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Identification of isoflