/N >3 were observed at the RT of each analyte or the ISs.

The ME was studied for each molecule by comparing the slopes of the calibration curves obtained after adding appropriate calibration standard solutions in flaxseed dry residues with the slopes calculated from the same standard solutions in H2O:methanol (40:60, v/v) (Salomone et al. 2011). To avoid the occurrence of the four target analytes in the dry residues, flaxseed extract was used. Three kinds of curves were prepared: one in MeOH 60 % (external calibration in solvent taken as reference); one with no dilution of hydrolyzed extract before the evaporation step; and one with a dilution factor of 10. Each curve was achieved with five points in triplicate, and the mean slopes were calculated and compared. During pretests, an important ion enhancement was found for DA (data not

Table 5.5. Validation parameters of the analytical method: retention time 'RT'; relative retention time 'RRT'; Q/q ratio; correlation coefficient 'R^2', slope and intercept of calibration curves in $MeOH_{60\%}$; LOD and LOQ calculated for the four target analytes; and maximum values for intra-/inter-assay precision (Max. prec.) and accuracy (Max. acc); are presented in the upper part of the table. Validation parameters for the complete method: matrix effect 'ME', recovery, repeatability, robustness and stability; are presented in the lower part of the table

Compound	RT [a] (min)	RRT [b]		Q/q [c]		Calibration curve in $MeOH_{60\%}$			LOD (ng.mL^{-1})	LOQ (ng.mL^{-1})	Max. prec.[d]		Max. acc.[e]	
		$Max._{(+2.5\%)}$	$Min._{(-2.5\%)}$	$Max._{(+20\%)}$	$Min._{(-20\%)}$	R^2	Slope	Intercept			intra	inter	intra	inter
DA	3.11 ±0.023	1.033	0.983	1.248	0.832	1.000	0.037	-0.0248	0.15	0.50	4.0	4.5	5.0 ± 3.8	3.3 ± 3.0
GE	3.72 ± 0.023	0.689	0.656	0.514	0.342	0.999	0.007	0.0017	1.34	4.46	3.9	3.7	6.9 ± 2.4	3.8 ± 2.3
FO	4.64 ± 0.051	0.860	0.818	0.919	0.613	0.998	0.013	0.0225	0.16	0.53	4.4	5.5	9.5 ± 4.3	6.1 ± 3.2
BA	5.28 ± 0.066	0.979	0.931	0.827	0.551	0.999	0.006	0.0056	0.15	0.50	8.2	7.6	11.4 ± 4.7	7.1 ± 4.2
$IS_{Flavone}$	5.52 ± 0.056	-	-	0.418	0.278	-	-	-	-	-	-	-	-	-
$Is_{Daidzein-d4}$	3.09 ± 0.023	-	-	0.662	0.441	-	-	-	-	-	-	-	-	-

Compound	ME (± %) [f]		Recovery [g]			Repeatability [i]		Robustness [l]		Stability [m]		
										T_0	Six days storage at:	
	No Dil.	Dil. 10	25 µg [h]	50 µg [h]	75 µg [h]	Sample 1 [j]	Sample 2 [k]	550 W	750 W		-20 °C	6°C
DA	-8.1	1.9	89.1 ± 3.93	82.8 ± 0.27	80.4 ± 1.85	126.6 (11.1%)	<LOQ	14.2 ± 0.8*	15.3 ± 2.3*	115.1 ± 7.52*	111.4 ± 15.18*	111.0 ± 8.30*
GE	20.1	14.7	92.3 ± 4.35	113.4 ± 5.06	116.5 ± 3.10	106.4 (18.8 %)	nd	56.7 ± 2.0*	57.5 ± 6.2*	86.5 ± 2.24*	93.5 ± 9.05*	89.3 ± 2.42*
FO	0.2	6.1	87.6 ± 2.76	80.4 ± 3.49	79.0 ± 3.40	502.1 (6.4 %)	7.6 (9.8 %)	484.7 ± 15.3*	473.4 ± 10.3*	474.8 ± 22.83*	486.7 ± 33.71*	474.8 ± 46.69*
BA	-19.5	-12.3	56.3 ± 4.76	47.3 ± 4.50	49.4 ± 4.07	212.6 (15.5 %)	19.9 (4.1 %)	234.6 ± 17.1*	251.9 ± 21.9*	186.4 ± 30.64*	219.3 ± 28.29*	188.3 ± 21.75*

[a] n ≥ 180 injections of standard solutions and samples over 2 months
[b] $RRT = RT_{analyte}/RT_{IS}$
[c] $Q/q = Signal_2/Signal_1$
[d] Precision is expressed in RSD %
[e] Accuracy is expressed in bias %
[f] $ME\ (\%) = \left(\frac{(Slope\ (matrix) - Slope\ (MeOH\ 60\%))}{Slope\ (MeOH\ 60\%)}\right) \times 100$
[g] $Recovery\ (\%) = \left(\frac{Amount\ found}{Amount\ added}\right) \times 100$
[h] Amount added by different volumes of analyte solution
[i] Expressed in µg.g^{-1} DM (RSD%, n=9 on 3 days) and nd = no detected
[j] Silage with a large proportion of *Leguminosae* and mixed with flaxseed meal
[k] Silage with a large proportion of grasses
[l] Expressed in µg.g^{-1} DM (mean ± SD, n = 3)
[m] Expressed in µg.g^{-1} DM (mean ± SD, n = 3)

Values marked by '*' are not significantly different with the Tukey's HSD test (p value > 0.05). LOQ for DA was 1.6 µg.g^{-1} DM.

shown). This was clearly linked to the enzymatic solution. To avoid this important matrix effect for DA, the use of the deuterated form of daidzein was selected. The percentage difference 'ME (±%)' between the slope of the calibration curve obtained by adding standard solutions in dry residues and the one arising from H_2O:methanol (40:60, v/v) is shown in Table 5.5. Ion enhancement and suppression were both observed, depending on the analyte, but in an acceptable range (±20 %) (Ferrer *et al.* 2011). Ion enhancement was observed for FO and GE with an ME just at the limit of the acceptable range for the last one in the undiluted extract. Between the undiluted and diluted extracts, the ME of DA increased from a negative value to a slightly positive value, always considered negligible. For BA, ion suppression was observed in both cases, also with a ME value just at the limit of acceptable range for the undiluted extract. In general, a decrease in ME was observed between the undiluted and ten times diluted extract. This finding leads us to recommend a dilution of the extract between the hydrolysis step and LC-MS/MS analysis. With the mix of forage plants used for this optimization process, dilution by a factor of 10 for DA and GE and more for the other two was necessary to put the signals in the range of calibration. The calibration curve in H_2O:methanol (40:60, v/v) was, therefore, used for measuring the target analytes in the forage samples.

In the absence of certified reference materials, the recovery was evaluated by comparing the concentration found in spiked samples and the expected concentration. For the same reason as previously mentioned, flaxseed was used to calculate the percentage recovery. Samples were spiked with the appropriate amounts of standard solutions to obtain final concentrations of 25, 50, and 75 ng.mL^{-1} (considering dilution by a factor of 10 after the enzymatic hydrolysis step). The analysis was carried out in triplicate. As shown in Table 5.5, values for three of the four target analytes fell within the acceptable range 70-120 % (Ellis 2008; Delgado-Zamarreño *et al.* 2012). For each analyte, the recovery value was fairly similar, whatever the concentration level added. BA showed a lower recovery (mean recovery of 51 ± 5.6 %). Nevertheless, the reproducibility over all levels was satisfactory. The low recovery for BA can be outweighed using a correction factor to get closer to the actual BA content in the sample.

The intrinsic variability of the complete sample work-up (extraction, hydrolysis, and UPLC®-MS/MS analysis) was assessed by performing a multiple independent analysis of two plant sample mixes (n = 9, within 3 days and by two analysts). The RSDs for the quantified amounts are given in Table 5.5, and the repeatability of the whole

analytical procedure was considered satisfactory for each isoflavone (RSD ≤20 %) (Ellis 2008). The maximum RSD was found for GE (present only in sample 1), with 18.8 %. The repeatability of all the analytical protocol was judged to be satisfactory for estimating the isoflavone content of different types of forage.

The stability of sample extracts during storage was also investigated. Extract from sample 1 analyzed on the first day of repeatability check was stored in two vials, one at -20 ± 1 °C and the other at 6 ± 1 °C. The sample extracts were reanalyzed in triplicate after 6 days of storage. No significant difference was found with the Tukey's HSD test between the isoflavone content found in the first analysis and after 6 days. There was, therefore, no isoflavone degradation in the methanolic extracts stored at -21 and 6 °C for at least 6 days.

Robustness of the extraction step was checked by modifying the ultrasound power. The extractions were performed in triplicate with two levels of power (750 and 550 W). The Tukey's HSD test revealed no significant difference between extraction yields obtained with the two ultrasound power levels (Table 5.5).

Together the results of the validation process reveal that the proposed method can be used to reliably identify and quantify the target compounds in forage samples with accurate sensitivity.

Use of the Method in an Experimental Setting

The method was used to determine the four isoflavones present in silages used in an experimental study intended to assess the impact of high isoflavones diets on cow's milk quality (unpublished data). As shown in Table 5.6, there are great differences between isoflavones found in the silages originating from the three meadows. P. Class and P. Dact have low and similar isoflavones contents compared with P. Wiame, which has contents above 100 $\mu g.g^{-1}$ DM for all target compounds. In P. Wiame, the minimum content was found for GE (105.8 ± 13.3 $\mu g.g^{-1}$ DM), and the maximum was found for FO (687.6 ± 110.4 $\mu g.g^{-1}$ DM). This finding is correlated with the presence of red clover (*Trifolium pratense* L.) in silages from P. Wiame, known for its high isoflavone contents, especially, FO and BA (Tsao et al. 2006; Andersen et al. 2009b; Saviranta et al. 2010; Mostrom & Evans 2012). For the same type of silage, the isoflavones contents found in each bale were rather similar, except for low contents (P. Class and P. Dact) where some compound were sometime not detected. This suggests that isoflavones are stable and does

Table 5.6. FO, BA, GE and DA (μg.g^{-1} DM) present in silage samples originating from experimental meadows

Origin of silage [c]	Isoflavones contents (μg.g^{-1} DM) [d]			
	FO	BA	GE	DA
P. Class				
Bale 1 (04/11/2013)	10.7 ± 2.1[b]	24.7 ± 0.9[b]	3.9 ± 0.2[b]	4.3 ± 0.6[b]
Bale 2 (18/11/2013)	11.3 ± 0.1[b]	19.6 ± 1.7[b]	6.2 ± 0.6[b]	8.1 ± 1.7[b]
Bale 3 (16/12/2013)	3.1 ± 0.3[b]	19.0 ± 0.3[b]	nd	nd
P. Dact				
Bale 1 (31/10/2013)	39.1 ± 4.6[b]	6.8 ± 0.4[b]	5.7 ± 0.4[b]	10.8 ± 1.7[b]
Bale 2 (18/11/2013)	14.3 ± 0.5[b]	24.1 ± 0.0[b]	3.5 ± 0.3[b]	2.8 ± 0.1[b]
Bale 3 (14/12/2013)	21.2 ± 2.4[b]	nd	3.1 ± 0.2[b]	nd
P. Wiame				
Bale 1 (30/10/2013)	541.8 ± 4.6[a]	436.5 ± 75.1[a]	131.3 ± 14.8[a]	304.3 ± 45.7[a]
Bale 2 (16/11/2013)	444.8 ± 43.5[a]	444.3 ± 66.0[a]	242.7 ± 47.9[a]	175.7 ± 7.9[a]
(17/11/2013)	548.2 ± 69.6[a]	548.8 ± 33.5[a]	198.4 ± 19.2[a]	254.2 ± 39.7[a]
(18/11/2013)	446.7 ± 49.6[a]	509.7 ± 4.2[a]	105.8 ± 13.3[a]	268.1 ± 31.0[a]
Bale 3 (25/11/2013)	687.6 ± 110.4[a]	535.4 ± 39.3[a]	150.3 ± 29.9[a]	397.4 ± 80.1[a]
Bale 4 (16/12/2013)	517.7 ± 59.4[a]	525.5 ± 83.5[a]	218.3 ± 31.0[a]	319.1 ± 2.8[a]
(17/12/2013)	465.7 ± 13.1[a]	516.9 ± 68.6[a]	256.6 ± 23.1[a]	229.7 ± 15.0[a]
(19/12/2013)	482.6 ± 39.6[a]	467.3 ± 25.4[a]	203.3 ± 0.2[a]	313.6 ± 19.3[a]

Values marked with the same letter are not significantly different with the Tukey's HSD test (p value > 0.05).
[c] The dates in brackets correspond to the sampling dates
[d] Expressed in mean ± SD (n = 2) and nd = no detected

not undergo transformation during silage storage period. A forage bale can be used several days to feed animals, so with two bales originating from P. Wiame, three sampling were realized over a 3 days period. The concentrations found remained similar for all isoflavones. The oxygen and light supplies with the opening of the bale also do not seem to affect the concentration of isoflavones in the forage. These exploratory analyses clearly confirm that grassland management affects the isoflavones contents present in silage. All the interpretations and assumptions above, however, must be confirmed by further studies. Even if isoflavones content seems to be stable during the silage storage period, it would be useful to investigate their evolution over a longer period from a well-characterized sample and under controlled conditions.

Conclusions

This study proposes a reliable and sensitive analytical method for simultaneous quantification of daidzein, genistein, formononetin, and biochanin A in forages grazed by ruminants. It is based on aglycones released by methanolic UAE, followed by enzymatic hydrolysis and UPLC®-MS/ MS analysis. The application of RSM enabled both the enzymatic hydrolysis and the extraction process to be optimized. The impact of several factors on the extraction and hydrolysis yield of four isoflavones was discussed, and a compromise in the conditions of application of these two processes was proposed. This analytical method was validated, following international recommendations, and is well suited to routine analysis. It can be used, among other things, to evaluate the modification of isoflavone content in forage silages.

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Compliance with Ethical Standards

Conflict of interest

The authors declare that there are no conflicts of interest.

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Supplementary data

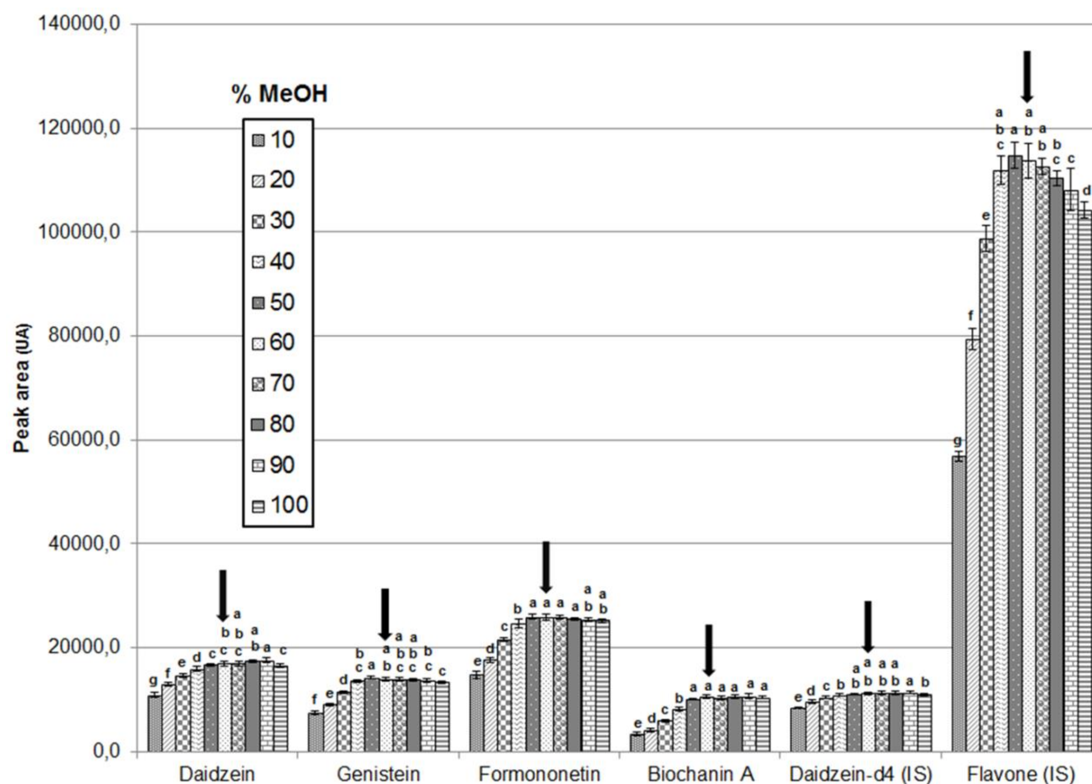

Figure S5.1. Peak area of isoflavones and ISs when solubilized in different methanol/water mixtures. Analytical concentration was set at 50 ng.mL^{-1} (n=3, m=3). Values marked by the same letter are not significantly different with the Tukey's HSD test (*p* value > 0.05). The arrow represents the selected solvent.

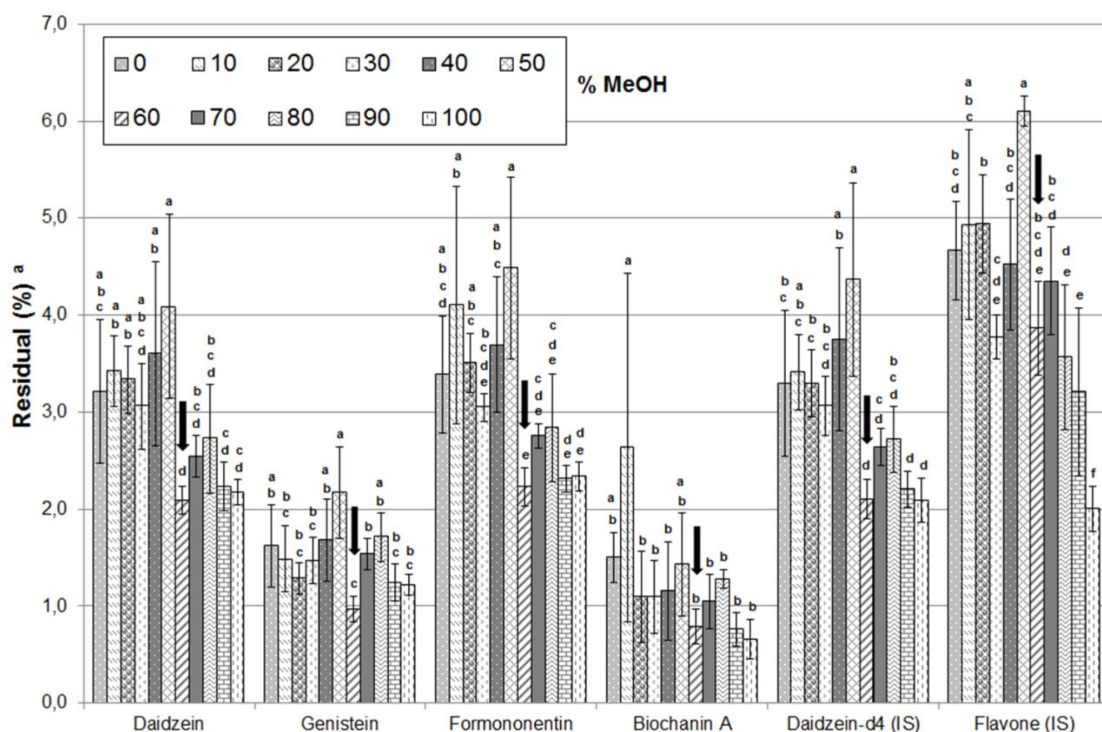

Figure S5.2. Residual of isoflavones and ISs when re-solubilized solutions with different methanol/water mixtures are used. The starting concentration was set at 500 ng.mL^{-1} for all compounds (a Residual [%] = [Arearesidual/AreaMeOH 60%]*100) (n=3, m=2). Values marked by the same letter are not significantly different with the Tukey's HSD test (*p* value > 0.05). The arrow represents the selected solvent.

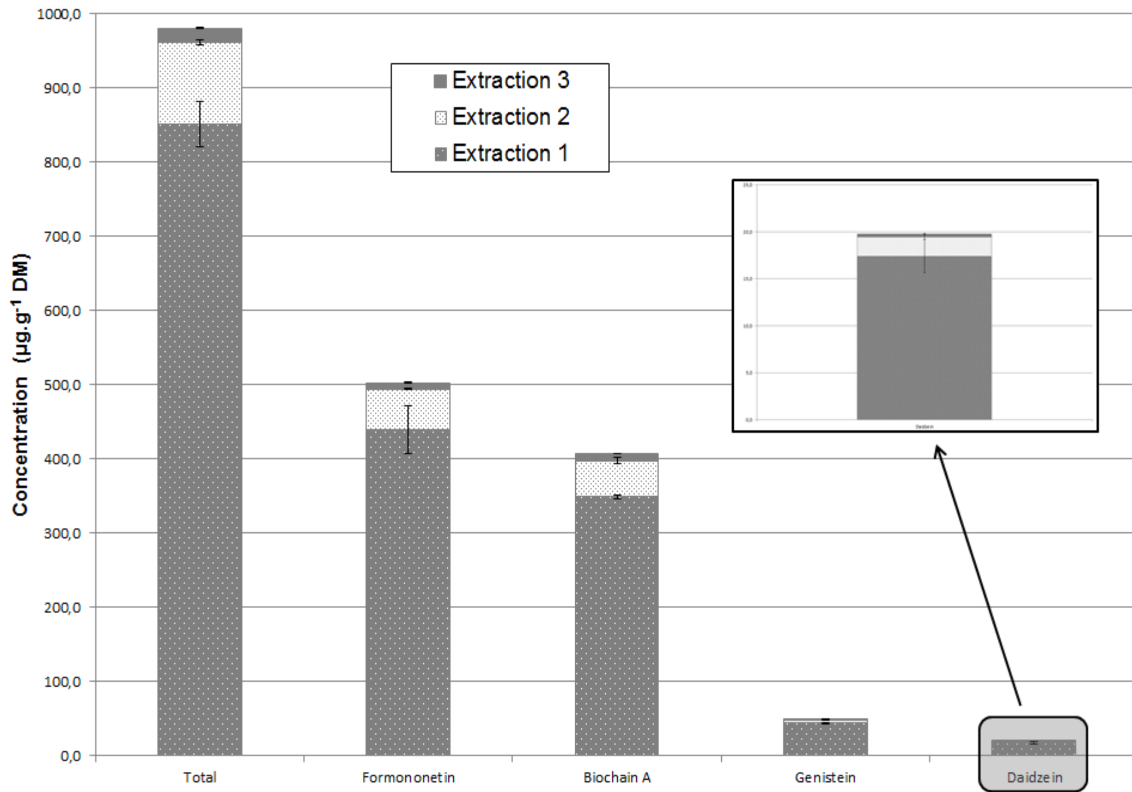

Figure S5.3. Sum of isoflavone concentrations found in a representative sample of cows feed after three consecutive extractions under selected UAE conditions.

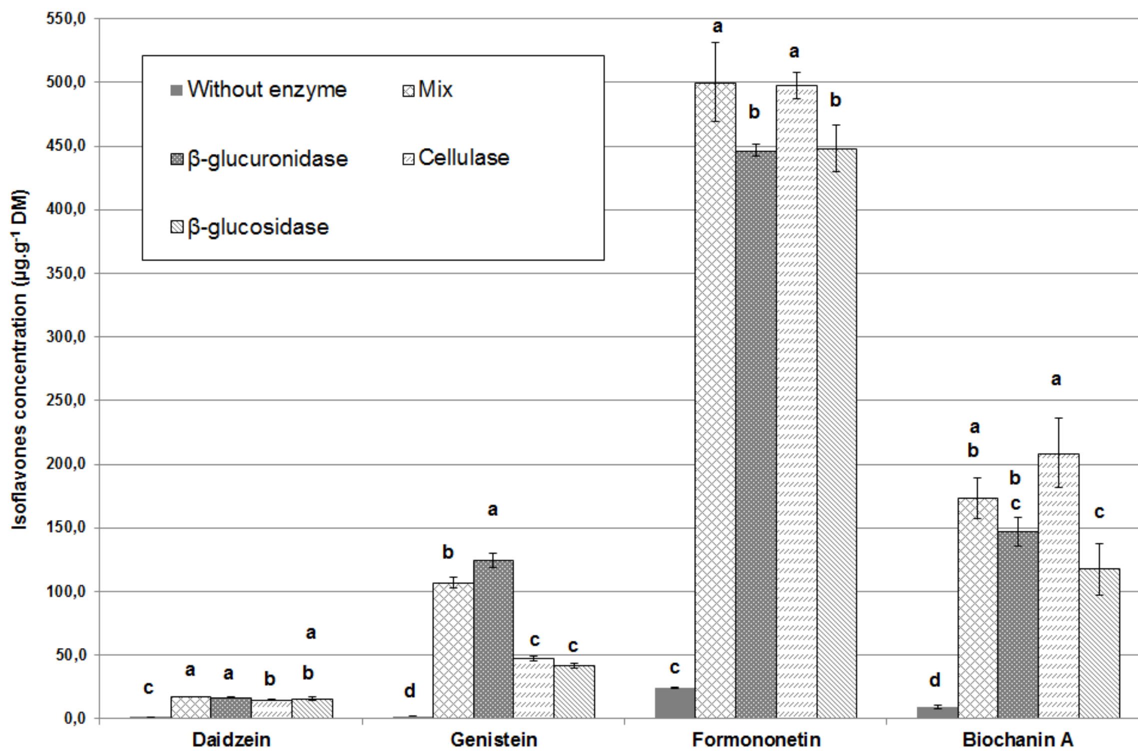

Figure S5.4. Effect of each enzyme on the isoflavone hydrolysis process from the forage mix (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. The hydrolysis conditions were: temperature 37°C; hydrolysis time 18 h; and pH 5. Values marked by the same letter are not significantly different with the Tukey's HSD test (*p* value > 0.05).

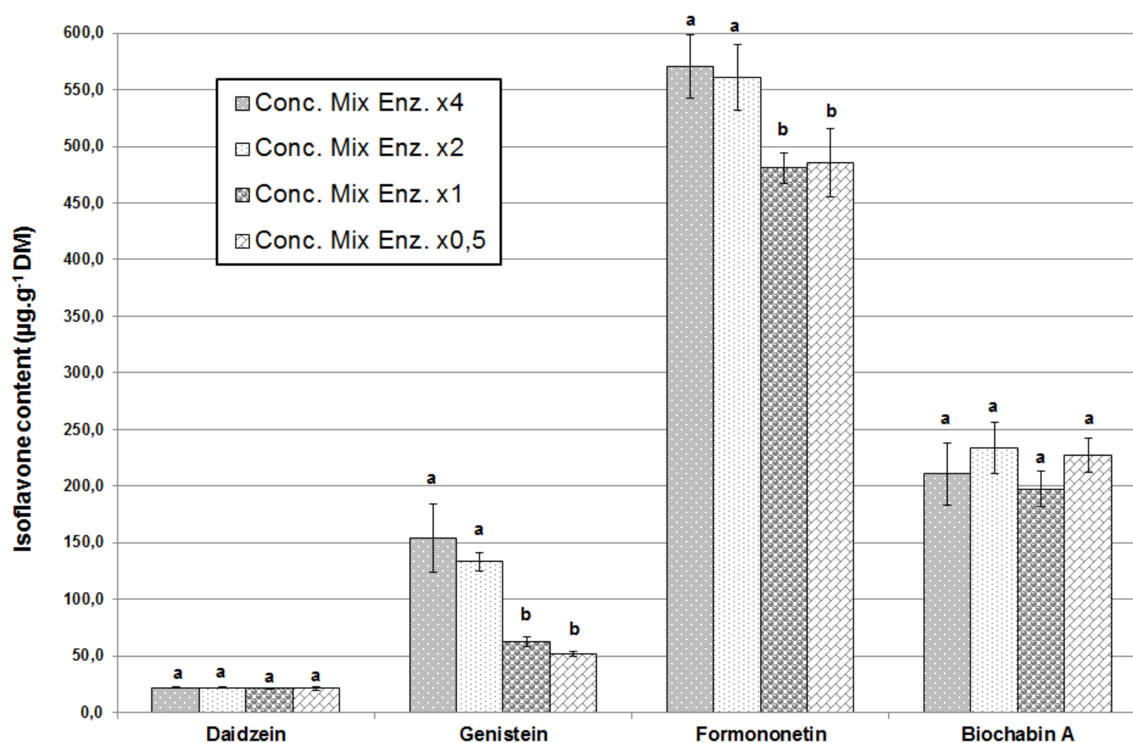

Figure S5.5. Effect of enzyme mix concentration on the isoflavone hydrolysis process from the forage mix (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. The hydrolysis conditions were: temperature 37°C; hydrolysis time 18 h; and pH 5. 'Mix Enz. No Dil'contained ≥ 12, ≥ 24, ≥ 10200 units.mL^{-1} of cellulase, *β*-glucosidase and *β*-glucuronidase, respectively, and was then diluted by a factor of 2 'Mix Enz. Dil 2', 4 'Mix Enz. Dil 4' and 8 'Mix Enz. Dil 8'. Values marked by the same letter are not significantly different with the Tukey's HSD test (*p* value > 0.05).

Table S5.1. MS/MS acquisition parameters used for the analysis of isoflavones and ISs

Compound	Ionization	Cone voltage (V)	Precursor ion (m/z)	Collision energy (V)	Product ions (m/z)	Dwell time (s)
DA	ESI$^+$	35	255	25	199 ^{Q}	0.03
				25	137 ^{q}	0.03
GE	ESI$^+$	50	271.1	25	153 ^{Q}	0.05
				25	215 ^{q}	0.05
FO	ESI$^+$	40	269.2	35	197 ^{Q}	0.35
				25	237.1 ^{q}	0.35
BA	ESI$^+$	40	285.2	35	213 ^{Q}	0.05
				30	269.1 ^{q}	0.05
Flavone *(IS)*	ESI$^+$	45	223.1	25	121 ^{Q}	0.07
				25	178 ^{q}	0.07
Daidzein-d4 *(IS)*	ESI$^+$	45	259.2	25	203 ^{Q}	0.05
				25	137 ^{q}	0.05

^{Q} product ion for quantification, ^{q} product ion for identification.

Table S5.2. Intra-/inter-assay precision (RSD%) and accuracy (bias%) for each isoflavone

Injected concentration	Instrumentation parameter		Day	DA	GE	FO	BA
15 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	4.0	3.4	2.3	4.7
			2	3.0	2.3	2.0	8.2
			3	3.4	1.8	3.4	4.6
		Inter-assay [d]		4.5	3.7	5.5	7.6
	Accuracy [b] (bias%)	Intra-assay [c]	1	5.0 ± 3.79	4.1 ± 3.29	4.4 ± 2.18	3.6 ± 4.52
			2	2.7 ± 1.88	3.1 ± 1.45	7.6 ± 2.11	8.8 ± 6.22
			3	2.2 ± 2.68	1.3 ± 1.15	4.1 ± 0.88	7.7 ± 4.90
		Inter-assay [d]		3.3 ± 2.96	2.8 ± 2.35	5.4 ± 2.35	6.7 ± 5.41
30 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	1.9	2.2	2.8	6.0
			2	3.7	2.2	4.0	4.2
			3	1.9	1.5	1.3	4.6
		Inter-assay [d]		3.6	3.1	4.1	7.4
	Accuracy [b] (bias%)	Intra-assay [c]	1	3.0 ± 1.86	1.8 ± 1.29	2.4 ± 2.35	5.4 ± 2.84
			2	3.9 ± 2.28	6.9 ± 2.37	9.5 ± 4.35	11.4 ± 4.71
			3	1.9 ± 1.57	2.8 ± 1.59	6.5 ± 1.39	4.5 ± 2.61
		Inter-assay [d]		2.9 ± 1.98	3.8 ± 2.27	6.1 ± 3.20	7.1 ± 4.24
50 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	2.2	3.7	4.4	5.9
			2	2.3	2.2	3.0	4.6
			3	1.4	3.9	1.6	5.9
		Inter-assay [d]		3.1	3.6	4.4	7.0
	Accuracy [b] (bias%)	Intra-assay [c]	1	4.1 ± 2.13	2.6 ± 2.57	3.4 ± 2.15	5.6 ± 4.48
			2	1.8 ± 1.42	4.6 ± 2.32	8.1 ± 3.21	6.6 ± 4.96
			3	1.5 ± 1.10	3.5 ± 1.16	5.9 ± 1.74	5.5 ± 3.27
		Inter-assay [d]		2.5 ± 1.91	3.6 ± 2.14	5.8 ± 3.01	5.9 ± 4.01
80 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	3.2	2.6	1.8	2.9
			2	2.3	1.5	2.0	5.0
			3	3.3	2.9	3.2	4.3
		Inter-assay [d]		2.8	2.4	2.5	4.9
	Accuracy [b] (bias%)	Intra-assay [c]	1	2.6 ± 1.84	2.1 ± 1.37	1.3 ± 0.96	3.3 ± 0.98
			2	2.0 ± 0.78	1.9 ± 1.53	2.6 ± 1.52	4.6 ± 2.62
			3	2.6 ± 1.68	2.6 ± 0.47	3.8 ± 1.37	5.4 ± 3.88
		Inter-assay [d]		2.4 ± 1.43	2.2 ± 1.16	2.6 ± 1.58	4.4 ± 2.71

[a] $RSD\% = \left(\frac{SD\ Conc._{Calc.}}{Mean\ Conc._{Calc.}}\right) \times 100$

[b] $Bias\% = Abs\left(\frac{(Conc._{Calc.} - Conc._{Nom.})}{Conc._{Nom.}}\right) \times 100$

[c] n=5

[d] n=15

Chapter VI

Changes in isoflavones concentration during ensiling and storage in laboratory-scale silos

Objectives

Currently, a number of studies are being conducted on the impact of a high-isoflavone diet on the quality of cow's milk. Knowledge about the effect of the ensiling process on isoflavone content, however, remains limited. The exploratory analyses of farm-scale silages described in *Chapter V* suggested that isoflavone concentration was stable during the silage storage period. Nevertheless, it would be useful to investigate their evolution from a well-characterized sample and under controlled conditions. This chapter describes the first study to be conducted on the evolution of the four selected isoflavones in red clover (*Trifolium pratense* L.) ensiled in laboratory-scale silos using vacuum-packed plastic bags. The analyses of the isoflavones were carried out using the reliable analytical method previously described. An overview of this original scoping study is presented in Fig. 6.1.

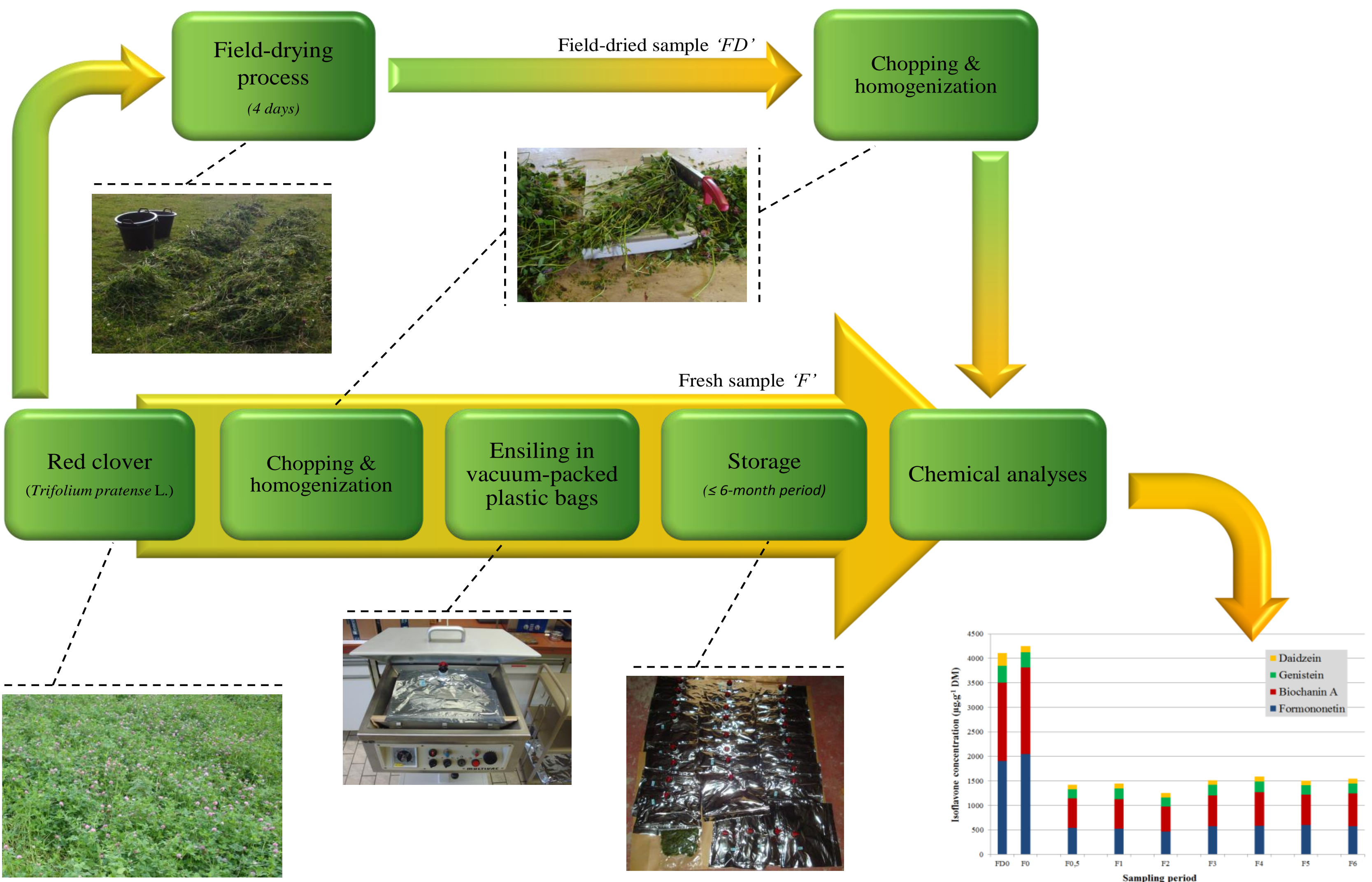

Figure 6.1. Graphical abstract of *Chapter VI.* Evolution of isoflavone concentration in red clover (*Trifolium pratense* L.) ensiled in laboratory-scale silos and after field-drying process.

Changes in the isoflavone concentration in red clover (*Trifolium pratense* L.) during ensiling and storage in laboratory-scale silos

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Reference: Daems, F., Decruyenaere, V., Agneessens, R., Lognay, G., Romnee, J.-M., Froidmont, É., 2016. Changes in the isoflavone concentration in red clover (*Trifolium pratense* L.) during ensiling and storage in laboratory-scale silos. *Animal feed Science & Technology*, 217, pp. 36-44.

Abstract

Isoflavones constitute one of the most common categories of nonsteroidal estrogen-like substances belonging to the broad group of phytoestrogens. The highest concentrations in the plant kingdom are found in the *Fabaceae* family. They have become a focus of research because of their estrogenic or anti-estrogenic effect and potential impact on human health. In recent years, several studies have focused on the impact of biotic and abiotic factors and farming management on the isoflavone concentration in plants and their impact on the composition of cow's milk. Nevertheless, knowledge about the effect of the ensiling process on isoflavone concentration remains limited. The objective of this work was to study the evolution of the concentrations of four compounds (daidzein, formononetin, genistein and biochanin A) in red clover (*Trifolium pratense* L.) ensiled at harvest. The impact of the field-drying process was also assessed. The plant material was ensiled in laboratory-scale silos using vacuum-packed plastic bags and stored over a 6-month period. The quality of the silages was checked throughout the experiment by determining the pH, lactic acid concentration, volatile fatty acids, crude proteins, cellulose and other chemical characteristics. The isoflavone concentration in fresh plant material was 2050, 1766, 306 and 127$\mu g.g^{-1}$ DM for formononetin, biochanin A, genistein and daidzein, respectively. After 4 days of drying in the field, no significant change in isoflavone concentration was found, except for daidzein, which increased twofold. The laboratory-scale silos experiment, however, showed a decrease in isoflavone concentration during the first 2 weeks, followed by stabilization over the 5 remaining months. The concentrations fell by 26, 39, 66 and 73% for daidzein, genistein, biochanin A and formononetin, respectively. Animals fed with silage would therefore absorb fewer isoflavones than those fed with fresh plant material or hay.

Keywords:

Isoflavones – Phytoestrogens – Laboratory-scale silos – Silages – Storage – Red clover

Introduction

Isoflavones are secondary plant metabolites and constitute one of the most common categories of phytoestrogens. They are structurally similar to 17β-estradiol, an endogenous steroid (Mostrom & Evans 2012; Vitale *et al.* 2013; Ko 2014). These nonsteroidal compounds have various functions in plants, such as attracting pollinators and seed-dispersing organisms. They also participate in plant defense mechanisms (Mostrom & Evans 2012). Numerous human health benefits have been attributed to isoflavones (Mostrom & Evans 2012; Vitale *et al.* 2013; Ko 2014), but many of them are also considered to be endocrine disruptors, with the potential to cause health problems (Patisaul & Jefferson 2010; Sirotkin & Harrath 2014). These polyphenolic compounds are found mainly in *Fabaceae* (Mostrom & Evans 2012). Among the forage plants of this family, red clover (*Trifolium pratense* L.) is known for its high concentrations of formononetin and biochanin A (Sivesind & Seguin 2005; Tsao *et al.* 2006; Mostrom & Evans 2012), but other isoflavones, as well as their glycosides forms (malonylglycoside and acetylglycoside), are also present (Sivesind & Seguin 2005; Saviranta *et al.* 2010; Mostrom & Evans 2012; Vitale *et al.* 2013). When ingested by ruminants, isoflavones are metabolized mainly by rumen micro-organisms and the main excretion route is through feces and urine, with only a small proportion being excreted in milk (Mostrom & Evans 2012; Kalač 2013; Njåstad *et al.* 2014). Formononetin is demethylated into daidzein and then reduced *via* hydrogenation and ring fission into equol; it can also metabolize into *O*-methyl equol or *O*-desmethylangolensin (Mostrom & Evans 2012; Kalač 2013; Njåstad *et al.* 2014). Equol is considered to be an antioxidant and could be used as a novel therapeutic agent against several diseases and cancers (Setchell & Clerici 2010b; Jackson *et al.* 2011; Mostrom & Evans 2012). The two other microbial metabolites, however, appear to have low estrogenic activity (Setchell & Clerici 2010b; Jackson *et al.* 2011; Mostrom & Evans 2012). Biochanin A is demethylated mainly into genistein and *via* ring cleavage into *para*-ethyl phenol (a compound that has no estrogenic activity) and organic acids (Mostrom & Evans 2012; Kalač 2013; Njåstad *et al.* 2014).

Several studies have been conducted recently on the phytoestrogen concentration in milk and on increasing the metabolites, especially equol (Kalač 2013; Daems *et al.* 2016a). Animal feeding experiments have shown that a diet with a high isoflavone concentration (including daidzein and formononetin) greatly increases milk equol content

(Kalač 2013; Daems *et al.* 2016a). Milk from dairy cows therefore appears to be a potential source of equol in human diets (Kalač 2011; Tsen *et al.* 2014). In order to increase the equol concentration in milk, it is necessary to feed cows with forages enriched with equol precursors (Kalač 2013; Adler *et al.* 2015). The isoflavone concentration in plants is not constant and is affected by many factors, including abiotic factors, diseases, species, genotype, plant part and plants development stage (Sivesind & Seguin 2005; Mostrom & Evans 2012; Kalač 2013). Post-harvest handling and conservation processes (such as silage) can also affect the isoflavone concentration in forages, but there is very little information on this subject. The effect of the ensiling process on isoflavone concentration is not well understood and there are contradictions in the available scientific literature. Sarelli *et al.* (2003) reported that the concentration of daidzein, genistein, formononetin and biochanin A in red clover silage was higher than that of raw materials, whereas Sivesind & Seguin (2005) found that formononetin concentration was lower in silage than in fresh red clover. The impact of the silage process on isoflavones therefore needs to be clarified.

Given that forages, specifically grass silage, constitute an important part of ruminant feeding in temperate regions, particularly when animals are kept indoors during winter (Huyghe *et al.* 2014), the study of isoflavone evolution during the silage process is relevant to the production of equol-enriched milk. Studying isoflavones in life-size silos would be a difficult task. Extended storage areas would be required and this could raise many analytical issues, such as sample homogenization, possible variation in the parameters studied between the different parts of the silage, and variability in grass composition in the grassland (Merry *et al.* 1995; Tsao *et al.* 2006; Kalač 2013). Laboratory-scale silage systems seemed to be a good alternative. It was assumed that laboratory-scale silos would provide a reliable prediction of what was happening in farm-scale silos (Hoedtke & Zeyner 2011; Cazzato *et al.* 2011). Two categories of laboratory-scale silos are commonly used: fixed-volume vessels (Cussen *et al.* 1995; Mustafa & Seguin 2003; Palić *et al.* 2011) and vacuum-packed plastic bags (Sinnaeve *et al.* 1994; Johnson *et al.* 2005; Cherney *et al.* 2006; Hoedtke & Zeyner 2011). Although the ensiling conditions are similar in the two techniques, the second one is increasingly preferred because it is easier to handle and enables the impact of packaging density to be studied and the fermentation gases to be analyzed (Johnson *et al.* 2005; Hoedtke & Zeyner 2011).

The objective of the present work was to study the evolution of four isoflavones (daidzein, formononetin, genistein and biochanin A) in red clover silage over a 6-month period in laboratory-scale silos using a vacuum-packing system. The impact of the field-drying process on isoflavones was also investigated.

Material and methods

The experiment was conducted in autumn 2013 at the Walloon Agricultural Research Centre (CRA-W) in Gembloux, Belgium.

Experimental forage

The red clover (*T. pratense* L.) came from a mixed species grassland sown in spring 2010 after winter barley. The grassland was in the third year of production and was cut three or four times a year. The sown mixture consisted of red clover (*T. pratense* L., 2n, var. Ruttinova), large-leaved white clover (*Trifolium repens* L., var. Alice) and cocksfoot (*Dactylis glomerata,* var. Beluga). The sown density was 24 kg per ha (cocksfoot 18 kg, red clover 3 kg, white clover 3 kg).

For the study, the grassland was mowed in September 2013, in the third cut and after a regrowth of 6 weeks. At this point, the clovers were at the flowering stage. After mowing, fresh forages were sampled from the field and the red clover was manually separated from the other plant species. The red clover sample was homogenized and divided into two parts. The first part was pre-wilted over 4 consecutive days in the field in order to assess the impact of drying on isoflavone concentration. The second part was not wilted, but was used fresh in the laboratory-scale silage experiment.

Laboratory-scale silos

The fresh red clover was chopped with a grass chopper and homogenized. The chopped forage was put manually into plastic bags-in-boxes (5 L capacity) with a metallized film (ref: 017.879.8, Brouwland, Beverlo, Belgium). The bags had been cut at the top to introduce 200 ±20 g of fresh chopped red clover (23.3 ± 1.03% of dry matter 'DM', n = 3). They were then sealed with a vacuum packaging machine (model: UNICA GAS, Lavezzini, Fiorenzuola, Italy), forming 28 micro-silos. They were stocked in a darkened room where the temperature was recorded (average = 18.9 ± 1.57 °C with 15.5

and 23.3 °C as the minimum and maximum values, respectively) throughout the study (6 months).

Sampling and sample processing

There were nine samplings in this study: one on the pre-wilted sample in the field, one on the fresh, chopped and homogenized sample, and seven conducted over the 6-month silage period (i.e., after 2 weeks, and then after 1, 2, 3,4, 5 and 6 months). For each silage sampling, four micro-silos were randomly selected. Their contents were chopped a second time, pooled and homogenized. The homogeneous samples were then put into several opaque plastic bags and stored at -20°C until analysis.

Table 6.1. Statistical performance of NIRS calibrations in the estimation of the chemical composition of forage (CRA-W data)

Constituant	N	mean	SD	SEC	R^2	SECV	RPD
Ash ($g.kg^{-1}$ DM)	3298	101.421	27.01	11.60	0.81	11.84	2.28
CP($g.kg^{-1}$ DM)	2810	149.03	51.32	8.42	0.97	8.69	5.91
CEL($g.kg^{-1}$ DM)	2014	267.37	52.00	14.83	0.92	15.30	3.40
NDF ($g.kg^{-1}$ DM)	1048	480.93	72.15	18.21	0.94	19.48	3.70
ADF ($g.kg^{-1}$ DM)	718	272.23	44.30	9.19	0.96	10.06	4.40
ADL ($g.kg^{-1}$ DM)	678	26.87	11.21	4.11	0.87	4.66	2.40
TSS ($g.kg^{-1}$ DM)	707	123.72	78.79	10.29	0.98	10.97	7.18
DMDcel	159	0.677	0.115	0.022	0.96	0.028	4.16

N: number of samples in the NIR database; SD: standard deviation in the reference database; SEC: standard error of calibration; R^2: coefficient of determination of NIR calibration; SECV: standard error of cross validation; CP: crude protein; CEL: cellulose (AFNOR, 1993); DMDcel: cellulase enzyme dry matter digestibility (Aufrère, 1982); NDF: neutral detergent fibre; ADF: acid detergent fibre; ADL: acid detergent fibre (AFNOR, 1997); TSS: total soluble sugar; RPD ratio: SD/SECV (Williams, 2001).

Chemical analyses

Micro-silage characterization

Silage quality was evaluated by determining the pH, lactic acid concentration and volatile fatty acid (VFA, including acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids) profile of the water phase (100 $g.L^{-1}$). The first parameter was estimated on the non-filtered solution by simply using a conventional glass pH electrode that had been previously calibrated. Lactic acid and VFA were measured using high performance liquid chromatography with ultraviolet detector (HPLC-UV). Briefly, the solutions were filtered through 22 µm filters and 25 µL was injected into an HPLC-UV (Waters, Zellik,

Belgium) equipped with an Aminex HPX-87H column (300 x 7.8 mm, 9 µm particle size) from Biorad (Hercules, USA). The column was maintained at 60 °C. The mobile phase was 0.01 N sulphuric acid from Chemlab (Zedelgem, Belgium). The isocratic elution was operated at a flow rate of 0.6 $mL.min^{-1}$. The eluting compounds were detected at 210 nm. An external calibration was done at concentrations ranging from 0.05 to 1 $mg.mL^{-1}$ ($R^2 \geq 0.99$) for all the targeted compounds. The results were expressed as $mg.g^{-1}$ DM.

The DM of the fresh forage, the pre-wilted forage and the ensiled forage was determined at 103 ± 2 °C until a constant weight was achieved (Reynaud *et al.* 2010). For the determination of the chemical composition, the samples were ground in a Cyclotec mill (1 mm screen) (FOSS Electric, Hillerad, Denmark). They were then placed in a quarter cup for near-infrared spectroscopy (NIRS) analyses (XDS-system spectrometer, FOSS Electric, Hillerad, Denmark) and the absorption data recorded as log 1/R from 1100 to 2498 nm every 2nm (WINISI 1.5, FOSS Tecator Infrasoft International LCC, Hillerad, Denmark). The chemical composition and cellulase enzyme DM digestibility (DMDcel) were then estimated using NIRS calibrations developed at CRA-W (Table 6.1). The performances of the NIRS calibrations were evaluated using statistical parameters such as R^2, standard error of cross validation (SECV) and the ratio of performance to deviation (RPD) (ratio of SECV to the standard deviation (SD) of the reference data, SD). Following the recommendations of Williams (2001) that: R^2 should be higher than 0.80 and the RPD ratio higher than 3, the NIRS calibrations were adequate for estimating the chemical composition and *in vitro* digestibility (DMDcel) of forage samples.

Isoflavone quantification

The isoflavone concentration was determined following the method optimized and validated by Daems *et al.* (2016b). Briefly, for each sampling, three chopped samples stored at -20 °C were freeze-dried at 800 mbar and 0°C for 48 h with a lyophilizator Martin Christ Delta 1 -24 LSC plus (Analis, Suarlée, Belgium). About 500 mg of each freeze-dried sample were mixed with 25 mL of H_2O:methanol (45:55, v/v) and extracted over l0 min using an ultrasonic bath set at 80 °C. The extract was then centrifuged (5 min at 3200g) and 1 mL of the supernatant was evaporated to dryness. The dry residues were solubi-lized with 1 mL of sodium acetate buffer (pH 6) and 2 mL of enzymatic solution. The solution was a mixture of β-glucosidase (from almonds, ≥6$U.mg^{-1}$), β-glucuronidase

(type H-2 from *Helix pomatia,* ≥85,000 units.mL^{-1}) and cellulase (from *Aspergillus niger,* ≥0.3 units.mg^{-1}) from Sigma-Aldrich (Diegem, Belgium). These enzymes were dissolved in 0.2 M sodium acetate buffer at pH = 6 to reach an enzymatic solution with ≥6, ≥12 and ≥5100 units.mL^{-1} of cellulase, β-glucosidase and β-glucuronidase, respectively. The hydrolysis was conducted overnight at room temperature and the mixture was centrifuged a second time. Supernatant was diluted with several H_2O:methanol (40:60, v/v) solutions, depending on the suspected isoflavone concentration, and 0.5 mL of each diluted extract was evaporated to dryness. The dry residues were then reconstituted in 1 mL of H_2O:methanol (40:60, v/v) with two internal standards (daidzein-d4 and flavone were set at 50 and 20 ng.mL^{-1}, respectively). The reconstituted solution was filtered (0.2 µm) and 10 µL was injected into an UPLC®-MS/MS (Waters, Zellik, Belgium) with an electrospray ionization operated in positive ionization mode and where the quantification was performed using multiple reaction monitoring. An external calibration was done at concentrations ranging from 5 to 125 ng.mL^{-1} ($R^2 \geq 0.99$) for the four targeted isoflavones. The limit of quantification (LOQ) of the method was 1.6 µg.g^{-1} DM for the targeted compounds. Due to the enzymatic hydrolysis step, the isoflavone concentration determined in this study represented the total concentration of the four targeted compounds, including the conjugated and aglycone forms.

The formononetin, biochanin A, genistein and flavone 'internal standard' were from Sigma-Aldrich (Diegem, Belgium). Daidzein was acquired from Cayman Europe (Tallinn, Estonia). Daidzein-d_4 was used as an internal standard and was purchased from C/D/N ISOTOPES (Pointe-Claire, Canada). The external calibration was done with the targeted compounds pooled in the same mix solution prepared in H_2O:methanol (40:60, v/v). Methanol (LC-MS reagent) was obtained from J.T. Baker (Devender, Netherlands). The dilution of organic solvents and the preparation of sodium acetate buffer solutions were carried out with deionized water that had been prepared using a Milli-Q® system (Millipore, Overijse, Belgium).

Statistical analysis

All the laboratory analyses were performed in triplicate, except the chemical characteristics estimated using NIRS calibrations. Statistical analyses and graphics were conducted using Microsoft® Excel software (Microsoft®, Diegem, Belgium) and the JMP® 7 statistical software package (SAS Institute, Cary, US). Analysis of variance

(ANOVA; α = 0.05) and the comparison of averages by Tukey's HSD (α = 0.05) test were used to compare the isoflavone concentration in the silage samples.

Table 6.2. Dry matter content (%) and chemical characteristics (g.kg^{-1} DM) of fresh, pre-wilted and ensiled red clover (NIRS analyses)

Red clover forage	DM	CP	CEL	Ash	NDF	ADF	ADL	TSS	DMDcel
Pre-wilted	30.7 ± 2.3	154.8	242.8	101.1	353.4	268.2	5.09	91.1	0.598
Fresh Silages	23.3 ± 1.0	154.2	240.4	100.7	364.9	260.3	51.6	88.1	0.599
2 wk.	18.1 ± 0.5	160.7	239.1	120.5	343.8	269.8	57.8	0	0.577
1 mo.	20.1 ± 0.5	164.7	226.4	126.1	316.9	261.3	56.4	0	0.597
2 mo.	19.9 ± 1.4	165.6	234.1	126.9	323.4	264.7	56.1	0	0.588
3 mo.	19.7 ± 1.2	163.7	253.0	124.4	345.5	280.1	55.8	0	0.567
4 mo.	17.7 ± 0.3	163.3	236.0	129.3	319.4	268.1	56.0	0	0.588
5 mo.	18.0 ± 0.6	162.0	243.2	127.7	327.5	273.1	56.0	0	0.577
6 mo.	18.1 ± 0.3	161.1	254.0	125.9	342.6	282.6	55.9	0	0.564

DM: dry matter: CP: crude protein: CEL: cellulose (AFNOR, 1993): DMDcel: cellulase enzyme dry matter digestibility (Aufrère, 1982): NDF: neutral detergent fibre: ADF: acid detergent fibre: ADL: acid detergent fibre (AFNOR, 1997): TSS: total soluble sugar.

Results

Red clover forage characteristics

The chemical characteristics of fresh and pre-wilted red clover forages are given in Table 6.2. The DM content was 23.3 and 30.7% for the fresh and pre-wilted forage, respectively. Apart from DM content, the chemical composition of fresh and pre-wilted forages appeared to be fairly similar. Crude protein (CP) yield was high, with more than 150 g.kg^{-1} DM, and the cellulose (CEL) was 240 g.kg^{-1} DM on average. Forage *in vitro* digestibility (DMDcel) was less than 0.6. Total soluble sugar (TSS) content was lower than 100 g.kg^{-1} DM.

Red clover silage chemical and fermentation characteristics (laboratory micro-silos)

The chemical characteristics of the red clover silages are given in Table 6.2. With the exception of TSS, the differences in chemical composition between fresh and ensiled red clover appeared to be small. Compared with the fresh material and after 6 months of storage as silage, the CP content was slightly higher (154 and 161 g.kg^{-1} DM for fresh and ensiled forages, respectively) and the fiber content also increased (240 and 254 g.kg^{-1} DM for fresh and ensiled forages, respectively). The DMDcel tended to decrease (from 0.60 to 0.56) after the 6-month storage period.

Table 6.3. Fermentation parameters of red clover silage over a 6-month period (mean ± sd)

Red clover forage	pH	Lactic acid (mg.g^{-1} DM)	Volatile fatty acids (mg.g^{-1} DM)		
			Acetic acid	Propionic acid	Butyric acid
Fresh	5.5 ± 0.1	0.0 ±0.0	0.8 ± 0.1	0.9 ± 0.1	0.0 ± 0.0
Silages					
2 wk.	4.5 ± 0.0	27.4 ± 1.9	9.7 ± 0.9	1.8 ± 0.2	0.0 ± 0.0
1 mo.	4.3 ± 0.1	33.6 ± 3.5	10.8 ± 1.2	1.9 ± 0.4	0.0 ± 0.0
2 mo.	4.5 ± 0.0	24.5 ± 0.4	8.7 ± 0.6	2.4 ± 0.1	4.5 ± 0.1
3 mo.	4.7 ± 0.0	26.3 ± 3.3	7.9 ± 1.2	2.8 ± 0.7	7.2 ± 0.6
4 mo.	4.6 ± 0.0	37.6 ± 3.6	12.1 ± 1.3	4.6 ± 0.7	9.0 ± 1.2
5 mo.	4.5 ± 0.0	32.5 ± 2.7	11.8 ± 0.8	6.3 ± 0.5	10.3 ± 0.9
6 mo.	4.6 ± 0.0	21.7 ± 0.9	8.5 ± 1.6	4.9 ± 0.7	10.3 ± 0.6

The pH, lactic acid content and VFA profiles of the silage during the experimental period are shown in Table 6.3. The pH value decreased rapidly, reaching 4.5 after 2 weeks and 4.3 after 1 month. After 2 months of storage, the pH value increased and remained stable for the last 4 months. The lactic acid content was low, with a mean value of 29 mg.g^{-1} DM. Acetic acid was present, but in low quantities (9.94 mg.g^{-1} DM, on average). There was no butyric acid in the silage in the first 2 months of storage, but it was present (9.2 mg.g^{-1} DM, on average) from 3 to 6 months, with a tendency to increase. Little propionic acid was detected (3.53 mg.g^{-1} DM), but, like butyric acid, it tended to increase depending on the length of storage. No iso-butyric, iso-valeric and valeric acids were detected at concentrations higher than the LOQ.

Isoflavones

The isoflavone concentrations found in fresh red clover forage, pre-wilted red clover and red clover silage are presented in Table 6.4. There was no difference among the isoflavones found in pre-wilted (30.7% DM) and fresh (23.3% DM) red clover samples, except for daidzein. The concentrations in fresh forage were 2050 ± 206, 1766 ± 349 and 306 ± 38 µg.g^{-1} DM for for-mononetin, biochanin A and genistein, respectively. Daidzein was less evident than the other three targeted isoflavones. The daidzein concentration in pre-wilted red clover (260 ± 3 µg.g^{-1} DM) was twice as high as that in fresh red clover (127 ± 17 µg.g^{-1} DM). The concentrations of all the targeted isoflavones fell drastically after only 2 weeks of storage in the micro-silos. This decrease was particularly marked for the two main isoflavones in red clover: a loss of 73% and 66% for formononetin and biochanin A, respectively. Formononetin concentration, for example, fell from 2050 to 544 µg.g^{-1} DM whereas the concentrations of daidzein and genistein

Table 6.4. Effect of pre-wilting and ensiling on the isoflavone concentration ($\mu g.g^{-1}$ DM) of red clover forage

Compound	Field-dried	Silages								Anova
		Fresh	2 wk.	1 mo.	2 mo.	3 mo.	4 mo.	5 mo.	6 mo.	*P*-value
Formononetin	1906.3 ± 96.1^{a}	2050.8 ± 205.7^{a}	544.3 ± 97.9^{b}	526.3 ± 71.7^{b}	465.5 ± 81.9^{b}	574.7 ± 116.3^{b}	583.4 ± 64.6^{b}	602.2 ± 81.4^{b}	576.8 ± 51.7^{b}	<0.0001*
Biochanin A	1600.0 ± 168.3^{a}	1766.4 ± 348.9^{a}	599.9 ± 176.7^{b}	600.8 ± 91.8^{b}	508.7 ± 160.1^{b}	628.6 ± 95.1^{b}	687.0 ± 128.8^{b}	617.7 ± 60.8^{b}	668.2 ± 27.7^{b}	<0.0001*
Genistein	340.7 ± 24.0^{a}	306.4 ± 38.2^{a}	185.5 ± 30.5^{b}	217.3 ± 18.5^{b}	191.5 ± 22.3^{b}	217.4 ± 34.2^{b}	217.9 ± 13.9^{b}	196.2 ± 3.9^{b}	200.5 ± 15.0^{b}	<0.0001*
Daidzein	260.2 ± 3.3^{a}	127.0 ± 17.1^{b}	94.2 ± 4.6^{c}	100.6 ± 11.3^{c}	92.1 ± 6.3^{c}	91.1 ± 2.9^{c}	100.3 ± 9.9^{c}	90.4 ± 11.9^{c}	101.0 ± 1.4^{c}	<0.0001*
Total	4107.2 ± 274.0^{a}	4250.7 ± 578.5^{a}	1423.9 ± 251.2^{b}	1445.1 ± 47.0^{b}	1257.9 ± 265.4^{b}	1511.8 ± 89.1^{b}	1588.6 ± 204.1^{b}	1506.5 ± 152.0^{b}	1546.5 ± 66.6^{b}	<0.0001*

Each analysis was done in triplicate and the results given as mean ± sd. Values marked by the same letter are not significantly different, using the Tukey's HSD test (p-value > 0.05).

diminished by 26% and 39%, respectively. After 2 weeks of storage in micro-silos, however, the isoflavone concentration was stable over the remainder of the storage period, with no significant difference in concentration between 2 weeks and 6 months of storage.

Discussion

Red clover (*T. pratense* L.) was selected for this study because it contains naturally high concentrations of formononetin and biochanin A (Sivesind & Seguin 2005; Tsao *et al.* 2006; Mostrom & Evans 2012), with daidzein and genistein in lower concentrations (Mostrom & Evans 2012; Butkutė *et al.* 2014). The four targeted isoflavones were therefore present in a single plant sample, facilitating the preparation of micro-silos because it was not necessary to mix several plant species in order to obtain the four compounds of interest that we wished to follow in our study. Conserving red clover by ensiling is not easy because of the low water soluble carbohydrate content of *Fabaceae* and the high buffering capacity (King *et al.* 2012). Our results showed that the TSS content of the fresh forage (88.1 $g.kg^{-1}$ DM) was similar to that reported by King *et al.* (2012). The fermentation parameters of red clover silage after a 1-month period of storage, however, indicated a good conservation process. Silage must have a sufficiently low pH in order to enable secondary fermentation to be inhibited and butyric acid to be produced. A pH of 4.20-4.35 is recommended for forages containing between 20 and 25% of DM (Pahlow *et al.* 2003). The lowest pH value in our study was 4.3, which was sufficient for anaerobically stable silage (Pahlow *et al.* 2003). From 2 to 6 months storage, the pH value increased and butyric acid was produced. The occurrence of butyric acid was probably due to Clostridium fermentation. As reported by Pahlow *et al.* (2003), Clostridium fermentation induces a pH increase and the production of butyric acid in silage.

In line with the work reported by King *et al.* (2012), ensiling led to an increase in the CP, cellulose and acid detergent fiber (ADF) content and a decrease in forage digestibility, with digestibility and cellulose being negatively correlated. The increase in ADF and the decrease in neutral detergent fiber (NDF) reflected the decrease in the hemicellulose fraction (NDF-ADF), which could be used as substrate for lactic fermentation (Pahlow *et al.* 2003). As a result of these achievements, and despite the use

of a matrix difficult to ensile, the vacuum-packed plastic bags seem to be appropriate for studying the ensiling process of forage at the laboratory scale.

The concentrations of isoflavone found in the fresh red clover (*T. pratense* L, 2n var. Ruttinova) used in this study are in line with previous studies, where the formononetin and biochanin A concentrations were high, the genistein concentration intermediate and the daidzein concentration comparatively low (Sarelli *et al.* 2003; Sivesind & Seguin 2005; Tsao *et al.* 2006; Saviranta *et al.* 2008; Zgórka 2009; Butkutė *et al.* 2014). In our case, the formononetin and biochanin A concentrations represented 90% of the total concentration of the four isoflavones found. According to Tsao *et al.* (2006), the concentration of formononetin and biochanin A in different parts of 13 red clover cultivars harvested at different growing stages also represented at least 90% of the total amount of the four isoflavones found. Usually, the concentrations of formononetin and biochanin A are higher than 1 $mg.g^{-1}$ DM and the concentrations of genistein and daidzein range from few dozen to hundreds of $\mu g.g^{-1}$ DM. It is, however, difficult to compare the concentrations reported in the different studies because isoflavones present in plants can be affected by many factors (Tsao *et al.* 2006; Reynaud *et al.* 2010; Saviranta *et al.* 2010; Mostrom & Evans 2012; Kalač 2013). Nevertheless, the percentage between the concentrations of formononetin-biochanin A and genistein-daidzein have also been reported in silage containing different ratios of red clover (Steinshamn *et al.* 2008; Mustonen *et al.* 2009; Höjer *et al.* 2012; Njåstad *et al.* 2014).

So far as we know, changes in isoflavone concentration during the ensiling process have not really been studied to date. Only three studies have touched on this subject (Sarelli *et al.* 2003; Sakakibara *et al.* 2004; Sivesind & Seguin 2005), but in each of them, the authors compared the concentration found in fresh plant material and after a fixed period of storage (maximum 4 months). No-one has studied the kinetic evolution of isoflavones over 6 months of ensiling. Our results showed, first, that wilting from 23 to 30% of DM leads to a slight decrease in the two major isoflavones and a slight increase in the genistein and daidzein concentrations. This was probably due to the demethylation of biochanin A and formononetin, which are transformed into genistein and daidzein, respectively (Mostrom & Evans 2012). Nevertheless, during this wilting period, no significant impact on the isoflavone concentration was statistically proven, except for daidzein, where the concentration doubled. The low amount of demethylated formononetin could have led to a significant increase in daidzein because its

concentration was low in the fresh matrix. These findings accord with those reported by Jones (1979), which showed that wilting three varieties of red clover under field conditions led to no significant difference in the formononetin concentration. This observation related, however, to a wilting period of only 8 h. Nevertheless, our results are not in accord with findings reported by Sarelli *et al.* (2003), Sakakibara *et al.* (2004) and Sivesind & Seguin (2005), who suggested that the wilting process reduced the isoflavone concentrations in red clover, in some case significantly, in other less so, depending on the outdoor conditions.

Unlike wilting in the field, there was a sharp decrease in the concentration of each isoflavone during the ensiling process. The decrease was less so for daidzein and genistein than for formononetin and biochanin A, which could be linked again to the demethylation of these two last compounds, compensating for some of the loss of daidzein and genistein. In the digestive system of ruminants, daidzein is metabolized into equol and *O*-desmethylangolensin by bacteria (Mostrom & Evans 2012; Kalač 2013). This metabolization pathway could therefore be a plausible explanation for the loss of daidzein. In our micro-silos, however, no trace of equol was detected. Nevertheless, the degradation of these four isoflavones during storage was most probably linked to the microbial activity inside the micro-silos. The evolution in isoflavone concentration and pH during the storage period followed the same trend. At the start of fermentation process, the conditions were probably conductive to some bacteria metabolizing isoflavones in other microbial products. When the pH fell slightly, the bacteria would be inactivated. This assumption requires further investigation.

The present results differ from those reported by Sarelli *et al.* (2003) and Sakakibara *et al.* (2004). In the first case, the authors found that isoflavone concentration was greater in ensiled red clover (3-4 months) than in wilted herbage before ensiling; in the second case, the authors found that this concentration remained stable for the 2 first months of the ensiling process. In contrast to these two studies, Sivesind & Seguin (2005) reported that the total isoflavone (formononetin and biochanin A) concentration was higher in fresh plants than in silage (mainly because of the decrease in formononetin). This finding was confirmed in our study. The difference between the studies could be due to differences in the ensiling process, such as differences in microbial population, pH, temperature, silage additives, plant phenological stage and botanical composition. Few of these factors were measured in the earlier studies. Even if the decrease in isoflavones

during ensiling seemed to be linked to the wilting process (Kalač 2013), our results did not confirm this assumption. No neoformation process of isoflavones has been observed (Sakakibara *et al.* 2004). The decrease in isoflavone concentration during fermentation in our study seemed to be linked to the fermentation process that occurred during the initial days of ensiling. On the basis of our results, feeding ruminants with silage, rather than fresh red clover, could reduce their isoflavone intake. Consequently, the fermentation process could contribute to reducing the estrogenicity of red clover, but the production of microbial metabolites such as equol in milk (potentially beneficial for health) could therefore also be reduced.

This initial exploratory study fills some of the gaps in the information on isoflavone concentrations change during ensiling, highlighted by Kalač (2013). It opens the door to future studies and to improving the understanding of isoflavone transformation in silage. The trends that emerged in our study need to be confirmed using silage and silos that are much closer to reality in the field (i.e., using a mixture of plant species, not only red clover). Other potential metabolites of isoflavones and silage parameters, such as microbial populations, need to be taken into account in order to explain the isoflavone transformations.

Conclusion

Despite the use of a matrix that was difficult to ensile, the silage pH and fermentation products levels observed in silages suggest that the vacuum-packed plastic bags technique correctly simulates the ensiling process at a laboratory scale. This technique was used for the first time to investigate changes in isoflavone concentration during the ensiling process. The results suggest that the field-drying process has no impact on isoflavone concentration, apart from daidzein, whose concentration is significantly increased by the drying process. They also clearly show that there is a significant decrease in isoflavone concentration during the first 2 weeks and that thereafter this concentration remains stable for the rest of the storage period. Therefore, animals fed red clover containing silage would absorb fewer amounts of isoflavones than those fed fresh plant material of the same origin. This initial exploratory study opens the door to future studies aimed at improving our understanding of the mechanisms involved in isoflavone transformation in silage.

Conflict of interest

Frédéric Daems, Virginie Decruyenaere, Richard Agneessens, Georges Lognay, Jean-Michel Romnee and Éric Froidmont declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Chapter VII

Optimization and validation of an analytical method for quantifying isoflavones and equol in digestive fluids and feces

Objectives

Studies on the impact of an isoflavone-rich feed on the quality of milk involve generally analytical methods developed mainly for quantifying these compounds in feed and milk samples. For other biological matrices relating to the isoflavone metabolism in cows, however, there are fewer analytical methods in the literature, especially for digestives fluids and feces. This chapter addresses the last two objectives of this thesis by presenting an analytical method that can simultaneously quantify four selected isoflavones and equol in rumen and duodenal fluids and in feces. The method was developed with the same response surface methodology used previously in the analytical method for analyzing isoflavones in forages. It is also based on compounds released by methanolic ultrasound-assisted extraction, followed by enzymatic hydrolysis and UPLC®-MS/MS analysis. In order to ensure the reliability of the results, a complete validation was performed following international recommendations (EMA 2015). An overview of the chapter is presented in Fig. 7.1.

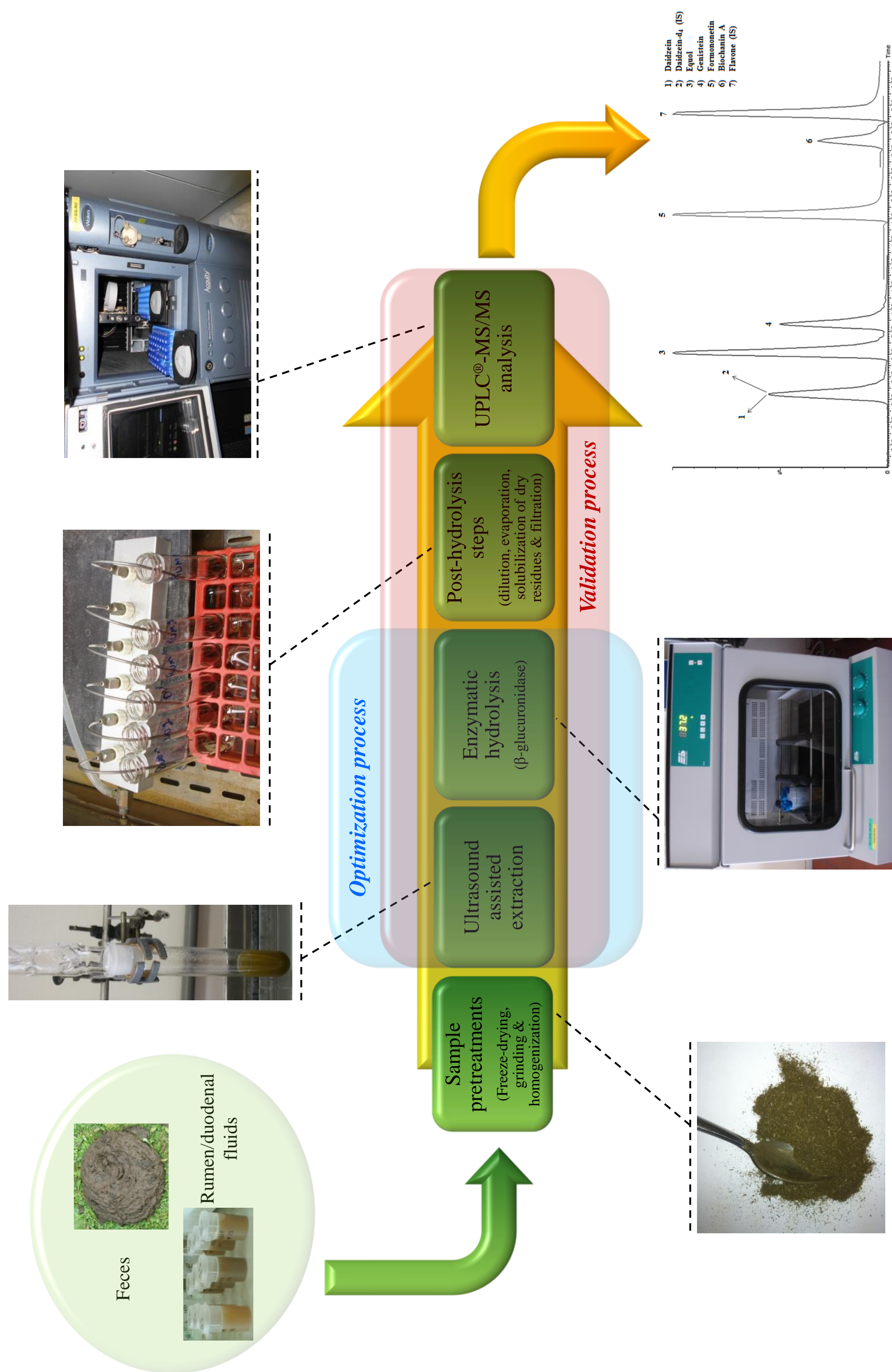

Figure 7.1. Graphical abstract of *Chapter VII*. Analytical procedure developed and validated for quantifying isoflavones and equol in cows' feces and rumen and duodenal fluids.

Article (in prep)

Optimized Quantitative Method for Determining Isoflavones and Equol in Bovine Digestive Fluids and Feces

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Abstract

Many studies have been conducted on the impact of animal feed on isoflavones and their metabolite concentrations in bovine milk, but few studies have focused on the development and validation of analytical protocols for quantifying these compounds in biological matrices other than milk and plants. The purpose of this study was to develop a method that would enable four isoflavones and equol in cows' feces and digestive fluids to be quantified simultaneously. The method is based on aglycones released by methanolic ultrasound-assisted extraction, followed by enzymatic hydrolysis and ultra-performance liquid chromatography tandem mass-spectrometry analysis. The sample preparation was optimized using the Box-Behnken design. The selected extraction conditions were 80 °C, 10 min and 50% methanol for digestive juices and 70 °C, 35 min and 60% methanol for feces. For hydrolysis, the selected conditions were 37°C, 1 h and a pH of 6 for both matrices. The analytical method showed a good linear regression model ranging from 5 to 125 ng.mL^{-1}. Both inter- and intra-day accuracy (≤ 8.5 and ≤ 12.3 %) and precision (≤ 11.1 and ≤ 15.2 %) were good. No matrix effect was found. There was good repeatability and extract stability for at least 4 days of storage at -20 and 6°C. All recoveries were in the acceptable range of 70-120% for both matrices, except for biochanin A in feces, where the rate was about 43%. This sensitive and reliable method will be useful for monitoring the passage of isoflavones and equol in the digestive system of ruminants.

Keywords

Isoflavones – Equol - UPLC®-MS/MS – Validation - Digestive fluids – Feces

Introduction

Isoflavones are a subgroup of phytoestrogens that have attracted widespread interest because of their association with a range of effects on human health. These natural plant substances are chemically similar to 17-β-estradiol, leading to the possibility of binding to estrogen receptors. They can also interact with the metabolism of steroid hormones (Pilsková *et al.* 2010; Mostrom and Evans 2012; Baber 2013; Vitale *et al.* 2013; Ko 2014; Sirotkin and Harrath 2014; Nemitz *et al.* 2016). The highest content is found under conjugated forms in plants of the Fabaceae family, such as clover (*Trifolium pratense* L.) and soybean (*Glycine max* L.) (Mostrom & Evans 2012; Ko 2014). When they are ingested by humans or animals, the conjugated forms are hydrolyzed before being absorbed by the body or metabolized by bacteria in digestive system first, and then absorbed (Mostrom & Evans 2012; Kalač 2013; Vitale *et al.* 2013; Ko 2014). Among the isoflavone microbial metabolites, equol is probably the most widely studied because it seems to have numerous health benefits (Setchell & Clerici 2010; Jackson *et al.* 2011; Mostrom & Evans 2012; Sánchez-Calvo *et al.* 2013). For example, it has an antioxidant capacity, improves the symptoms associated with menopause and is chemopreventive in prostate cancer (Setchell & Clerici 2010; Sánchez-Calvo *et al.* 2013). In the human population, however, not everyone would be able to benefit from its positive effects because of the lack of specific bacteria in their digestive systems. In Western populations, only one third of people are thought to be equol producers (Setchell & Clerici 2010; Jackson *et al.* 2011; Sánchez-Calvo *et al.* 2013; Legette *et al.* 2014). An alternative strategy for making equol's health benefits available to everyone would be the supply of this isoflavan in the human diet via food of animal origin. Among the foods commonly consumed, milk from dairy cows is an interesting potential source of equol (Křížová *et al.* 2011; Tsen *et al.* 2014). In recent years, a number of studies have focused on the impact of a high-isoflavone diet on the quality of cow's milk, especially on its enrichment with equol (Kalač 2013; Daems *et al.* 2016a). In order to conduct this type of research, it is necessary to have reliable analytical methods.

Many analytical methods for quantifying isoflavones in plants have been proposed in scientific literature (Wang 2002; Wu *et al.* 2004; Grynkiewicz *et al.* 2005; Stalikas 2007; Vacek *et al.* 2008; Valls *et al.* 2009; Rostagno *et al.* 2009; Ko 2014; Raju *et al.* 2015). An increasing number of analytical methods have been described recently for quantifying isoflavones and equol in milk (Daems *et al.* 2016a). For other biological

matrices aimed at improving the understanding of isoflavone metabolism in cows, however, fewer analytical methods have been proposed, especially with regard to digestives fluids and feces. So far as we know, in recent years only two studies have focused on analytical methods for the quantification of isoflavones in these two matrices (Njåstad *et al.* 2014; Kasparovska *et al.* 2016a). Kasparovska *et al.* (2016a) proposed a liquid-liquid extraction with organic solvents, followed by analysis using liquid chromatography (LC) and mass spectrometry with time of flight (MS-TOF) detection. Njåstad *et al.* (2014) proposed an enzymatic hydrolysis after the solid-liquid extraction step because they focused only on equol and aglycone forms of isoflavones. They also used liquid chromatography, but coupled with a tandem mass spectrometry detector (LC-MS/MS). Prior to that, Dickinson *et al.* (1988) had proposed high performance liquid chromatography (HPLC) with a UV-visible detector for quantifying two isoflavones in rumen fluid. Wang *et al.* (1994) recommended a radioimmunoassay (RIA) for quantifying formononetin in the same matrix. These two analytical techniques are not often used for detecting and quantifying isoflavones and their metabolites. Absorbance detection methods have low sensitivity and specificity (Ko 2014). With regards to immunoassay methods, they need a long time to generate key reagents, a full characterization of anti-analyte antibody specificity and are used for a single analyte determination (Ko 2014). Generally, for biological matrices (other than plants), acceptable performances were achieved using methods with solid phase or solvent extraction, followed by separation and detection with LC-MS (Ko 2014; Raju *et al.* 2015). The LC-MS approach is now a fast-moving field that requires relatively simple sample pretreatment, achieves high sensitivity and selectivity and reduces instrumental analysis time (Ko 2014; Daems *et al.* 2016a).

The aim of this study was to develop and validate a reliable and sensitive analytical method that enabled daidzein, formononetin, genistein, biochanin A and equol in cows' feces and rumen and duodenal fluids to be quantified simultaneously. Sample preparation was independently optimized for each matrix using response surface methodology.

Materials and method

Chemicals and reagents

Methanol (an LC-MS reagent) for LC-MS/MS analysis and methanol ('MeOH'; HPLC grade) for extraction were obtained from J.T. Baker (Devender, Netherlands). Formic acid (ULC-MS grade, 99%) was obtained from Biosolve (Valkenswaard, Netherlands). Deionized water was prepared using a Milli-Q® system (Millipore, Overijse, Belgium).

Equol (EQ; CAS number: 94105-90-5), formononetin (FO; 485-72-3), biochanin A (BA; 491-80-5), genistein (GE; 446-72-0) and flavone (IS_{FL}; 525-82-6) were bought from Sigma-Aldrich (Diegem, Belgium). Daidzein (DA; 486-66-8) was acquired from Cayman Europe (Tallinn, Estonia). Daidzein-d4 ($IS_{DA\text{-}d4}$; 1219803-57-2) was purchased from C/D/N ISOTOPES (Pointe-Claire, Canada). Individual molecule stock solutions (100 $\mu g.mL^{-1}$) were prepared in methanol and stored at -20°C.

Cellulase (from *Aspergillus niger*, ≥ 0.3 $units.mg^{-1}$, 9012-54-8), *β*-glucosidase (from almonds, ≥ $6U.mg^{-1}$, 9001-22-3) and *β*-glucuronidase (type H-2 from *Helix pomatia*, ≥ 85,000 $units.mL^{-1}$, 9001-45-0) were obtained from Sigma-Aldrich (Diegem, Belgium). Sodium acetate (127-09-3) was obtained from Merck KGaA (Darmstadt, Germany) and used to prepare the enzymatic solution. For optimization of the hydrolysis step in the experimental design, the enzymes were dissolved in three sodium acetate buffer solutions (0.2 M) with a pH set at 4.0, 5.0 and 6.0.

Samples

Rumen and duodenal fluids, as well as feces, came from an experiment (approval number: 1218) involving cannulated Holstein dry cows conducted at the Walloon Agricultural Research Centre in Gembloux, Belgium. Feces were collected on the floor of the booth during the experiment. Digestive fluids, however, were collected by the cannula with a pump. Samples used for the development and validation of the proposed method came from two sampling periods using the same cow. The first sampling was taken when the cow was being given feed containing ≥ 87% dehydrated red clover (samples with a suspected high isoflavone concentration) and the second sampling was taken 1 month later when the cow was being given feed without red clover (samples with a suspected low isoflavone concentration). The samples were freeze-dried at 850 mbar and 0°C (at the

tray level) for at least 72 h with a Martin Christ Gamma 1-20 (Analis, Suarlée, Belgium) freeze-dryer and then finally ground to powder. The powder was put into several vacuum opaque plastic bags and stored at -20°C until analysis. For optimization of the sample preparation with response surface methodology, samples (with a suspected high isoflavone concentration) of the rumen and duodenal fluids were mixed together (50/50) to form a single homogeneous sample of digestive fluids. For the validation process and the estimation of extraction efficiency, these two types of digestive fluids were not pooled, except for stability and process efficiency.

Analytical procedure

The analytical procedure used by Daems *et al.* (2016b) for quantifying isoflavones in forages was optimized in order to determine these same compounds and EQ in digestive fluids and feces. An overview of the sample preparation optimization and validation process is shown in Fig. 7.1. Briefly, 500 ± 5 mg of freeze-dried samples were initially mixed with 25 mL of extraction solvent and the compounds of interest were extracted using ultrasound-assisted extraction (UAE), with 1 mL of extract being evaporated to dryness and the residues then being submitted to enzymatic hydrolysis. Depending on the suspected compound concentrations, the mixtures were either not diluted or diluted by a factor 50 and 10 with H_2O:methanol (40:60, v/v). They were then evaporated a second time, reconstituted with H_2O:methanol (40:60, v/v), IS_{FL} at 20 $ng.mL^{-1}$ and $IS_{DA\text{-}d4}$ at 50 $ng.mL^{-1}$, filtered and subjected to UPLC®-MS/MS analysis (Waters, Zellik, Belgium). The LC system was equipped with a Waters ACQUITY UPLC® HSS T3 column (2.1 × 100 mm, 1.8 µm particle size). The targeted compounds were detected using a Waters Quattro Premier XE (Micromass® Waters, Zellik, Belgium) with an electrospray ionization interface operating in positive ionization mode (ESI^+). All the data were collected and processed using MassLynx® software with a Quanlynx® program (Micromass® Waters, Zellik, Belgium).

Experimental design

The conditions for UAE and enzymatic hydrolysis for the digestive juices and feces were optimized using Box-Behnken experimental designs that involved three factors and three levels per factor. The levels chosen for each factor in this study were based on the single-factor experiments and conditions described in the scientific literature. The complete design consisted of 16 assays, including four replicates of the

center point (Tables 7.1 and 7.2). The experimental data were analyzed by quadratic stepwise regression to fit the second-order equation, which was then used for UAE and hydrolysis optimization.

Enzymatic hydrolysis

Before starting the optimization with response surface methodology, three one-variable-at-a-time assays were conducted on the digestive fluids and feces. The impact of the three most widely used enzymes for hydrolyzing conjugated forms of isoflavones in biological matrices, their concentrations and the hydrolysis duration were investigated. Initially, for evaluating the impact of each enzyme, the best conditions reported by Daems *et al.* (2016b) were used.

The enzymatic hydrolysis step was then optimized using a Box-Behnken design. The three factors and levels selected were: hydrolysis temperature (X_1; 20, 40 and 60°C); hydrolysis time (X_2; 30, 120 and 210 min); and pH (X_3; 4, 5 and 6). The 16 combinations are presented in Table 7.1. After the statistical treatments, the best hydrolysis conditions were validated by performing seven replicates.

Table 7.1. Box-Behnken design and the response for isoflavone yield hydrolyzed from the digestive fluids extract

Assay	X_1 (°C)	X_2 (min)	X_3 (U pH)	Isoflavone yield (µg.g^{-1} DM)					
				Observed values					
				Total	EQ	FO	DA	BA	GE
1	60	120	4	*628,7*	580,9	26,3	9,9	7,3	4,2
2	60	120	6	*824,1*	769,3	29,3	11,8	9,0	4,7
3	60	30	5	*772,4*	723,5	26,4	11,5	6,8	4,1
4	20	120	6	*823,8*	767,3	30,6	11,7	9,6	4,6
5	60	210	5	*713,5*	672,5	22,8	11,0	4,0	3,1
6	20	120	4	*655,6*	612,7	24,6	10,0	4,9	3,3
7	40	120	5	*821,9*	771,8	28,0	10,5	7,2	4,3
8	40	210	6	*846,6*	787,3	32,7	11,9	9,7	5,0
9	40	30	4	*667,7*	623,4	25,6	9,6	5,3	3,7
10	40	120	5	*769,4*	720,9	27,0	11,2	6,3	4,0
11	40	210	4	*676,5*	636,9	22,6	10,1	4,2	2,7
12	40	120	5	*815,8*	765,0	28,5	11,3	7,3	3,7
13	40	120	5	*774,4*	726,1	27,1	10,6	6,7	3,8
14	20	30	5	*836,1*	779,5	31,5	11,4	9,2	4,4
15	20	210	5	*842,0*	789,3	29,4	11,2	7,7	4,3
16	40	30	6	*832,8*	771,7	33,1	12,5	10,2	5,2

Total correspond to the sum of the five compounds. X_1, X_2 and X_3 correspond to temperature, duration and pH, respectively.

Table 7.2. Box-Behnken design and the response for isoflavone yield extracted from digestive fluids and feces

Assay	X_1 (°C)	X_2 (min)	X_3 (%)	Isoflavone yield (µg.g^{-1}DM)										
				Observed values										
				Digestive juice						Feces				
				Total	EQ	FO	DA	BA	GE	*Total* [a]	EQ	FO	DA	BA
1	80	40	10	*810,3*	767,8	22,3	10,8	6,0	3,5	*164,1*	144,8	6,6	6,6	6,0
2	80	40	90	*722,5*	667,7	28,6	10,3	12,2	3,7	*302,6*	277,6	9,3	10,3	5,4
3	80	10	50	*935,7*	875,5	30,9	11,0	13,6	4,7	*312,4*	283,6	11,3	9,9	7,6
4	30	40	90	*697,0*	640,4	29,9	9,8	12,8	4,0	*293,6*	274,3	6,5	8,8	3,9
5	80	70	50	*954,0*	888,4	35,5	11,5	13,5	5,1	*308,5*	275,0	11,3	9,6	12,6
6	30	40	10	*756,2*	721,7	19,6	9,0	3,6	2,4	*85,0*	77,6	2,3	3,8	1,3 [b]
7	55	40	50	*947,8*	885,8	34,5	11,2	11,7	4,6	*298,5*	274,5	9,1	8,8	6,1
8	55	70	90	*599,6*	554,0	24,7	8,1	9,7	3,1	*294,0*	270,9	9,1	9,4	4,5
9	55	10	10	*827,9*	785,0	22,8	9,4	7,2	3,5	*147,7*	136,0	3,9	5,8	2,1
10	55	40	50	*930,2*	866,7	33,1	11,3	14,1	5,0	*300,9*	276,1	9,7	8,8	6,2
11	55	70	10	*805,9*	765,0	22,1	9,9	5,7	3,1	*160,1*	143,5	6,6	6,7	3,3
12	55	40	50	*900,9*	842,3	30,8	10,6	12,6	4,7	*285,8*	259,8	10,0	9,2	6,7
13	55	40	50	*879,7*	817,2	33,1	11,2	13,5	4,6	*314,8*	286,7	10,3	9,0	8,8
14	30	10	50	*811,9*	758,4	28,5	10,5	10,5	3,9	*271,4*	251,2	6,7	8,1	5,4
15	30	70	50	*880,9*	824,0	30,6	11,3	10,7	4,3	*266,4*	245,5	7,5	8,2	5,3
16	55	10	90	*684,4*	628,6	30,1	9,6	12,1	3,9	*293,1*	274,4	6,7	8,5	3,5

Total correspond to the sum of the five compounds. X_1, X_2 and X_3 correspond to temperature, duration and MeOH proportion, respectively. [a] Total not included GE because only 2 assays (3 and 5) have a concentration upper than the fixed LOQ (1.6 µg.g^{-1} DM); [b] This concentration is just lower than the fixed LOQ (1.6 µg.g^{-1} DM).

All the assays were conducted on extracts obtained under the UAE conditions used by Daems *et al.* (2016b). Analyte concentration was expressed in $\mu g.g^{-1}$ dry matter (DM), where DM was estimated following the procedure described by (Reynaud *et al.* 2010).

UAE

UAE was optimized in the same way for the two matrices. The three factors and levels selected were: extraction temperature (X_1; 30, 55 and 80°C); duration (X_2; 10, 40 and 70 min); and solvent proportions (X_3; 10, 50 and 90% MeOH/water). The dried residues were hydrolyzed under the best hydrolysis conditions established earlier. The 16 combinations are presented in Table 7.2. The experimental results were analyzed using the aforementioned method and the model was validated. Extraction efficiency was then checked by performing three consecutive extractions on samples with suspected high isoflavone concentration.

Method validation

The validation was performed following European Medicines Agency (EMA) guidelines (2015). The LC-MS method was validated by investigating the following parameters: specificity, linearity range, limits of detection (LOD) and of quantification (LOQ), precision and accuracy. Calibration curves used for external standard calibration were generated by plotting the ratio between the peak areas and that of IS (Aa/Ais, IS_{DA-d4} [50 ng mL^{-1}] used for DA and IS_{FL} [20 ng mL^{-1}] for the other analytes) to the corresponding isoflavone concentrations ($ng.mL^{-1}$). Process efficiency (matrix effect [ME] and recoveries), extract stability and method repeatability were also investigated. Repeatability was investigated using three samples (feces, and rumen and duodenal fluids) with a suspected high concentration and three others with a suspected low concentration. Robustness was indirectly investigated throughout the Box-Behnken experimental assays.

Statistical analysis

Most of the analyses were performed at least in triplicate. The Box-Behnken design was performed only once, except for the validation of the models, which were performed seven times. JMP® statistical software package (SAS institute, Cary, US) was

used to obtain the coefficients of the quadratic polynomial model and to determine the best conditions for the hydrolysis and UAE steps. Analysis of variance (ANOVA; $\alpha = 0.05$) and the comparison of averages by Tukey's HSD ($\alpha = 0.05$) test were used to compare process and validation parameters.

Results and discussion

UPLC®-MS/MS method

UPLC®-MS/MS was chosen as the most appropriate analytical method for quantifying isoflavones and their metabolites in biological matrices because it provided reliable, accurate and fast analysis with good sensitivity (Ko 2014; Raju *et al.* 2015; Daems *et al.* 2016a). The elution gradient and column used by Daems *et al.* (2016b) enabled the five targeted compounds and two ISs to be separated accurately in line with the expected order of elution proposed by Valls *et al.* (2009). The first compound (DA) appeared at 3.30 min and the last one (IS_{FL}) at 5.80 min (Fig. 7.2a). The total runtime of 8 min was slightly shorter than the times reported in two recent studies (Njåstad *et al.* 2014; Kasparovska *et al.* 2016a), allowing a higher throughput of sample analysis. As shown in Fig. 7.2a, only peaks of DA and $IS_{DA\text{-}d4}$ co-eluted, but with the use of multiple reactions monitoring (MRM) there was no problem in discriminating between these two analytes.

The optimization of MRM conditions was achieved by infusing standard solutions of isoflavones, EQ and ISs directly in the MS/MS. The compounds were detected using a parent ion $[M+H]^+$ and the two most abundant characteristic daughter ions (DA 255 > 199, 137; $IS_{DA\text{-}d4}$ 259 > 203, 137; EQ 243 > 133, 123; GE 271 > 153, 215; FO 269 > 197, 237; BA 285 >213, 269; and IS_{FL} 223 > 121, 178) (Fig. 7.2b). For each compound, the most abundant fragment was selected for quantification (Q) and the second one for confirmation (q). The fragments found for each compound were similar to those reported by Antignac *et al.* (2003), Justino *et al.* (2009), Prokudina *et al.* (2012) and Raju *et al.* (2015).

Optimization of hydrolysis

An enzymatic hydrolysis step was included in the sample preparation process because: (1) in the digestive tract, aglycones are thought to be the most biologically active form that can be absorbed and metabolized by the organism, and they then become the most interesting forms to monitor in the overall passage of these compounds from the

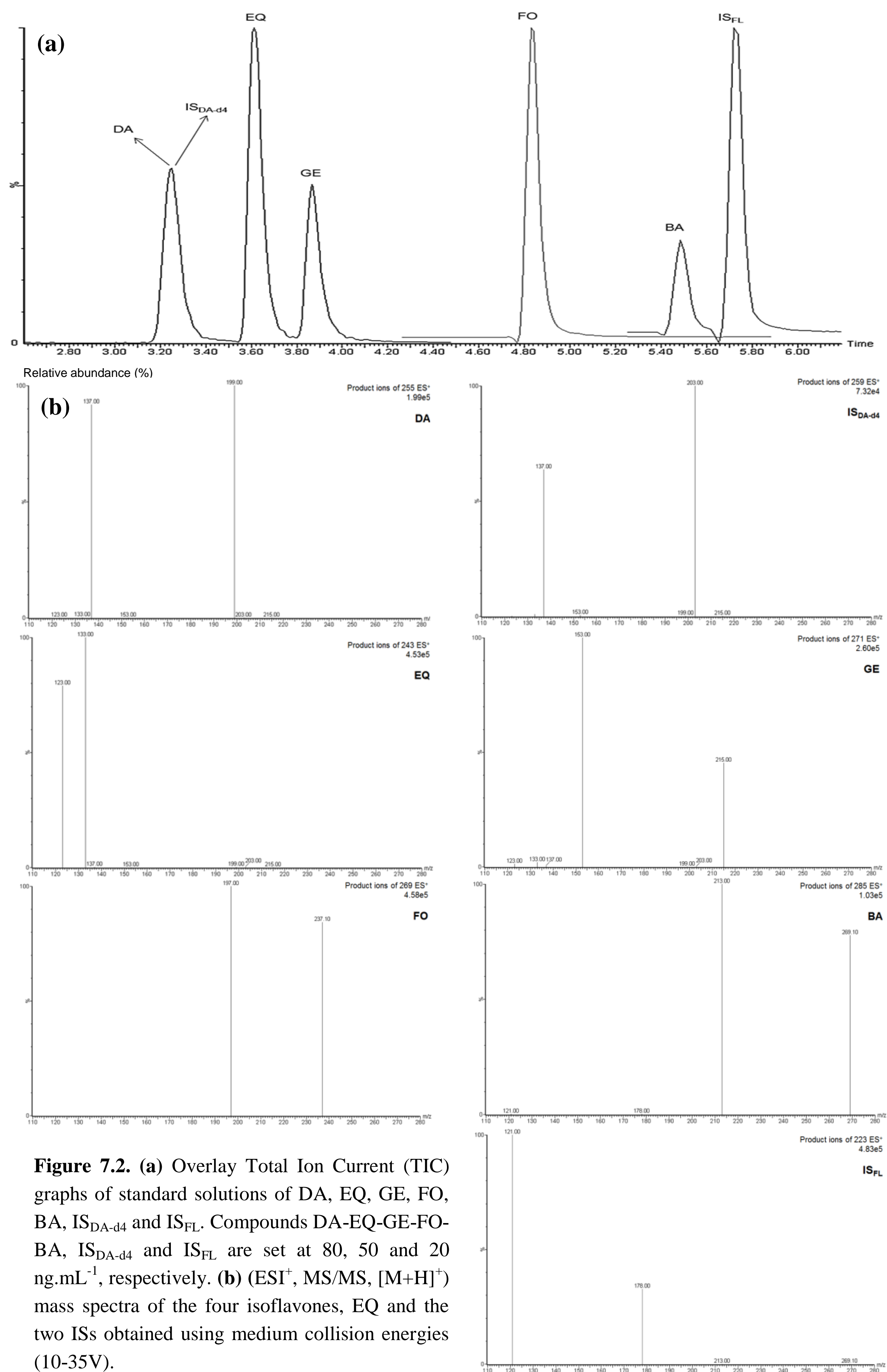

Figure 7.2. (a) Overlay Total Ion Current (TIC) graphs of standard solutions of DA, EQ, GE, FO, BA, $IS_{DA\text{-}d4}$ and IS_{FL}. Compounds DA-EQ-GE-FO-BA, $IS_{DA\text{-}d4}$ and IS_{FL} are set at 80, 50 and 20 ng.mL^{-1}, respectively. **(b)** (ESI^+, MS/MS, $[M+H]^+$) mass spectra of the four isoflavones, EQ and the two ISs obtained using medium collision energies (10-35V).

plant matrix to animal products; (2) a hydrolysis step reduces the number of compounds being followed, facilitating their separation and reducing the runtime; and (3) some of the target compounds seem to be sensitive to acidic conditions (Mostrom & Evans 2012; Ko 2014; Daems *et al.* 2016a). Among the available enzymes, *β*-glucuronidase/arylsulfatase from *Helix pomatia* is the most commonly used one in the hydrolysis of isoflavones and their microbial metabolites in biological samples. It was therefore used in this study, as well as two other enzymes (*β*-glucosidase and cellulase) suggested by Konar *et al.* (2012) and Daems *et al.* (2016b) hydrolyzing conjugated forms in plant matrices.

First, in the case of digestive juices, the need for hydrolysis was evaluated by testing sample preparation with and without this step. In the same experiment, hydrolysis with each enzyme alone or in combination was also tested (Fig. S7.1). No significant difference was revealed between the concentration in extracts hydrolyzed with the mixture of the three enzymes and *β*-glucuronidase used alone. The concentrations for each compound with these two enzymatic solutions were, however, significantly higher than those for the two other enzymes, as well as in the process without a hydrolysis step. These findings showed that isoflavones and EQ are present mainly in conjugated forms (especially, glucuronides) in a cow's digestive system (Daems *et al.* 2016a). Unlike the plant matrix (Konar *et al.* 2012; Daems *et al.* 2016b), cellulase and *β*-glucosidase do not appear to be necessary for hydrolyzing the conjugated forms of isoflavones. Therefore, in order to reduce the analysis cost, only *β*-glucuronidase was selected for the hydrolysis of isoflavones in digestive juices. The concentration of the *β*-glucuronidase solution was also investigated. A solution containing ≥ 10,200 units.mL^{-1} of this enzyme was prepared and then diluted 2, 4 and 8 times by the buffer solution at pH 6. The experiment showed that the enzymatic solution concentration had no impact on the hydrolysis yield of the targeted compounds (Fig. S7.2). An enzymatic solution with ≥ 1,275 units.mL^{-1} of *β*-glucuronidase from *Helix pomatia* was therefore selected. Finally, in order to facilitate the selection of the duration variable in our Box-Behnken design, three hydrolysis durations were tested. No difference was found among the isoflavone concentrations after 1, 3 and 16 h hydrolysis (Fig. S7.3).

The same three experiments were conducted on feces. For this matrix, a hydrolysis step did not seem necessary, except for FO, where the results were the same as for digestive fluids (Fig. S7.4). For samples with or without a hydrolysis step, the FO concentrations were low, ranging between 4 and 9 µg.g^{-1} DM. GE and BA were detected

but their concentrations were close to the LOQ. No impact of the β-glucuronidase solution concentration or hydrolysis duration was evident (Fig. S7.5 and Fig. S7.6). At this stage, the usefulness of a hydrolysis step for feces was therefore questionable. It was decided to optimize the hydrolysis step for digestive fluids and to use the same conditions for feces, as a precautionary measure.

These initial experiments also showed that EQ was the major compound. In the digestive fluids samples, EQ concentrations ranged from 600 to 900 $\mu g.g^{-1}$ DM, whereas for DA and FO they ranged from 10 to 50 $\mu g.g^{-1}$ DM, and for BA and GE were even lower. In the feces, EQ concentrations were three times lower than in the digestive fluids, but EQ remained the major compound. DA and FO concentrations never exceeded 12 $\mu g.g^{-1}$ DM and BA was near the LOQ (1.6 $\mu g.g^{-1}$ DM). GE was detected, but it was often below the fixed LOQ (1.6 $\mu g.g^{-1}$ DM). GE was therefore not considered in the Box-Behnken design used for UAE optimization in feces. The high concentration of EQ compared with the other compounds could have influenced the choice of sample preparation conditions, and this observation was taken into account in our Box-Behnken designs.

Digestive fluids

The results of the Box-Behnken designs are shown in Table 7.1. The second-order polynomial models for each individual compound and the total amount are given in supplementary data (Eqs. 7.1-6) and Table 7.3 gives their statistical significance. As shown, all the ANOVA p-values indicated that the models were significant (p-value < 0.05). The lack-of-fit tests were not significant (p-value > 0.05) in any of the compounds, indicating no lack of matching between the data and the models. The goodness-of-fit was checked by the adjusted coefficient of determination (R^2 *Adj*). There is no specific rule relating to the minimum value accepted for this coefficient as long as the quality of the model has been proved. The lowest and highest R^2 *Adj* values were found for GE (0.588) and DA (0.828), respectively. These results suggested that the models adequately represented a large part of the real relationship between the chosen variables in the range of selected values.

As shown in Table 7.3, only pH (X_3) had a significant impact on hydrolysis yield. It showed a significant linear effect (p-value < 0.05) on the hydrolysis of all the targeted compounds. A significant quadratic effect (p-value < 0.05) was also observed for EQ.

Table 7.3. Model-fitting results for enzymatic hydrolysis optimization (in the upper part of the table) and isoflavone concentrations predicted under optimal and selected hydrolysis conditions, as well as the actual concentration obtained (in the lower part of the table)

Model-fitting

Statistical parameters	Total	EQ	FO	DA	BA	GE
R^2	0.924	0.926	0.784	0.931	0.798	0.780
R^2 Adj	0.810	0.816	0.676	0.828	0.663	0.588
ANOVA *p*-value	0.0095	0.0088	0.0041	0.0072	0.0094	0.0340
Lack-of-fit *p*-value	0.3059	0.3427	0.0531	0.7672	0.0533	0.1562
Factors with significant effect	X_3^{***} $X_3X_3^{*}$	X_3^{***} $X_3X_3^{*}$	X_3^{***}	X_3^{***}	X_3^{***}	X_3^{***}

Verification of the prediction model

Compound	Condition	Enzymatic hydrolysis variables			Y (µg.g^{-1} DM)		
		X_1 (°C)	X_2 (min)	X_3 (U pH)	Predicted [x]	Pred. Inter. [y]	Obtained [z]
Total	Optimal	20	210	5.8	865.1 ± 85.6	776.1 – 954.1	744.5 ± 18.4
	Selected	37 [m]	60 [n]	6 [o]	841.0 ± 56.0	758.1 – 923.8	
EQ	Optimal	20	210	5.7	806.4 ± 74.5	725.3 – 887.5	693.9 ± 17.9
	Selected	[m]	[n]	[o]	781.5 ± 51.5	705.4 – 857.7	
FO	Optimal	20	30	6	33.8 ± 3.9	28.2 – 39.4	27.9 ± 0.6
	Selected	[m]	[n]	[o]	31.8 ± 2.4	27.5 – 36.1	
DA	Optimal	60	30	6	12.6 ± 1.0	11.7 – 13.6	10.6 ± 1.0
	Selected	[m]	[n]	[o]	12.1 ± 0.6	11.3 – 13.0	
BA	Optimal	20	30	6	11.3 ± 2.6	8.4 – 14.3	8.3 ± 0.4
	Selected	[m]	[n]	[o]	10.3 ± 1.4	7.6 – 13.0	
GE	Optimal	20	210	6	5.3 ± 1.1	4.1 – 6.4	3.9 ± 0.2
	Selected	[m]	[n]	[o]	4.9 ± 0.6	3.9 – 6.0	

* Significant at 0.05, ** Significant at 0.01, *** Significant at 0.005. X_1, X_2, X_3 and Y correspond to temperature, duration, pH and isoflavone yield, respectively.
Total considers the five compounds.
[m] Selected temperature
[n] Selected duration
[o] Selected pH
[x] Predicted content ± half confidence interval (α=0.05)
[y] Prediction interval of 95%
[z] Mean value ± standard deviation (n=7)

Equations 7.1 to 7.6 enabled the best hydrolysis conditions for total and individual compounds to be established. The optimal pH was 6.0 for all isoflavones, except for EQ, which had an optimum of 5.7. Given that EQ was the major compound, the optimal pH was estimated to be 5.8 (Table 7.3). This optimal pH is in the same order of magnitude of the pH recommended by Daems *et al.* (2016b) for the hydrolysis of isoflavones in plant matrices using the combination of the three enzymes cited earlier. It is, however, slightly higher than the pH usually used by enzyme suppliers and by Njåstad *et al.* (2014) for the hydrolysis of isoflavones with Cellulase Onozuka R-10 from *Trichoderma viride*. The optimal temperatures (X_1) were estimated to be 60°C and 20°C for DA and the four other compounds, respectively. This variable, however, had no significant impact on hydrolysis

yield. Njåstad *et al.* (2014) conducted hydrolysis at an ambient temperature, whereas the enzyme suppliers use a temperature fixed at 37°C. Finally, for the duration variable (X_2), there was no significant impact on hydrolysis yield. This finding is in line with the earlier observations from the one-variable-at-a-time assays. The optimal durations were estimated to be 30 min and 210 min for FO-DA-BA and EQ-GE, respectively. These durations remain, however, far below the overnight duration proposed by Njåstad *et al.* (2014).

As shown in Table 7.3, the optimal hydrolysis conditions for total and individual compounds were not exactly the same (Table 7.3). A compromise was therefore needed. The optimal hydrolysis conditions were finally fixed as follows: temperature of 37°C for 60 min at a pH of 6. The optimal temperature and hydrolysis duration were very different for each individual compound, but these two factors had no significant impact on hydrolysis yield. The temperature commonly requested by enzyme suppliers was therefore selected and, a duration of 60 min was considered satisfactory to ensure complete hydrolysis. This duration had already been recommended by Andersen *et al.* (2009b) for other biological matrices and allows the whole process to be completed in one day. The pH was substantially the same for each compound and was therefore set at 6.0. The predicted yield for each compound with the selected conditions was within the prediction interval obtained with the optimal conditions (Table 7.3). This observation shows that the selected conditions represented a good compromise. In order to verify the predictive capacity of the model, seven additional hydrolyses under the selected conditions were performed. For FO, BA and GE, the concentrations were within the confidence interval, confirming the models used. For EQ and DA, however, three extracts had a concentration slightly below the lower limit of the confidence interval, thus affecting the total isoflavone yield. These yields, however, remained very close to the prediction intervals, confirming the validity of the models designed in this study.

UAE optimization

The samples were initially freeze-dried in order to facilitate storage and solid-liquid extraction. UAE with a combination of MeOH with water was selected as the most suitable extraction technique (Londoño-londoño *et al.* 2010; Raju *et al.* 2015; Daems *et al.* 2016b). The UAE was optimized for the two matrices with the same response surface methodology used previously.

Digestive fluids

The results are given in Table 7.2, with the second-order equations in the supplementary data (Eqs. 7.7-12) and details of the statistical significance of these equations in Table 7.4. As shown, the ANOVA and lack-of-fit *p*-values were < 0.05 and > 0.05, respectively. These findings indicated that the models were significant and that there was a match between the data and the proposed models. All the R^2 *Adj* values were ≥ 0.70, indicating that all the models provided an accurate representation of the relationship between the selected variables and extracted yields. Among the three variables tested, only the solvent proportion (X_3) had significant linear and quadratic effects on extraction yield, except for DA, where only a linear effect was observed. An optimum extraction yield for each compound was obtained for a solvent proportion containing 40-63% MeOH. These results are in line with those reported by Rostagno *et al.* (2003), Raju *et al.* (2015) and Daems *et al.* (2016b). For the two other variables, however, there was no significant effect on extraction. The optimal extraction temperature (X_1) was about 80°C. These results, suggest that these compounds are not sensitive to heating treatment. For duration (X_2), however, the optimal time was between 19 and 50 min for BA and FO, respectively.

With regard to hydrolysis optimization, the optimal UAE conditions for individual compounds differed slightly (Table 7.4). A compromise was therefore needed. The selected UAE conditions for the digestive fluids were fixed as follows: temperature 80°C; duration 10 min; and solvent proportion 50% of MeOH. Temperature and duration had no significant impact on extraction yield. The optimal temperature for each compound was almost identical and this temperature was therefore selected for the final conditions. It was the same as that recommended by Daems *et al.* (2016b) for extracting isoflavones from freeze-dried forages. Duration was arbitrarily set at 10 min in order to reduce sample preparation time, while taking care that the predicted values (with the selected conditions) were in the predicted interval (with optimal conditions). For the last variable with the most significant impact on extraction yield, it was not possible to obtain an extraction with the optimal solvent proportion for each compound. The medium of the optimal range was therefore selected, while taking care that the predicted values (with the selected conditions) were in the predicted interval (with optimal conditions). Seven replicates of extractions under the selected conditions were then performed in order to verify the predictive capacity of the model. The concentration was 874.4 ± 26.2 $\mu g.g^{-1}$

DM, which was within the confidence interval. This finding was identical for each individual compound (Table 7.4). The models designed for UAE optimization were therefore valid.

Table 7.4. Model-fitting for extraction of isoflavones from digestive fluid, showing results for UAE optimization (in the upper part of the table) and isoflavone concentrations predicted under optimal and selected UAE conditions, as well as the actual concentration obtained (in the lower part of the table)

Model-fitting

Statistical parameters	Total	EQ	FO	DA	BA	GE
R^2	0.916	0.921	0.894	0.877	0.920	0.894
R^2 Adj	0.789	0.803	0.734	0.692	0.801	0.735
ANOVA *p*-value	0.0128	0.0106	0.0241	0.0357	0.0109	0.0240
Lack-of-fit *p*-value	0.1344	0.1612	0.1303	0.1192	0.2073	0.0641
Factors with significant effect	$X_3X_3^{***}$ X_3^{**}	$X_3X_3^{***}$ X_3^{**}	$X_3X_3^{***}$ X_3^{**}	X_3^{***}	X_3^{***} $X_3X_3^{***}$	$X_3X_3^{***}$

Verification of the prediction model

Compound	Condition	UAE variables			Y (µg.g^{-1} DM)		
		X_1 (°C)	X_2 (min)	X_3 (%)	Predicted [x]	Pred. Inter. [y]	Obtained [z]
Total	Optimal	80	29.8	42.8	957.4 ± 75.4	836.1 – 1078.6	874.4 ± 26.2
	Selected	80 [m]	10 [n]	50 [o]	945.3 ± 103.5	817.5 – 1073.1	
EQ	Optimal	80	32	40.7	895.6 ± 69.2	783.8 – 1007.3	817.6 ± 25.1
	Selected	m	n	o	883.6 ± 95.5	765.7 – 1001.6	
FO	Optimal	76.7	49.3	55.8	33.6 ± 3.4	27.5 – 39.7	30.5 ± 0.9
	Selected	m	n	o	31.8 ± 5.3	25.2 – 38.3	
DA	Optimal	80	41.8	43.6	11.8 ± 0.8	10.5 – 13.2	11.0 ± 0.7
	Selected	m	n	o	11.5 ± 1.1	10.1 – 12.9	
BA	Optimal	80	18.7	62.7	14.0 ± 2.6	10.3 – 17.7	11.2 ± 1.5
	Selected	m	n	o	13.6 ± 3.1	9.8 – 17.4	
GE	Optimal	80	33.6	49.2	4.9 ± 0.6	3.9 – 5.9	4.1 ± 0.3
	Selected	m	n	o	4.8 ± 0.8	3.8 – 5.9	

* Significant at 0.05, ** Significant at 0.01, *** Significant at 0.005. X_1, X_2, X_3 and Y correspond to temperature, duration, MeOH proportion and isoflavone yield, respectively
Total considers the five compounds
[m] Selected temperature
[n] Selected duration
[o] Selected MeOH proportion
[x] Predicted content ± half confidence interval (α=0.05)
[y] Prediction interval of 95%
[z] Mean value ± standard deviation (n=7)

Finally, extraction efficiency was checked by performing three consecutive extractions under the selected conditions. Depending on the compounds, 80.2-100% and 86.1-91.0% were recovered after one extraction from duodenal and rumen fluid samples (Table S7.1), respectively. These values, as well as the good reproducibility observed, were judged acceptable for a single extraction.

Feces

The Box-Behnken design results are given in Table 7.2, with the second-order equations in the supplementary data (Eqs. 7.13-17) and the statistical significance of these equations in Table 7.5. As shown, the five models were significant (ANOVA *p*-values < 0.05) and there was no indication of a lack-of-fit between the data and the models proposed (lack-of-fit *p*-values > 0.05). The lower and higher R^2 *Adj* values were 0.84 (BA) and 0.97 (EQ). This means that all the equations generated provided a good representation of the relationship between the selected variables and the extraction yield for each compound. As found previously for digestive juices, solvent proportion (X_3) had a significant impact on the extraction yields. Significant linear and quadratic effects were highlighted for all the compounds, except for BA, where no linear effect was found. Depending on the molecule, the optimal solvent proportion was between 48 and 71% of MeOH (Table 7.5). This range is slightly higher than that for the digestive juices, but is in line with the extraction solvent proportion usually used for these compounds (Rostagno *et al.* 2003; Raju *et al.* 2015; Daems *et al.* 2016b). Temperature (X_1) had a significant linear impact on the extraction yield of all the compounds and the optimal temperature was 80°C. EQ was the only compound with an optimal extraction temperature estimated to be 65°C. For duration (X_2), no significant effect was found, except for FO, where there was a significant linear effect. For this variable, the optimal duration varied between 10 and 70 min for EQ and FO-DA-BA, respectively. Again, a compromise was needed that took into account the variables with the greatest impact.

The selected UAE conditions for feces were fixed as follows: temperature 70°C; duration 35 min; and solvent proportion 60%. Again, the predicted yields under the selected conditions were within the prediction interval obtained with the optimal conditions, showing that these selected conditions represented a good compromise (Table 7.5) and, after extraction under the selected conditions, all the concentrations were within the confidence intervals obtained with these selected conditions. The models designed for UAE optimization with feces were, therefore also valid.

The extraction efficiency for EQ and DA was 90% and 87%, respectively (Table S7.1). A single extraction was therefore judged sufficient for these two compounds. For FO and BA, however, the values were 62% and 58%, respectively, and a second extraction was therefore recommended.

Table 7.5. Model-fitting for extraction of isoflavones from feces, showing results for UAE optimization (in the upper part of the table) and isoflavone concentrations predicted under optimal and selected UAE conditions, as well as the actual concentration obtained (in the lower part of the table)

Model-fitting

Statistical parameters	Total	EQ	FO	DA	BA
R^2	0.987	0.986	0.963	0.948	0.935
R^2 Adj	0.966	0.966	0.906	0.922	0.839
ANOVA p-value	<.0001	<.0001	0.0013	<.0001	0.0061
Lack-of-fit p-value	0.3718	0.3696	0.1599	0.0541	0.7158
Factors with significant effect	X_3^{***} $X_3X_3^{***}$ X_1^{***} $X_1X_3^{*}$	X_3^{***} $X_3X_3^{***}$ X_1^{**} $X_1X_3^{*}$	$X_3X_3^{***}$ X_1^{***} X_3^{***} X_2^{*}	X_3^{***} $X_3X_3^{***}$ X_1^{***}	$X_3X_3^{***}$ X_1^{***}

Verification of the prediction model

Compound	Condition	UAE variables			Y (µg.g^{-1} DM)		
		X_1 (°C)	X_2 (min)	X_3 (%)	Predicted [x]	Pred. Inter. [y]	Obtained [z]
Total	Optimal	69.5	10	68.2	325.2 ± 23.0	291.6 – 358.9	317.2 ± 18.1
	Selected	70 [m]	35 [n]	60 [o]	320.9 ± 15.8	289.1 – 352.7	
EQ	Optimal	64.7	10	70.8	299.8 ± 20.6	268.6 – 331.0	288.7 ± 17.9
	Selected	[m]	[n]	[o]	292.4 ± 14.7	262.7 – 322.0	
FO	Optimal	80	70	56.9	11.8 ± 1.7	9.7 – 13.8	11.0 ± 0.4
	Selected	[m]	[n]	[o]	10.8 ± 0.9	8.9 – 12.6	
DA	Optimal	80	70	69.7	10.4 ± 0.7	9.3 – 11.6	9.4 ± 0.3
	Selected	[m]	[n]	[o]	9.8 ± 0.4	8.7 – 10.8	
BA	Optimal	80	70	48.3	11.8 ± 2.3	9.0 – 14.7	8.1 ± 0.6
	Selected	[m]	[n]	[o]	7.9 ± 1.3	5.3 – 10.5	

* Significant at 0.05, ** Significant at 0.01, *** Significant at 0.005. X_1, X_2, X_3 and Y correspond to temperature, duration, MeOH proportion and isoflavone yield, respectively
Total considers the four compounds
[m] Selected temperature
[n] Selected duration
[o] Selected MeOH proportion
[x] Predicted content ± half confidence interval (α=0.05)
[y] Prediction interval of 95%
[z] Mean value ± standard deviation (n=7)

Method validation

The reliability of the UPLC®-MS/MS analytical method was checked initially in terms of specificity, linearity of external calibration curve, LOD and LOQ, precision and accuracy (Table 7.6). The target analytes stored in methanol were pooled in the same solution mixture prepared in H_2O:methanol (40:60, v/v) and working dilutions were created, as required for calibration. This solvent proportion was based on recommendations by Daems *et al.* (2016b). ISs were prepared separately using the same procedure.

Table 7.6. Validation parameters of UPLC®-MS/MS method: retention time (RT); relative retention time (RRT); Q/q ratio; calibration range, correlation coefficient (R^2) value, slope and intercept of calibration curves in MeOH60%; LOD and LOQ calculated; and maximum values for intra-/inter-assay precision (max. prec.) and accuracy (max. acc.)

Compound	RT [a](min)	RRT [a,b]		Q/q [a,c]		Calibration curve in $MeOH_{60\%}$ [d]			LOD	LOQ	Max. prec.[e]		Max. acc.[f]	
		$Max._{(+2.5\%)}$	$Min._{(-2.5\%)}$	$Max._{(+20\%)}$	$Min._{(-20\%)}$	R^2	Slope	Intercept	($ng.mL^{-1}$)	($ng.mL^{-1}$)	intra	inter	intra	inter
DA	*3.32 ± 0.06*	1.033	0.982	1.207	0.805	0.995	0.0272	0.0250	0.07	0.24	8.9	8.8	9.6 ± 5.6	8.4 ± 6.3
EQ	*3.69 ± 0.07*	0.649	0.617	1.095	0.730	0.996	0.0087	0.0092	0.21	0.70	8.7	7.2	11.4 ± 3.0	7.0 ± 4.8
GE	*3.94 ± 0.06*	0.693	0.659	0.547	0.364	0.998	0.0071	0.0110	0.29	0.96	10.3	7.7	9.5 ± 2.4	5.6 ± 5.2
FO	*4.93 ± 0.08*	0.867	0.825	0.925	0.617	0.995	0.0088	0.0270	0.06	0.20	3.4	4.1	9.7 ± 2.3	5.8 ± 3.2
BA	5.57 ± 0.08	0.981	0.933	0.793	0.529	0.994	0.0041	0.0066	0.07	0.22	15.2	11.1	12.3 ± 1.9	8.5 ± 7.1
IS_{DA-d4}	3.29 ± 0.06	-	-	0.636	0.424	-	-	-	-	-	-	-	-	-
IS_{FL}	5.83 ± 0.10	-	-	0.406	0.271	-	-	-	-	-	-	-	-	-

[a] n ≥ 220 injections of standard solutions and samples over 4 months

[b] $RRT = RT_{analyte}/RT_{IS}$

[c] $Q/q = Signal_2/Signal_1$

[d] Range 5-125 $ng.mL^{-1}$

[e] Maximum precision (expressed in RSD%) found among the four concentrations (Table S7.2)

[f] Maximum accuracy (expressed in bias%) found among the four concentrations (Table S7.2)

The specificity was characterized by retention time (RT), relative retention time (RRT) and the *Q/q* ratios (Table 7.6). Throughout the optimization and validation process, the RRTs and *Q/q* ratios of each analyte remained in the ±2.5% and ±20% tolerance ranges, respectively (Antignac *et al.* 2003; Moreno-González *et al.* 2013). The calibration curves were established by injecting six replicates of seven concentration points between 5 and 125 ng.mL^{-1}. An ANOVA with lack-of-fit test was used to assess whether or not the least squares linear regression model was adequate ($\alpha = 0.05$) (De Bock *et al.* 2012). Checks were also done to ensure that the relative standard deviation (RSD) for each concentration point never exceeded 20% for points near the LOQ or 15% for the others (EMA 2015), and that the correlation coefficient (R^2) was always ≥ 0.99 (Fiechter *et al.* 2013). LOD and LOQ were determined as the concentration at a signal-to-noise (S/N) of 3 and 10, respectively. Among the available samples, however, none was free of the five target compounds. The S/N of each analyte was therefore calculated in standard solutions near the lower calibration concentration (LCC) (n=5). Depending on the analyte, the LODs and LOQs were estimated to be between 0.1-0.3 and 0.2-1.0 ng.mL^{-1}, respectively (Table 7.6). These values were confirmed by analyzing samples (digestive juices and feces with suspected low target analyte concentrations) diluted and spiked at concentrations equal to or slightly higher (≤ 20%) than the calculated limits (n=4) (Salomone *et al.* 2011). The variation in the peak area did not exceed 20%, confirming the limits estimated. The LOQs calculated were lower than the fixed LCC. Nonetheless, an LCC set at 5 ng.mL^{-1} was judged satisfactory for this type of analysis. The maximum inter-/intra-essay precision values were 11.1% (BA at 5 ng.mL^{-1}) and 15.2% (BA at 5 ng.mL^{-1}) (Tables 7.6 and S7.2), respectively. These values did not exceed the maximum allowed value of 15-20% (Ellis 2008; EMA 2015). The maximum bias between the measured and assigned values for inter-/intra-assays were 8.5 ± 7.1% (BA at 5 ng.mL^{-1}) and 12.3 ± 1.9% (BA at 80 ng.mL^{-1}) (Tables 7.6 and S7.2), respectively. These values were also lower than the allowed value of 15% (Ellis 2008; EMA 2015).

This first part of the validation process indicated that the UPLC®-MS/MS method was reliable. For the four isoflavones, all the parameters checked were of the same order of magnitude as those reported by Daems *et al.* (2016b), except for LOD and LOQ. The limits in our study were lower than those reported earlier, especially for GE, which was four times smaller. This difference could be due to the extract from which S/N was estimated. In their validation process, Daems *et al.* (2016b) used a flaxseed extract for

estimating the S/N ratios, whereas in the present study H_2O:methanol (40:60, v/v) was used. N was less important than in the flaxseed matrix and the limits estimated were therefore the smallest. The LOQs for DA, EQ and GE were slightly lower than those reported by Kasparovska *et al.* (2016a), but the calibration ranges were similar.

In the second part of the validation process, the reliability of the whole analytical method was checked in terms of recovery, ME, stability and repeatability. For the recovery test, known amounts of all the analytes were added to samples with suspected low isoflavone and EQ concentrations. The analysis was carried out in triplicate and the results are shown in Table 7.7. For the digestive fluids, all the values fell within the acceptable range of 70 to 120% (Ellis 2008; Delgado-Zamarreño *et al.* 2012). For the feces, FO and GE, recoveries were close to the lower limit value (70%). BA, however, had a lower recovery (43.0 ± 3.2%) than the four other analytes. For each analyte and regardless of the matrix, the recovery value was fairly similar, whatever the concentration level added, and the RSD% never exceeded 9% (feces, BA at 30 µg added) for the same concentration. With this good reproducibility at all levels, the low BA recovery in feces could be overcome by using a correction factor (2.3). The DA, EQ and GE recovery values for the digestive juices in our study were of the same order of magnitude as those reported by Kasparovska *et al.* (2016a); our RSDs, however, were lower.

ME was studied by comparing the slopes of the calibration curves obtained by spiking the dry residues of hydrolyzed extracts (without dilution and with a dilution factor of 10 and 50) with the slopes of those arising from the standard solutions (Salomone *et al.* 2011; Daems *et al.* 2016b). The three dilution factors corresponded to the most widely used dilution factor during routine analysis. Each curve was achieved in triplicate, and the mean slopes were calculated and compared (Table 7.7). All the ME values calculated were within an acceptable range (± 20%) (Ferrer *et al.* 2011). Given this information, the calibration curve in H_2O:methanol (40:60, v/v) was therefore judged adequate for measuring isoflavones and EQ in samples of cows' digestive juices and feces.

For practical reasons, sample extracts are sometimes stored until assayed. It is therefore important to determine the appropriate storage conditions and how long these extracts can be stored without excessive degradation prior to analysis. Two samples with a suspected high analyte concentration were analyzed in triplicate. The extracts were then stored in two vials, one at 6 ± 1°C and the other at -20 ± 1°C. The extracts were re-analyzed after 4 days of storage. As shown in Table 7.7, whatever the storage

Table 7.7. Validation parameters for the whole method: recovery, matrix effect (ME) and stability

Compound	Recovery [a, b]						ME [d]						Stability [b, e]					
	Digestive juice			Feces			Digestive juice			Feces			Digestive juice [f]			Feces [f]		
													T_0 [g]	4 days storage at:		T_0 [g]	4 days storage at:	
	30 µg [c]	50 µg [c]	75 µg [c]	30 µg [c]	50 µg [c]	75 µg [c]	No Dil.	Dil. 10	Dil. 50	No Dil.	Dil. 10	Dil. 50		- 20 °C	6 °C		- 20 °C	6 °C
DA	*96.0 ± 6.2*	*100.5 ± 5.8*	*108.9 ± 1.9*	*91.3 ± 2.0*	*85.0 ± 3.3*	*87.0 ± 2.3*	*4.3*	*0.8*	*-14.9*	*-0.2*	*-0.3*	*-11.3*	*29.3±0.8**	*30.4±1.1**	*30.0±1.1**	*8.9±0.1**	*8.9±0.5**	*9.1±0.3**
EQ	*96.6 ± 3.7*	*101.9 ± 3.7*	*104.3 ± 4.1*	*90.2 ± 3.7*	*87.8 ± 4.0*	*88.1 ± 2.7*	*-12.8*	*4.5*	*7.3*	*-12.8*	*-2.2*	*1.4*	*974.3±22.6**	*906.8±51.0**	*931.5±14.1**	*200.8±2.0**	*202.4±11.3**	*208.0±5.0**
GE	*92.5 ± 5.8*	*96.8 ± 3.1*	*100.6 ± 0.9*	*71.2 ± 2.0*	*68.5 ± 0.5*	*68.7 ± 2.4*	*10.4*	*4.7*	*-2.9*	*3.5*	*3.3*	*0.0*	*5.3±0.2**	*5.2±0.7**	*5.6±0.4**	*2.5±0.2**	*2.5±0.0**	*2.6±0.8**
FO	*91.6 ± 4.4*	*92.1 ± 2.6*	*93.4 ± 4.2*	*71.7 ± 1.0*	*71.0 ± 1.5*	*69.3 ± 0.7*	*15.1*	*3.9*	*0.4*	*6.9*	*2.5*	*-1.4*	*27.5±1.0**	*28.2±1.7**	*28.6±0.6**	*14.2±0.2**	*14.2±0.7**	*14.5±0.1**
BA	74.3 ± 2.3	77.9 ± 1.2	85.7 ± 1.4	45.9 ± 4.1	42.5 ± 1.5	40.7 ± 0.6	7.8	3.9	-3.0	5.7	4.0	-2.5	*22.6±0.4**	*23.3±0.6**	*22.0±1.3**	*11.7±0.4**	*12.0±0.6**	*12.1±1.0**

[a] $\text{Recovery (\%)} = \left(\frac{\text{Amount found}}{\text{Amount added}}\right) \times 100$

[b] *n=3*

[c] *Amount added by different volumes of analyte solution*

[d] $\text{ME (\%)} = \left(\frac{(\text{Slope (matrix)} - \text{Slope (MeOH 60\%)})}{\text{Slope (MeOH 60\%)}}\right) \times 100$

Values marked by '' are not significantly different with the Tukey's HSD test (p-value > 0.05)*

[e] *Expressed in µg.g*$^{-1}$ *DM (mean ± SD)*

[f] *Samples with a suspected high analyte concentration*

[g] T_0 *correspond to the first analysis*

temperature, there was no significant difference between the analyte concentration found in the first analysis and after 4 days of storage.

The repeatability of the whole method was assessed by performing a multiple independent analysis of samples with suspected low and high analyte concentrations (n=3 per day and over 3 days). The maximum RSD was 13.8%, which was found for EQ near the LCC value in the feces sample (Table 7.8). For DA and EQ, the values in this study were far lower than those reported by Kasparovska *et al.* (2016a) with liquid-liquid extraction, except for GE, where these values were similar.

Table 7.8. Repeatability of the whole method applied to samples of feces, rumen and duodenal fluids with suspected low and high isoflavone concentrations

Compound	Feed ration rich in red clover [a, b]			Feed ration without red clover [a, c]		
	Rumen fluid	Duodenal fluid	Feces	Rumen fluid	Duodenal fluid	Feces
DA	26.6 ± 0.9 (3.4 %)	17.7 ± 0.9 (5.1 %)	7.8 ± 0.4 (5.6 %)	< LOQ [d]	1.9 ± 0.1 (7.3 %)	< LOQ [d]
EQ	940.9 ± 31.0 (3.3 %)	509.7 ± 27.3 (5.3 %)	223.8 ± 11.0 (4.9 %)	5.7 ± 0.3 (5.3 %)	5.9 ± 0.3 (4.8 %)	1.8 ± 0.2 (13.8 %)
GE	4.4 ± 0.2 (3.8 %)	1.8 ± 0.1 (8.3 %)	1.5 ± 0.1 (8.1 %)	nd [e]	< LOQ [d]	nd [e]
FO	25.1 ± 0.9 (3.6 %)	31.6 ± 1.4 (4.4 %)	11.2 ± 0.4 (3.9 %)	< LOQ [d]	< LOQ [d]	nd [e]
BA	19.8 ± 0.9 (4.3 %)	17.3 ± 1.3 (7.4 %)	7.8 ± 0.6 (8.2 %)	< LOQ [d]	< LOQ [d]	nd [e]

[a] Expressed in $\mu g.g^{-1}$ DM (n=9 on 3 days)

[b] Samples have been taken from a cow feeding silage with ≥ 87 % of dehydrated red clover

[c] Samples have been taken from the same cow feeding silage with 0 % of dehydrated red clover

[d] LOQ for all compounds was 1.6 $\mu g.g^{-1}$ DM

[e] nd = no detected

Taken together, the results of the validation process showed that the proposed method could be used to reliably quantify, with accurate sensitivity, the isoflavone and EQ content in rumen and duodenal fluids, as well as in feces. So far as we know, only one study has proposed a validated method for analyzing the isoflavone and EQ content of rumen fluid (Kasparovska *et al.* 2016). Njåstad *et al.* (2014) also proposed a LC-MS/MS method for quantifying phytoestrogens in feed, omasal digesta, milk, urine and feces, but their study did not present a full validation process. Both these studies, however, suggested that the rumen is the main location of isoflavone metabolism and the feces one

of the main excretion routes. With the samples used in this study, it was possible to confirm that feed is an important factor affecting the EQ level in digestive juices and feces. When feed with a suspected high isoflavone content was used, the EQ concentration was high, whereas in feed with a suspected low isoflavone content there was very little EQ in the biological samples (Table 7.8). The study also showed that feces are an important EQ excretion route, with more than 20 % of the EQ estimated in rumen fluid being found in feces (Table 7.8). The present method, used in combination with other validated LC-MS/MS methods for quantifying isoflavones in forages (Daems *et al.* 2016b) and EQ in milk (Daems *et al.* 2015), enables the transformation of some isoflavones into EQ and their transfer between forages and dairy cow milk to be monitored with reliability and enough sensitivity. The transfer of these analytical methods to urine and blood matrices could be interesting topics for future studies, enabling researchers to acquire sensitive and reliable analytical tools to help them better understand the passage and metabolization of these phytoestrogenic compounds between forages and the milk.

Conclusion

This paper described the development and validation of an analytical protocol based on methanolic UAE, followed by enzymatic hydrolysis and UPLC®-MS/MS analysis, which enables four isoflavones and EQ in cows' feces and rumen and duodenal fluids to be simultaneously quantified. Sample preparation was optimized using response surface methodology and the method was validated in accordance with international recommendations. This sensitive, fast and reliable method is well suited to routine analysis and will be useful for studies on the transfer of isoflavones between forage plants and milk.

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Supplementary data

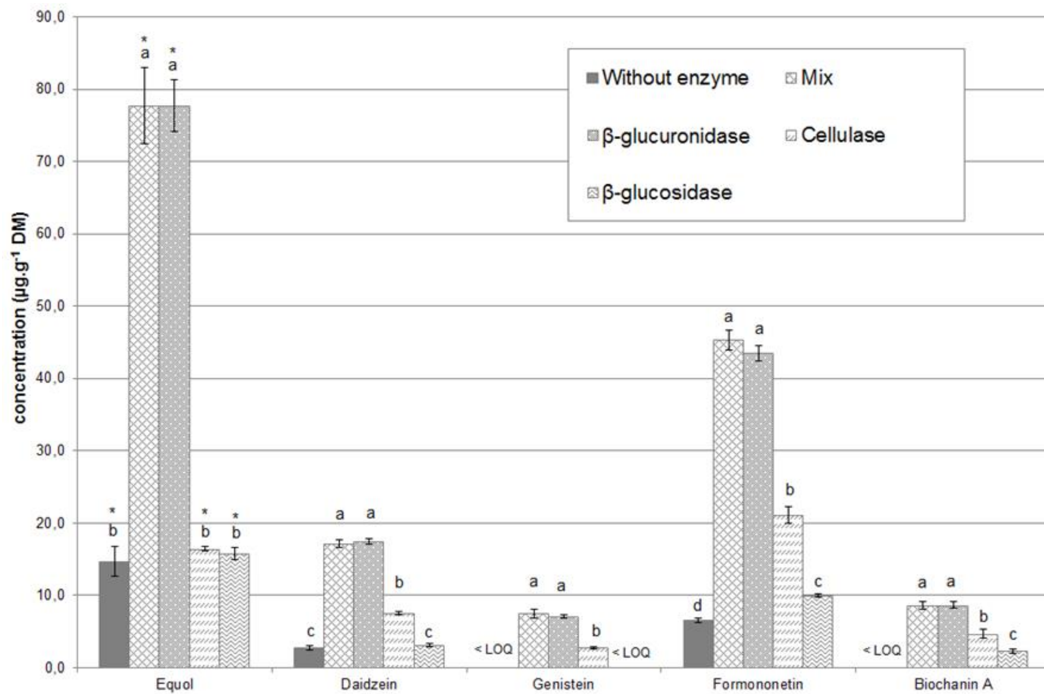

Figure S7.1. Effect of each enzyme on the isoflavone hydrolysis process from the digestive fluids (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. Enzymatic concentrations were set at ≥ 6, ≥ 12 and ≥ 5100 units.mL^{-1} of cellulase, β-glucosidase and β-glucuronidase, respectively. The hydrolysis conditions were: temperature 20 ± 2°C; time 18 h; and pH 6. Values marked by the same letter are not significantly different with the Tukey's HSD test (p-value > 0.05). * EQ concentrations were divided by a factor of 10.

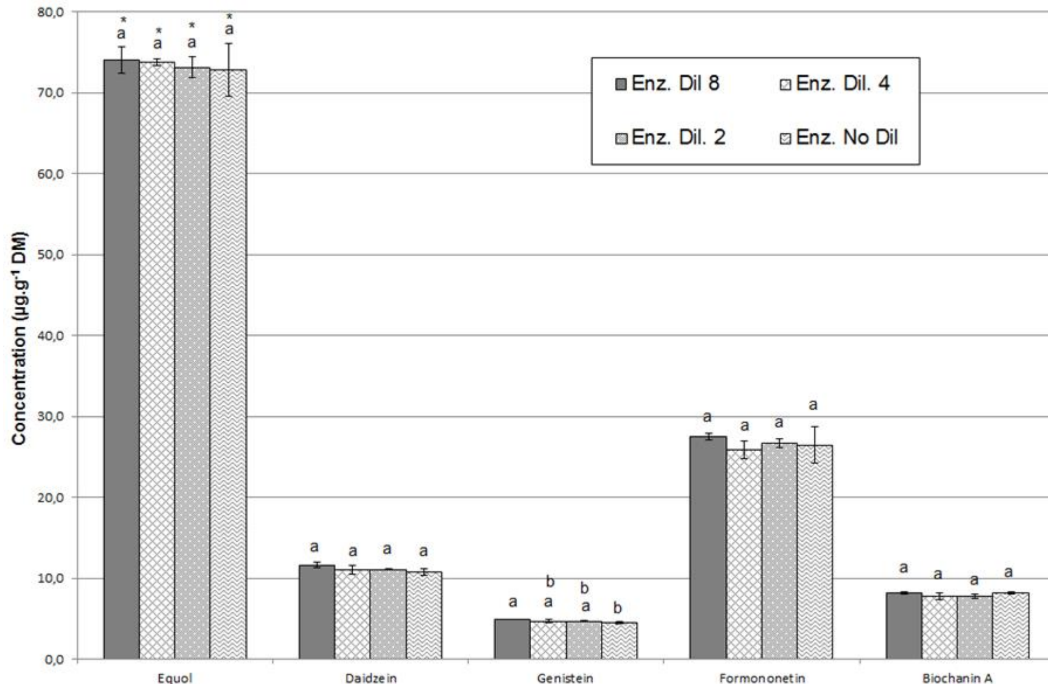

Figure S7.2. Effect of β-glucuronidase concentration on the isoflavone hydrolysis process from the digestive fluids (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. The hydrolysis conditions were: temperature 20 ± 2°C; time 18 h; and pH 6. 'Enz. No Dil'contained ≥ 10,200 units.mL^{-1} of β-glucuronidase and was then diluted by a factor of 2 (Enz. Dil 2), 4 (Enz. Dil 4) and 8 (Enz. Dil 8). Values marked by the same letter are not significantly different with the Tukey's HSD test (p-value > 0.05). * EQ concentrations were divided by a factor of 10.

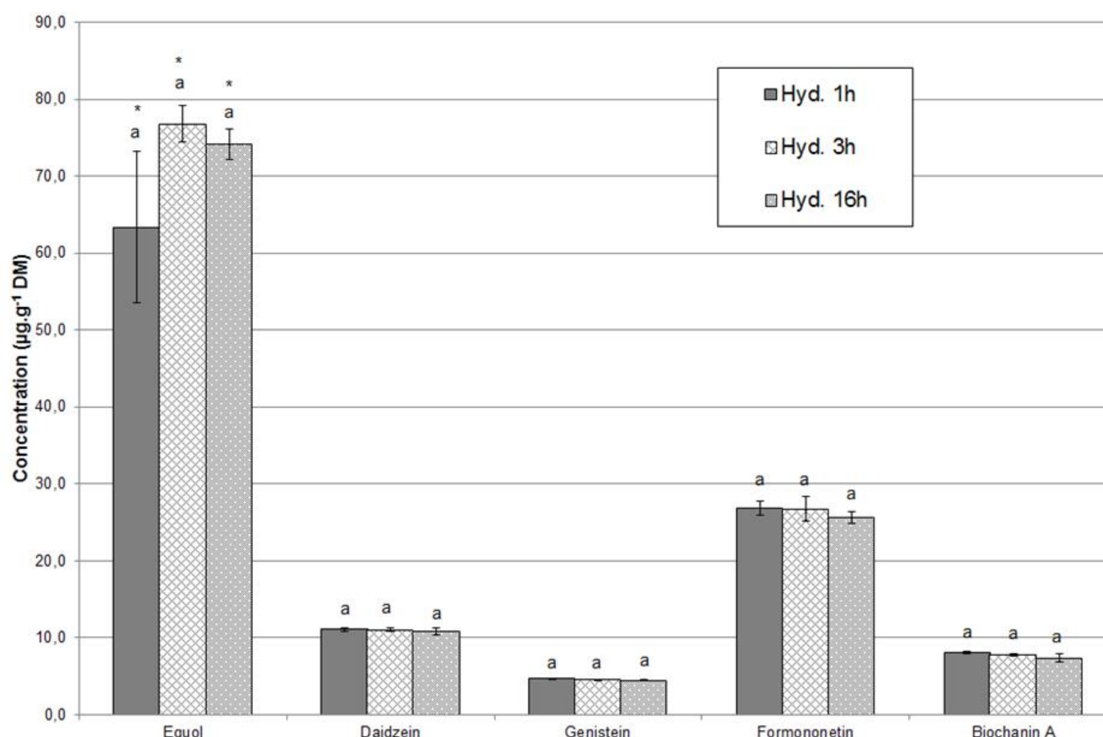

Figure S7.3. Effect of duration on the isoflavone hydrolysis process from the digestive fluids (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. The hydrolysis conditions were: temperature 20 ± 2°C and pH 6. β-glucuronidase concentration was ≥ 1,275 units.mL^{-1}. Values marked by the same letter are not significantly different with the Tukey's HSD test (*p*-value > 0.05). * EQ concentrations were divided by a factor of 10.

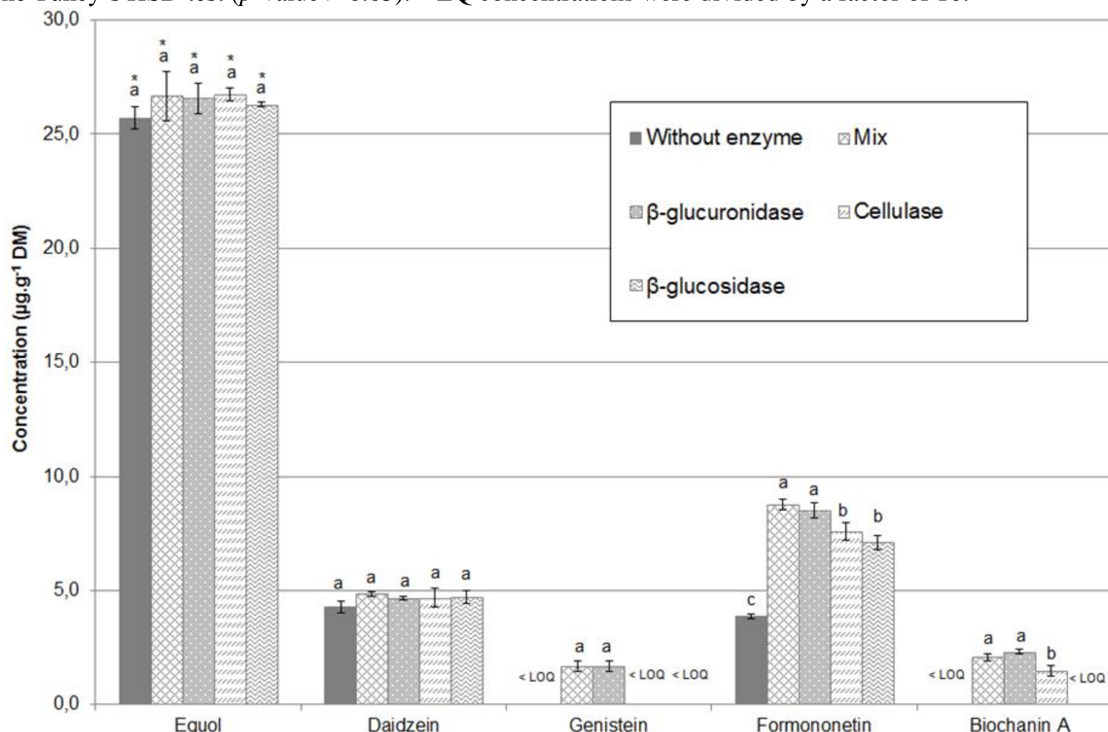

Figure S7.4. Effect of each enzyme on the isoflavone hydrolysis process from the feces (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. The hydrolysis conditions were: temperature 20 ± 2°C; time 18 h; and pH 6. Enzymatic concentrations were set at ≥ 6, ≥ 12 and ≥ 5100 units.mL^{-1} of cellulase, β-glucosidase and β-glucuronidase, respectively. Values marked by the same letter are not significantly different with the Tukey's HSD test (*p*-value > 0.05). * EQ concentrations were divided by a factor of 10. Note: the genistein (GE) and biochanin A (BA) concentrations found were near the fixed LOQ (1.6 µg.g^{-1} DM).

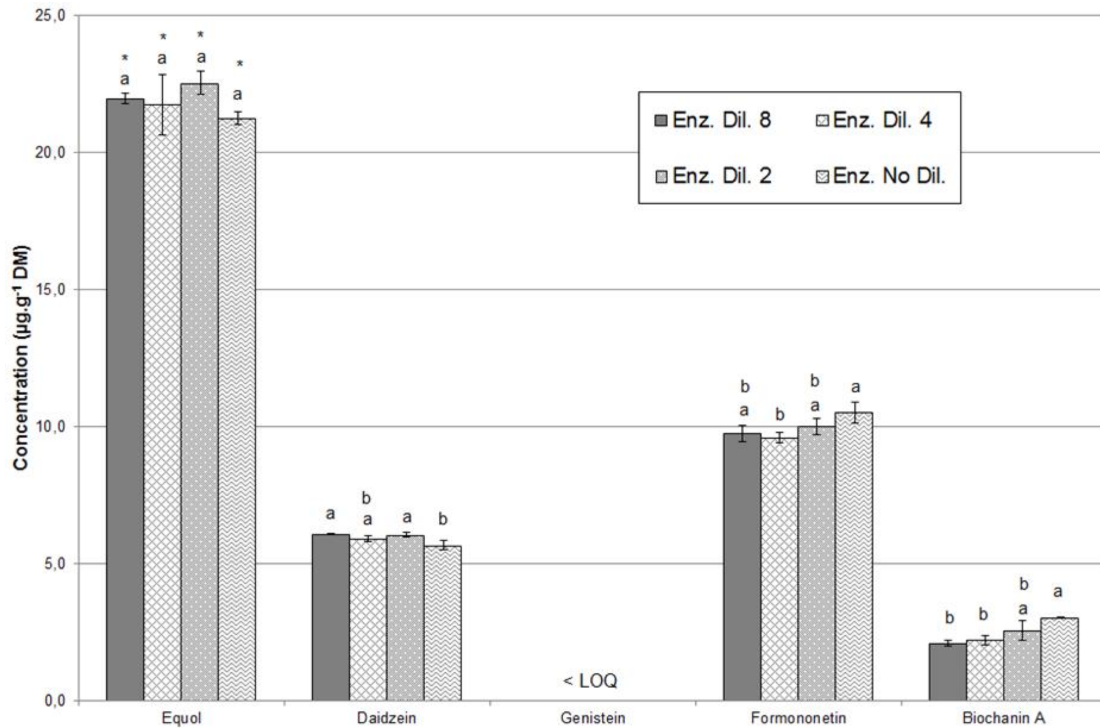

Figure S7.5. Effect of β-glucuronidase concentration on the isoflavone hydrolysis process from the feces (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. The hydrolysis conditions were: temperature 20 ± 2°C; time 18 h; and pH 6. 'Enz. No Dil'contained ≥ 10,200 units.mL^{-1} of β-glucuronidase and was then diluted by a factor of 2 'Enz. Dil 2', 4 'Enz. Dil 4' and 8 'Enz. Dil 8'. Values marked by the same letter are not significantly different with the Tukey's HSD test (*p*-value > 0.05). * EQ concentrations were divided by a factor of 10. LOQ for genistein (GE) was 1.6 µg.g^{-1} DM.

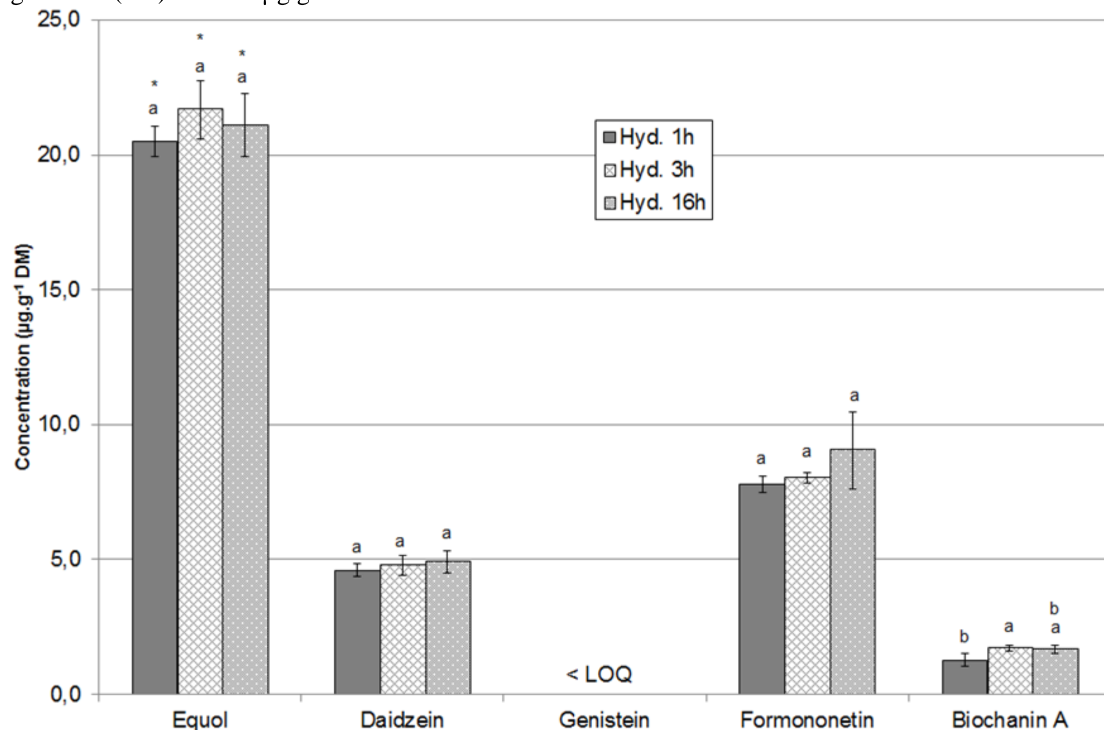

Figure S7.6. Effect of duration on the isoflavone hydrolysis process from the feces (n=3). The extraction conditions were: temperature 80°C; duration 10 min; MeOH concentration 55%; and solid-liquid ratio 0.5g.25mL^{-1}. The hydrolysis conditions were: temperature 20 ± 2°C and pH 6. β-glucuronidase concentration was ≥ 1,275 units.mL^{-1}. Values marked by the same letter are not significantly different with the Tukey's HSD test (*p*-value > 0.05). * EQ concentrations were divided by a factor of 10. LOQ for genistein (GE) was 1.6 µg.g^{-1} DM.

Table S7.1. Sum of isoflavone and EQ concentrations found in a representative sample after three consecutive extractions under selected UAE conditions

Compound	Extraction 1		Extraction 2		Extraction 3	
	Concentration found [a]	Extraction efficiency	Concentration found [a]	Extraction efficiency	Concentration found [a]	Extraction efficiency
	Duodenal fluid					
EQ	884.9 ± 26.6	88.8 %	99.5 ± 4.7	10.0 %	12.3 ± 1.0	1.2 %
FO	24.4 ± 0.9	85.4 %	4.2 ± 0.0	14.6 %	< LOQ [b]	-
DA	28.3 ± 2.2	89.1 %	3.5 ± 0.1	10.9 %	< LOQ [b]	-
BA	19.5 ± 0.3	80.2 %	4.8 ± 0.2	19.8 %	< LOQ [b]	-
GE	4.2 ± 0.1	100.0 %	< LOQ [b]	-	nd [c]	-
Total	*961.3 ± 26.8*	*88.6 %*	*112.0 ± 4.7*	*10.3 %*	*12.3 ± 1.0*	*1.1 %*
	Rumen fluid					
EQ	442.6 ± 13.9	90.1 %	42.7 ± 2.0	8.7 %	5.8 ± 0.4	1.2 %
FO	27.1 ± 1.5	89.5 %	3.2 ± 0.1	10.5 %	< LOQ [b]	-
DA	18.3 ± 0.6	91.0 %	1.8 ± 0.2	9.0 %	< LOQ [b]	-
BA	15.7 ± 2.4	86.1 %	2.5 ± 0.4	13.9 %	< LOQ [b]	-
GE	< LOQ [b]	-	nd [c]	-	nd [c]	-
Total	*503 ± 17.9*	*90.0 %*	*50.2 ± 2.3*	*9.0 %*	*5.8 ± 0.4*	*1.0 %*
	Feces					
EQ	205.3 ± 6.9	90.1 %	20.4 ± 0.4	8.9 %	2.3 ± 0.1	1.0 %
FO	11.3 ± 0.3	62.3 %	4.3 ± 0.1	23.9 %	2.5 ± 0.1	13.8 %
DA	11.0 ± 0.3	87.0 %	1.6 ± 0.1	13.0 %	< LOQ [b]	-
BA	9.3 ± 0.5	57.8 %	4.3 ± 0.2	26.4 %	2.5 ± 0.5	15.7 %
GE	1.7 ± 0.0	100.0 %	< LOQ [b]	-	nd [c]	-
Total	*238.6 ± 7.2*	*86.3 %*	*30.6 ± 0.4*	*11.1 %*	*7.3 ± 0.5*	*2.6 %*

[a] Expressed as μg.g^{-1} DM (Mean value ± standard deviation)
[b] LOQ is fixed at 1.6 μg.g^{-1} DM for all compounds
[d] no detected

Table S7.2. Intra-/inter-assay precision (RSD%) and accuracy (bias%) for the four isoflavones and EQ

Injected concentration	Instrumentation parameter		Day	DA	GE	EQ	FO	BA
5 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	8.5	10.3	8.7	3.2	12.2
			2	7.8	6.4	8.1	2.4	15.2
			3	8.9	5.6	3.6	1.2	6.3
		Inter-assay [d]		8.8	7.7	7.1	3.4	11.1
	Accuracy [b] (bias)	Intra-assay [c]	1	9.6 ± 5.6	7.2 ± 7.4	7.9 ± 7.1	8.0 ± 3.0	8.5 ± 8.0
			2	9.1 ± 8.4	5.6 ± 5.0	6.1 ± 6.4	6.8 ± 2.2	12.1 ± 7.3
			3	6.7 ± 5.4	4.1 ± 3.1	3.4 ± 1.6	2.6 ± 1.2	4.9 ± 5.1
		Inter-assay [d]		8.4 ± 6.3	5.6 ± 5.2	5.8 ± 5.5	5.8 ± 3.2	8.5 ± 7.1
15 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	3.6	2.5	4.4	1.5	2.1
			2	4.1	6.6	3.3	2.1	1.7
			3	2.7	3.3	3.4	2.6	1.0
		Inter-assay [d]		5.1	5.3	7.2	4.1	2.7
	Accuracy [b] (bias%)	Intra-assay [c]	1	6.2 ± 3.4	2.0 ± 1.1	2.9 ± 4.2	4.3 ± 1.6	4.6 ± 2.0
			2	3.1 ± 2.1	5.4 ± 3.3	6.6 ± 3.1	3.4 ± 2.0	4.0 ± 1.7
			3	3.0 ± 2.5	5.8 ± 3.1	11.4 ± 3.0	3.2 ± 2.0	0.8 ± 0.5
		Inter-assay [d]		4.1 ± 2.9	4.4 ± 3.0	7.0 ± 4.8	3.6 ± 1.8	3.1 ± 2.2
30 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	4.2	3.7	1.9	1.5	3.1
			2	2.7	4.9	2.8	3.4	2.2
			3	2.7	2.4	3.6	1.9	1.3
		Inter-assay [c]		3.8	4.6	5.8	3.8	4.4
	Accuracy [b] (bias%)	Intra-assay [c]	1	2.9 ± 3.0	6.2 ± 3.8	1.5 ± 1.3	8.3 ± 1.6	2.2 ± 1.9
			2	4.3 ± 2.6	3.7 ± 2.7	2.2 ± 2.0	2.9 ± 1.3	1.6 ± 1.3
			3	2.6 ± 0.8	1.7 ± 1.4	9.3 ± 3.3	3.5 ± 2.0	8.3 ± 1.4
		Inter-assay [d]		3.2 ± 2.3	3.9 ± 3.2	4.3 ± 4.2	4.9 ± 2.9	4.0 ± 3.4
50 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	3.0	2.2	1.4	2.1	2.2
			2	2.5	2.0	1.5	1.5	1.0
			3	2.1	3.9	2.0	1.9	2.4
		Inter-assay [d]		4.9	4.3	4.1	3.7	5.2
	Accuracy [b] (bias%)	Intra-assay [c]	1	3.3 ± 2.7	9.5 ± 2.4	2.2 ±1.4	9.7 ± 2.3	3.4 ± 2.3
			2	5.7 ± 2.3	1.7 ±1.5	4.3 ± 1.5	1.6 ± 1.3	0.7 ± 0.7
			3	3.4 ± 2.2	4.2 ± 3.6	4.5 ± 1.9	5.6 ± 2.0	12.1 ± 2.7
		Inter-assay [d]		4.1 ± 2.5	5.1 ± 4.1	3.7 ± 1.9	5.6 ± 3.8	5.4 ± 5.4
80 ng.mL^{-1}	Precision [a] (RSD%)	Intra-assay [c]	1	1.9	3.6	2.2	1.6	1.3
			2	2.3	1.6	5.0	2.3	1.9
			3	2.2	2.1	4.8	1.3	1.7
		Inter-assay [d]		5.6	4.0	5.3	3.5	5.9
	Accuracy [b] (bias%)	Intra-assay [c]	1	3.2 ± 1.9	6.2 ± 3.8	1.7 ± 1.2	3.9 ± 1.7	3.2 ± 1.4
			2	7.7 ± 2.2	1.4 ± 1.3	4.7 ± 4.0	3.0 ± 2.2	2.0 ± 1.5
			3	3.1 ± 1.6	2.0 ± 11	4.9 ± 4.6	2.5 ± 1.4	12.3 ± 1.9
		Inter-assay [d]		4.7 ± 2.9	3.2 ± 3.2	3.8 ± 3.6	3.2 ± 1.8	5.8 ± 4.9

[a] $RSD\% = \left(\frac{SD\ Conc_{\cdot Calc.}}{Mean\ Conc_{\cdot Calc.}}\right) \times 100$

[b] $Bias\% = Abs\left(\frac{(Conc_{\cdot Calc.} - Conc_{\cdot Nom.})}{Conc_{\cdot Nom.}}\right) \times 100$

[c] n=5
[d] n=15

Equations

Eq.S7.1) $Y_{Hyd.-Total} = 795.375 - 27.35X_1 - 3.8X_2 + 87.35X_3 - 16.2X_1X_2 + 6.8X_1X_3 + 1.25X_2X_3 - 13.6125X_1^2 + 9.2375X_2^2 - 48.7125X_3^2$

Eq.S7.2) $Y_{Hyd.-DA} = 10.9 - 0.0125X_1 - 0.1X_2 + 1.0375X_3 - 0.075X_1X_2 + 0.05X_1X_3 - 0.275X_2X_3 + 0.1X_1^2 + 0.275X_2^2 - 0.15X_3^2$

Eq.S7.3) $Y_{Hyd.-EQ} = 745.95 - 25.325X_1 - 1.5125X_2 + 80.2125X_3 - 15.2X_1X_2 + 8.45X_1X_3 + 0.525X_2X_3 - 13.5125X_1^2 + 8.7625X_2^2 - 49.8875X_3^2$

Eq.S7.4) $Y_{Hyd.-GE} = 3.9625 - 0.0625X_1 - 0.2875X_2 + 0.7X_3 - 0.225X_1X_2 - 0.2X_1X_3 + 0.2X_2X_3 + 0.2125X_3^2$

Eq.S7.5) $Y_{Hyd.-FO} = 27.84375 - 1.4125X_1 - 1.1375X_2 + 3.325X_3 - 0.75X_1X_3 + 0.65X_2X_3$

Eq.S7.6) $Y_{Hyd.-BA} = 6.9 - 0.5375X_1 - 0.7375X_2 + 2.1X_3 - 0.325X_1X_2 - 0.75X_1X_3 + 0.625X_3^2$

Eq.S7.7) $Y_{UAE-Dig.juice-Total} = 917.65 + 34.5625X_1 - 2.4375X_2 - 62.1X_3 - 12.675X_1X_2 - 7.15X_1X_3 - 15.7X_2X_3 - 0.9875X_1^2 - 18.0375X_2^2 - 167.1625X_3^2$

Eq.S7.8) $Y_{UAE-Dig.juice-DA} = 11.075 + 0.375X_1 + 0.0375X_2 - 0.1625X_3 - 0.075X_1X_2 - 0.325X_1X_3 - 0.5X_2X_3 + 0.3625X_1^2 - 0.3625X_2^2 - 1.4625X_3^2$

Eq.S7.9) $Y_{UAE-Dig.juice-EQ} = 853 + 31.8625X_1 - 2.0125X_2 - 68.6X_3 - 13.65X_1X_2 - 4.7X_1X_3 - 13.65X_2X_3 - 0.0875X_1^2 - 16.3375X_2^2 - 153.5125X_3^2$

Eq.S7.10) $Y_{UAE-Dig.juice-GE} = 4.725 + 0.3X_1 - 0.05X_2 + 0.275X_3 - 2.22e^{-16}X_1X_2 - 0.35X_1X_3 - 0.1X_2X_3 - 0.1125X_1^2 - 0.1125X_2^2 - 1.2125X_3^2$

Eq.S7.11) $Y_{UAE-Dig.juice-FO} = 32.875 + 1.0875X_1 + 0.075X_2 + 3.3125X_3 + 0.625X_1X_2 - 1X_1X_3 - 1.175X_2X_3 - 0.6625X_1^2 - 0.8375X_2^2 - 7.1125X_3^2$

Eq.S7.12) $Y_{UAE-Dig.juice-BA} = 12.975 + 0.9625X_1 - 0.475X_2 + 3.0375X_3 - 0.075X_1X_2 - 0.75X_1X_3 - 0.225X_2X_3 - 0.4625X_1^2 - 0.4375X_2^2 - 3.8625X_3^2$

Eq.S7.13) $Y_{UAE-Feces-Total} = 300 + 21.4X_1 + 0.55X_2 + 78.3X_3 + 0.275X_1X_2 - 17.525X_1X_3 - 2.875X_2X_3 - 11.3625X_1^2 + 1.0375X_2^2 - 77.3125X_3^2$

Eq.S7.14) $Y_{UAE-Feces-DA} = 8.95 + 0.9375X_1 + 0.2X_2 + 1.7625X_3 - 0.325X_1X_3 - 1.4625X_3^2$

Eq.S7.15) $Y_{UAE-Feces-EQ} = 274.275 + 16.55X_1 - 1.2875X_2 + 74.4125X_3 - 0.725X_1X_2 - 15.975X_1X_3 - 2.75X_2X_3 - 11.5375X_1^2 + 1.0875X_2^2 - 69.1625X_3^2$

Eq.S7.16) $Y_{UAE-Feces-FO} = 9.775 + 1.9375X_1 + 0.7375X_2 + 1.525X_3 - 0.2X_1X_2 - 0.375X_1X_3 - 0.075X_2X_3 - 0.4875X_1^2 - 0.0875X_2^2 - 3.1125X_3^2$

Eq.S7.17) $Y_{UAE-Feces-BA} = 6.95 + 1.9625X_1 + 0.8875X_2 + 0.575X_3 + 1.275X_1X_2 - 0.8X_1X_3 - 0.05X_2X_3 + 0.7875X_1^2 - 0.0125X_2^2 - 3.5875X_3^2$

Chapter VIII

General discussion, conclusion and perspectives

1 *Objectives*

2 This last chapter contains a brief statement of context in which this thesis was
3 realized, analytical advances achieved, as well as their first applications in the
4 understanding of the evolution of isoflavones concentration in silages and for estimating
5 the equol concentration in commercial cow's milk in Belgium. These advances are
6 discussed and some promising future studies are proposed. Finally, a general conclusion
7 will close these research works. Summary of the main points of the thesis are shown in
8 Fig. 8.1.

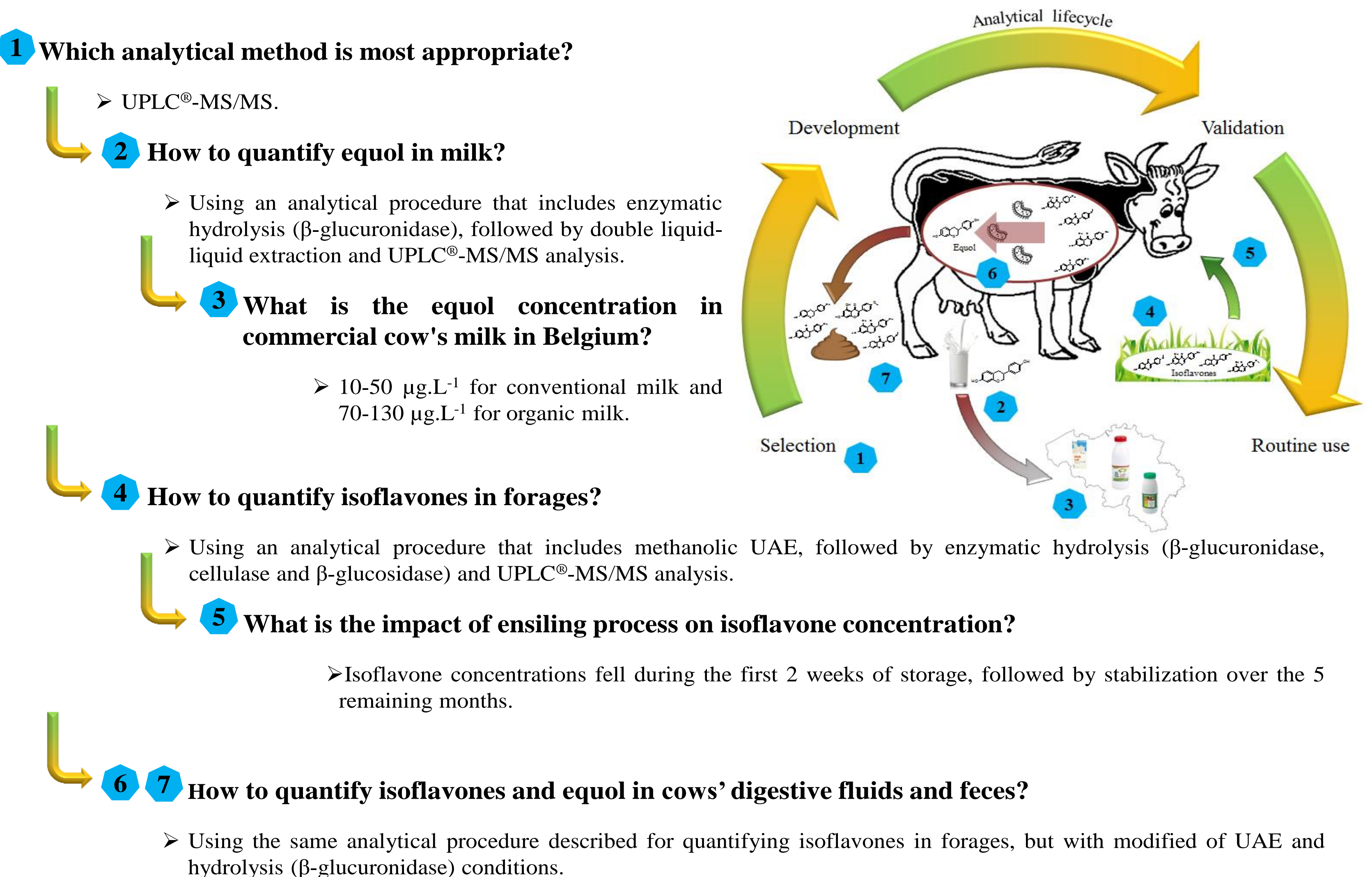

Figure 8.1. Graphical abstract of *Chapter VIII.* Summary of the main points of the thesis.

Context

Isoflavones are a subgroup of the phytoestrogens which have a widespread interest because of their association with a large variety of effects on human health. These natural plant substances are chemically similar to 17β-estradiol, giving the possibility to bind to estrogen receptors. They can also interact with the metabolism of steroid hormones (Pilsková *et al.* 2010; Mostrom & Evans 2012; Baber 2013; Vitale *et al.* 2013; Ko 2014; Sirotkin & Harrath 2014; Nemitz *et al.* 2016). The highest contents are found under conjugated forms in plants of the *Fabaceae* family, such as clover (*Trifolium pratense* L.) and soybean (*Glycine max* L.) (Mostrom & Evans 2012; Ko 2014). For example, soy is known for its high concentration of daidzein and genistein which can range between 1000 and 3500 $mg.kg^{-1}$ of dry weight. Red clover also contains these two compounds, but it is most widely known for its high concentration of formononetin and biochanin A which can range between 3000 and 15000 $mg.kg^{-1}$ of dry weight (Mostrom & Evans 2012). When they are ingested by animals (or humans), the conjugated forms are hydrolyzed before being absorbed by the body or metabolized by bacteria found in digestive system first, and then absorbed. Unconjugated forms seem to be highly absorbed by the digestive system and seem to be the most biologically active forms (Mostrom & Evans 2012; Kalač 2013; Vitale *et al.* 2013; Ko 2014; Nemitz *et al.* 2016). Their action on health are rather complex and may be related to large number of factors. They appear to have antioxidant activity and could offer alternative therapies for a range of hormone-dependent conditions, including cancer, cardiovascular disease, osteoporosis and menopausal symptoms (Mortensen *et al.* 2009; Pilsková *et al.* 2010; Mostrom & Evans 2012; Baber 2013; Vitale *et al.* 2013; Sánchez-Calvo *et al.* 2013; Ko 2014; Nemitz *et al.* 2016; Zhang *et al.* 2016). Nevertheless, these diphenolic compounds can also be considered as endocrine disruptors with the potential to have adverse health effects (Afssa 2005; Patisaul & Jefferson 2010; Wocławek-Potocka *et al.* 2013; Maggioni *et al.* 2013; Sirotkin & Harrath 2014; Wielogórska *et al.* 2015). These contradictory trends offer an attractive prospect for research.

Among the isoflavone microbial metabolites, equol is probably the most widely studied because it seems to have numerous health benefits (Setchell & Clerici 2010a; Jackson *et al.* 2011; Mostrom & Evans 2012; Sánchez-Calvo *et al.* 2013). It comes from the microbial metabolization of daidzein and formononetin (Mostrom & Evans 2012). In the human population, however, not everyone would be able to benefit from its positive

effects because of a lack of specific bacteria in their digestive systems. In Western populations, only one third is considered as "equol's producer" (Setchell & Clerici 2010a; Jackson *et al.* 2011; Sánchez-Calvo *et al.* 2013; Legette *et al.* 2014). An alternative strategy for making equol's health benefits available to all population would be the supply of this isoflavan in the human diet via food of animal origin. From the commonly foods consumed, milk from dairy cows is an interesting potential source of equol (Křížová *et al.* 2011a; Tsen *et al.* 2014). Moreover, to enrich naturally cow's milk with equol could also lead to the establishment of a new production sector. This could be achieved by increasing the amount of plants from *Fabaceae* family in the grasslands. Indeed, this plants family is known as a great source of equol precursors which are metabolized in equol in the cow's digestive system and then partly excreted in milk (Kalač 2013; Njåstad *et al.* 2014). A quality-differentiated milk will then be naturally produced that could be interesting for the consumers who are seeking products with potential health benefits (Palma *et al.* 2015). Moreover, plants from *Fabaceae* family have numerous potential agronomic properties and could help to increase the fixation of atmospheric nitrogen in the grasslands (Pflimlin *et al.* 2003). Currently, a number of researches are directed towards the study of the impact of a high-isoflavones diet on cow's milk quality, and also trying to fully understand their metabolization inside the animal body (Kalač 2013; Daems *et al.* 2016a; Kasparovska *et al.* 2016a; Kasparovska *et al.* 2016b). Numerous studies, however, are still needed, both agronomic and impact on human health fields, before claiming that it is a potential new quality-differentiated milk with health beneficial compounds.

The present thesis contributed therefore to study the feasibility to produce equol enriched cow's milk in Belgium through the establishment of analytical tools and their use in first experimental trials.

Discussion and perspectives

Analytical point of view

In order to improve knowledge in this field and ensure validity and reliability of the future results, the present thesis allowed developing and fully validating four analytical procedures for quantifying isoflavones (daidzein, formononetin, genistein and biochanin A) and equol in biological matrices (Fig. 8.1). Three of them were based on aglycones released by methanolic ultrasound assisted extraction, followed by enzymatic

hydrolysis and UPLC®-MS/MS analysis. They are aimed to quantify these compounds in forage, digestive fluids (ruminal and duodenal fluids) and feces. As far as we know, for digestives fluids and feces, it is the first time that an analytical procedure for simultaneously quantifying these five estrogenic compounds was fully validated. For the third matrix, there already exist several analytical methods for quantifying isoflavones in plants. Nevertheless, the originality of the method consists in the fact that it has been developed and validated taking into account the wide variety of forage plants likely to be grazed by cows, and the wide variation in isoflavones concentration. The sample preparation step of these analytical procedures have been optimized using the response surface methodology. The last of the four analytical procedures was dedicated solely to the predominant compound, among the five targeted compounds, found in milk: equol. This isoflavan was initially released by an enzymatic hydrolysis, followed by a double liquid-liquid extraction and analyzed by UPLC®-MS/MS. As far as we know, it was the first time that a quantification method of equol in milk was fully validated for routine work. The main advantage of the analytical method is the short sample analysis runtime required, which is important for high sample throughput.

The four analytical protocols proposed in this thesis included a hydrolysis step. Integrating a hydrolysis step depends on the objectives sought. If the interest is focused on the metabolization pathways of these phytoestrogens, a hydrolysis step should be avoided. The detection of all the conjugated forms could allow describing the complex metabolization pathways of these compounds. In addition, analysis of both conjugated forms and aglycones could allow to estimate the ratio between the two forms and to observe if one of them is more easily absorbed by the organism. However, as in the present thesis, if it's the overall content of the most biological forms which is desired, a hydrolysis step is therefore strongly advised (Daems *et al.* 2016a). It may be noted that the need of a hydrolysis step confirms that isoflavones and equol are essentially present under conjugated forms (Mostrom & Evans 2012), except for feces where the selected compounds seem to be under aglycone forms. As only *β*-glucuronidase/sulfatase from *Helix pomatia* was efficient to hydrolyze the conjugated forms of digestive juice, feces and milk samples, this confirms that most of the isoflavones and equol are conjugated with glucuronic acid or sulfate moiety in these biological samples (Nielsen *et al.* 2012; Mostrom & Evans 2012; Ko 2014). For forage samples, a mix of three enzymes (*β*-glucuronidase/sulfatase, *β*-glucosidase and cellulase) was needed, confirming that

isoflavones are usually found in other conjugated forms as glucosides, acetylglucoside, and malonylglucosides in plants matrix (Mostrom & Evans 2012; Konar *et al.* 2012; Ko 2014). As part of our researches, the lower calibration concentration was arbitrarily set at 5 $ng.mL^{-1}$ (1.6 $\mu g.g^{-1}$ of dry matter and 2 $ng.ml^{-1}$ for solid (all compounds) and milk (only equol) samples, respectively). This level was considered satisfactory for determining the samples with low or high concentration. Nevertheless, if smaller concentrations turn out to be biologically active and must be detected in samples (especially for food security), our researches have shown that it was possible to decrease the limit of quantification below the ppb level. If this becomes necessary, the use of a supplementary purification step before instrumental analysis (for example, SPE (Flachowsky *et al.* 2011; Wielogórska *et al.* 2015) or Quechers (Bustamante-Rangel *et al.* 2013)) may be realized. In this case, according to the level of quantification selected, more variation may be tolerated for some validation parameters (Ellis 2008; EMA 2015).

The choice to use UPLC®-MS/MS for quantifying the five targeted compounds in several biological matrices has been proved very appropriate. This instrumental analysis allows fewer sample manipulations than GC analysis, and high precision and sensitivity. UV detection could have been used for detection of the four isoflavones in forages where the concentration is higher than in the other biological matrices and where less sensitivity is therefore needed. Nevertheless, UV detection has been questioned for his specificity for distinguishing compounds of very similar structures as they often exhibit fairly similar UV absorption characteristics (Vacek *et al.* 2008; Raju *et al.* 2015). In addition, this detection method is unsuitable for equol measurement which exhibits poor UV absorption characteristics (Setchell & Clerici 2010a; Ko 2014). In order to keep the same instrumental analysis conditions for identifying and quantifying the five selected compounds in the majority of biological matrices, UV detection seemed therefore less attractive. In addition, during our analysis, it was found that the isoflavone concentration could be as low as a few parts per billion 'ppb' according to the sample origin, thus confirming the need of a sensitive method. With its short analysis time, low solvent consumption, high resolution, and high selectivity and sensitivity properties, UPLC®-MS/MS with an ESI and working in MRM mode, appears to be the fastest and easiest method to implement and to produce reliable results.

If improvements must be achieved, this will be done rather at the sample preparation step than at the instrumental one. Indeed, there is a growing demand for faster

and environment friendly analytical methods (Rostagno *et al.* 2009; Chemat *et al.* 2015). By using UPLC® technology, these requirements are achieved (Gumustas *et al.* 2013). For samples preparation step, if a hydrolysis step is needed, it will be difficult to decrease the time needed for a total hydrolysis of conjugate forms. For extraction of compounds of interest, however, the use of more eco-friendly extraction methods could be considered (Rostagno *et al.* 2009; Mustafa & Turner 2011; Talmaciu *et al.* 2015). Zuo *et al.* (2008) developed an interesting sample preparation procedure for isoflavones extraction from soybean meal based on supercritical CO_2 extraction modified by a mixture of methanol and water. Klejdus *et al.* (2010) proposed a method with a sonication pretreatment before the extraction of isoflavones from algae by supercritical CO_2. This technology reduces extraction time and solvent consumption, but requires more expensive and complex equipment than conventional solvent extraction techniques.

Beside the improvement of the sample preparation steps, it would be also interesting to transfer the analytical method used for digestive juices and feces to other biological matrices, such as blood, urine and milk. That would allow to follow the pathway of estrogenic compounds in cow with a single instrumental analysis. It would be also interesting to extend the number of estrogenic compounds quantifiable with this method. For example, *O*-desmethylangolensin (equol's less known cousin) is also a bacterial metabolite of daidzein for which several biological actions have been observed *in vitro* (Frankenfeld 2011), or whether, lignans which are included in the large family of phytoestrogens and that can also be transformed in microbial metabolites with potential human health impact (Gagnon *et al.* 2009; Zhou *et al.* 2009; Landete 2012).

Research field point of view

In the first stage of this research project, called *PhytoHealth*, the results not allow to conclude if the equol found in milk could have an impact on human health. Nevertheless, the analytical tools developed and validated have already been used successfully in two scoping studies and are included in this thesis (Fig. 8.1). In another project, called *GrassMilk*, these analytical methods were used and have allowed to show that proportion of red clover (*Trifolium pratense* L.) in the feed ration was positively correlated with milk concentration of equol (unpublished data in this thesis).

Currently, a number of researches are directed towards a better understanding of the impact of a high-isoflavones diet on cow's milk quality, especially to increase equol

concentration (Kalač 2013; Daems *et al.* 2016a). To create quality differentiated milk implies to be able to produce this new type of milk throughout the year, even in winter period when cows are inside the barn. Given that forages silage constitute an important part of ruminant feeding in temperate regions (Huyghe *et al.* 2014), it was therefore important to study the change of equol precursors in silage during the storage period. This lack of information was highlighted by Kalač (2013). Therefore, a study about the kinetic evolution of the isoflavones in red clover (*Trifolium pratense* L.) silage over 6-month period in laboratory-scale silos using a vacuum-packing system was for the first time done. The impact of the field drying process was also assessed. The results suggest that 4 days field-drying process has no impact on isoflavone concentration, except for daidzein, whose concentration increased twofold. These results are in accord with those reported by Jones (1979), but not with those reported by Sarelli *et al.* (2003), Sakakibara *et al.* (2004) and Sivesind & Seguin (2005), who suggested that wilting process reduced the isoflavone concentrations in clover. Despite good conservation parameters, the isoflavones concentrations in laboratory-scale silos were sharply decreased during the first 2 weeks of storage, followed by stabilization over the 5 remaining months. These results differ again from those reported by Sarelli *et al.* (2003) and Sakakibara *et al.* (2004). For the first one, isoflavone concentration was higher in ensiled red clover than in wilted herbage before ensiling; for the second, the concentration remained stable for the 2 first months of the ensiling process. Our results, however, are in accord with a third study (Sivesind & Seguin 2005) who reported that isoflavone concentration also decreases between fresh and ensiled samples. The difference between the studies could be due to differences in the ensiling process, such as differences in pH, silage additives, temperature, microbial population, plant phenological stage and botanical composition. These observed differences are really interesting and the trends that emerged in our study need to be confirmed using silages that are much closer to reality in the field. On the basis of our results, feeding cows with silage, rather than fresh plants or hay, could reduce their equol precursor's intake; and, therefore, also reduced the excretion of equol in milk. This assumption needs to be confirmed with field trials, because many other factors, such as growth stage of forages, quality of fermentation process inside the silos or bacterial flora in the cows' digestive system, could influence the production of equol.

Thanks to the method dedicated for quantifying equol in milk, it has been possible for the first time to assess the equol concentration in Belgian commercial cow's milk that we consume daily. Around fifty samples of all types of cow's milk available in

supermarkets and few raw samples from dairy farms were collected and analyzed in February 2013. This study showed that equol was found in all the samples analyzed, with a concentration ranging from 10 to 50 $\mu g.L^{-1}$ for conventional milk and from 70 to 130 $\mu g.L^{-1}$ for organic milk. As previously stated in other studies (Hoikkala *et al.* 2007; Mustonen *et al.* 2009; Höjer *et al.* 2012; Adler *et al.* 2015), the higher use of grass products to feed animals, often rich in *Fabaceae* species that are a great source of equol precursors, could explain this striking difference between equol concentration of organic and conventional milks. Equol concentration reached an average of 191 and 411 $\mu g.L^{-1}$ in organic milks from France (Antignac *et al.* 2004) and Finland (Hoikkala *et al.* 2007), respectively. In their animal feeding experiments, Höjer *et al.* (2012) reached 1539 $\mu g.L^{-1}$ (conversion factor: 1kg = 1.03L of milk (Mathieu 1998)). These concentrations are encouraging given that only dairy products and eggs appear to constitute a potential source of equol in Western diet, meat products and fishes does not seem to contain this isoflavan (Kuhnle *et al.* 2008).

In order to create a livestock sector with equol-enriched milk, one need to know whether a normal consumption of milk might contain interesting equol content for human health. With these first research works, it has not yet been possible to give an answer to this question. It will nevertheless be possible to estimate the daily average consumption of equol. In 2011, the daily per capita intake of milk and dairy products (excluding butter, suspected to have no equol (Kuhnle *et al.* 2008)) was estimated at 1082 g in Finland (the biggest milk consumer country in Europe that year), 616 g in Belgium, 703 g in USA, 598 g in Canada, 630 g in Australia and 195 g in Japan (FAOSTAT 2011a). Taking the highest equol concentration found in Belgian commercial milks analyzed (129 $\mu g.L^{-1}$), the average daily intake was about 77 µg for a Belgian. If one takes into account the highest equol concentration found by Höjer *et al.* (2012) in their animal feeding experiments (1494 $\mu g.kg^{-1}$ of equol in milk from cows feeding red clover-grass silage diet), the intake would be about 1617, 919, 1051, 894, 941 and 292 µg of equol per day for Finland, Belgium, USA, Canada, Australia and Japan, respectively. It was therefore found that it seems possible to increase equol concentration in milk by modifying the farm management and changing the cow feedstuffs. Equol concentration in milk, however, remains relatively low compared with the 10 mg per day recommended by Usui *et al.* (2013) to decrease the overweight and obesity problems. This recommended quantity represent an intake of $\geq$ 6 and $\geq$ 77 L of milk with the higher equol

concentration found by Höjer *et al.* (2012) and in samples analyzed (Daems *et al.* 2015), respectively. At this stage, it is obvious that this daily milk consumption is not realistic. Another approach is based upon an assumption that "equol's producers" have fewer health problems than "equol non-producers". According to Jackson *et al.* (2011), individuals with a plasma equol concentration of > 5-10 $\mu g.L^{-1}$ are classified as "equol's producers". If we take 5 $\mu g.L^{-1}$ as reference concentration for “equol’s producers”, this concentration representing approximately 12.5 µg on a bodily level (with an assumption that the human body contains approximatively 2500 mL of plasma). On this basis, ≥ 97 mL of milk with the higher equol concentration found in our Belgian commercial milks analyzed would be sufficient to provide enough daily equol content, like an “equol’s producer”. If one takes into account the highest equol concentration found by Höjer *et al.* (2012), ≥ 8 mL would be enough. In this case, this daily milk consumption is completely realistic and milk enriched with equol would become an interesting source of equol for “equol non-producers”. Nevertheless, these are a very simplistic approaches that nor does it take into consideration several factors, like absorption or metabolization of equol in individuals, as well as his bioaccumulation in the body. Nowadays, there is no fixed limit of equol concentration to define if an individual is "equol's producers" or not. This issue will need to be clarified in futher studies.

The potential ways to bring equol to “equol non-producers” is a last subject that it would be interesting to approach in this discussion section. As mentioned previously, only a part of the population (about 30% of the European and about 80% of the Asian population (Setchell & Clerici 2010a; Jackson *et al.* 2011; Sánchez-Calvo *et al.* 2013)) is able to produce equol in their digestive system. For the segment of the population that is not able to produce equol, two potential ways might be pursued to bring the health benefits of this compound to these individuals: by chemical synthesis or metabolization by specific bacteria. The chemical synthesis of equol (Heemstra *et al.* 2006; Li *et al.* 2009; Setchell *et al.* 2009b) and its spiking in foods commonly consumed or food supplements could be a potential way. As far as we know, this way has not yet been proposed and, however, it is not in line with the idea of a natural intake. Nevertheless, the chemical synthesis of equol could offer a large-scale production of pure compound for its use in clinical and animal studies (Setchell & Clerici 2010a). For the second way, with the solution to use of equol-producing bacteria, an enrichment of human diet with equol prior to consumption and/or modulation of the intestinal microbiota via the ingestion of

viable bacteria capable of in situ generation of this compound (Di Cagno *et al.* 2010), could also be considered. Decroos *et al.* (2006) found that administration of equol-producing bacteria to a simulator of the gastrointestinal microbial ecosystem 'SHIME', inoculated with an "equol non-producer" fecal sample, resulted in the formation of equol. Nevertheless, little information is currently available and further *in vivo* studies are needed to confirm these results. With this finding, probiotic supplements may eventually prove to be an effective mean to ensure equol production for "equol non-producers" which consume isoflavones rich food. Currently, enrichment of human diet or food complements with equol prior to consumption is the way which seems to be more often chosen. Natural enrichment of foods of animal origin seems to be conceivable. Like the objective of the present thesis, equol precursors found in forages were metabolized by specific bacteria to equol in cow's digestive system, and partly excreted in the milk produced (Kalač 2013). But it is not the only one, other animal products, like eggs, could also be a supplementary source of equol in human diet (Saitoh *et al.* 2004; Kuhnle *et al.* 2008; Abiru *et al.* 2012). In Asia, however, soy fermented products was described as equol source. Indeed, soybean is known as a great source of equol's precursors (Mostrom & Evans 2012). Recent studies found that stinky tofu (a popular traditional fermented soy food in Taiwan) contained substantial amounts of equol (Abiru *et al.* 2012; Jou *et al.* 2013; Rui *et al.* 2014). Abiru *et al.* (2012) analyzed 16 stinky tofu samples purchased during different seasons and the average equol concentration ranged from 0.34 to 2.68 $mg.100g^{-1}$. Other studies investigated the use of equol-producing bacteria to convert isoflavones into equol during soy milk fermentation (Tsangalis *et al.* 2002; Tsangalis *et al.* 2004). Tsangalis *et al.* (2002) have obtained an equol concentration of 5.37 $mg.100\ L^{-1}$ in their soymilk fermented by *Bifidobacterium animalis* for 48h of incubation at 37°C. Other studies even tried to use tablets of equol, produced from a mix of bacteria a soy germ isoflavones, to address healthy problems (Yee *et al.* 2008; Setchell *et al.* 2009a). Even if their consumption in Europe is marginal compared to Asia (FAOSTAT 2011b), soybean fermented products are therefore an interesting source of equol for "equol non-producers". But, like animal products, the biological activity of the resulting equol-enriched products was not clinically tested. The *in vivo* studies about relationship between equol intake and impact on human health, however, remains limited and they are often tested on few fixed dose provided to patients. Nowadays, more *in vitro* researches, together with clinical intervention studies, are still needed to assess the real role of equol

in human health (Magee 2011). Producing an equol-enriched food, as well as food supplements, is a really interesting research field with a broad range of potential studies.

Conclusion

The present work contributed to studying the feasibility to produce cow's milk fortified with equol through the development and the validation of four analytical procedures:

- One quantification method for equol in milk based on an enzymatic hydrolysis, followed by a double liquid-liquid extraction and analyzed by UPLC®-MS/MS method.
- One quantification method for four isoflavones in forage based on an UAE, followed by an enzymatic hydrolysis and analyzed by UPLC®-MS/MS method.
- One quantification method for four isoflavones and equol in digestive fluids based on an UAE, followed by an enzymatic hydrolysis and analyzed by UPLC®-MS/MS method.
- One quantification method for four isoflavones and equol in feces based on an UAE, followed by an enzymatic hydrolysis and analyzed by UPLC®-MS/MS method.

The development and validation of these analytical methods bring new reliable tools which will allow continuing improvements of scientific knowledges about the passage of isoflavones from forages to cow's milk. They have already proved their usefulness in two original experimental studies. One of them estimated for the first time the equol concentrations in commercial cow's milk in Belgium and highlighted most important concentrations in milks originating from organic farming than conventional farming. In the second one, the study of change of isoflavone concentration over the storage period in laboratory-scale silos allowed for the first time to highlight a major loss of isoflavone concentration at the beginning of the ensiling process, which suggested that the reactions (chemical and/or bacterial) generated at the beginning of the ensiling process would be the cause of the decrease of isoflavones concentration.

The establishment of a new quality differentiated animal product, based on cow's milk naturally fortified with equol, is a really interesting research field with a broad range

of potential studies. Analytically, the constant evolution in analytical technologies should be take into account and allow to propose faster and environment friendly methods, while keeping a good sensitivity. The present works compliment a research field already well documented and are paving the way for a new directions for reflection, where the medical world will need to be included to the agricultural reflection. Future studies are needed in order to improve our understanding of the metabolization pathways of isoflavones and to assess the real impact of these secondary plant substances and their microbial metabolites on human and animal health. Furthermore, it would be interesting to evaluate if the equol conjugated with a glucuronide fragment (major form of equol in milk) is absorbed similarly than aglycon form by human body. It will also be necessary to study the opportunity to setting up a controlled agricultural sector producing milk fortified with equol. This will require exploring the inter-individual variation among cows in terms of the efficiency of metabolizing these estrogenic compounds. In animal feeding experiments it would also be interesting to investigate the impact of feeding modifications on the quality of milk produced and the health of the cows, as well as the possibility to produce this quality differentiated milk throughout the year. The impact of various milk processing on concentrations of isoflavones and their metabolites should still clearly be elucidated. And, finally, if the production of milk fortified with equol is interesting, it will be necessary to provide product specifications and, eventually, other analytical tools to realize field monitoring. As you can see, this is a really interesting research field which leaves the door wide open for numerous further studies.

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Annex A

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Annex B

List of scientific productions

The following scientific productions were realised during this PhD thesis. All these communications are compiled in: http://orbi.ulg.ac.be/.

Publications

- Daems, F., Romnee, J.-M., Rasse, C., Froidmont, É., Lognay, G., 2016. Optimized Quantitative Method for Determining Isoflavones and Equol in Bovine Digestive Fluids and Feces. *Analytical Letters*. *(under revision)*
- Daems, F., Decruyenaere, V., Agneessens, R., Lognay, G., Romnee, J.-M., Froidmont, É., 2016. Changes in the isoflavone concentration in red clover (*Trifolium pratense* L.) during ensiling and storage in laboratory-scale silos. *Animal Feed Science and Technology*, 217, pp.36-44.
- Daems, F., Romnee, J.-M., Rasse, C., Froidmont, É., Heuskin, S., Lognay, G., 2016. Quantification of four isoflavones in forages with UPLC®-MS/MS, using the box-behnken experimental design to optimize sample preparation. *Chromatographia*, 79, pp.711-725.
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- Daems, F., Romnee, J.-M., Lognay, G., Froidmont, É., 2015. UPLC®-MS/MS, an analytical tool for the accurate and rapid quantification of phytoestrogen metabolites in milk. *Biotechnology, Agronomy, Society and Environment*, 19(2), pp.121-122.
- Daems, F., Jasselette, C., Romnee, J.-M., Planchon, V., Lognay, G., Froidmont, É., 2015. Validating the use of an ultra-performance liquid chromatography with tandem mass spectrometry method to quantify equol in cow's milk. *Dairy Science & Technology*, 95(3), pp.303-319.
- Daems, F., Franckson, D., Jasselette, C., Lognay, G., Romnee, J.-M., Froidmont, É., 2014. Monitoring of phytoestrogens content in red clover silage. *Rencontres Recherches Ruminants*, 21, pp.80.
- Franckson, D., Daems, F., Jasselette, C., Romnee, J.-M., Froidmont, É., 2014. Impact of grass silage composition on milk nutritional quality. *Rencontres Recherches Ruminants*, 21, pp.81.

- Daems, F., Franckson, D., Jasselette, C., Lognay, G., Romnee, J.-M., Froidmont, É., 2013. Determination of equol content in milks marketed in Wallonia (Belgium). *Rencontres Recherches Ruminants*, 20, pp.180.

Article outside the scope of the PhD thesis

- Daems, F., Béra, F., Lorge, S., Fischer, C., Brostaux, Y., Francis, F., Lognay, G., Heuskin, S., 2016. Impact of climatic factors on the release of E-β-caryophyllene from alginate beads. *Biotechnology, Agronomy, Society and Environment*, 20(2), pp.130-142.

Article published in a journal without a review committee

- Franckson, D. & Daems, F., 2015. Le trèfle violet, une plante à exploiter dans la ration hivernale. *CRA-WINFO*, N°46, pp.1.

Oral presentations

International

- Daems, F., Romnee, J.-M., Lognay, G., 2016. Use of response surface metodology to optimize samples preparations in laboratory. *Chimiometrie XVII*, Namur, Belgium.
- Daems, F., Lognay, G., Romnee, J.-M., Froidmont, É., 2015. Validation d'une méthode d'analyse quantitative de l'équol par UPLC®-MS/MS dans le lait de vache - Application aux laits commercialisés en Wallonie (Belgique). *83èmes Congrès de l'Acfas*, UQAR, Rimouski, Canada.
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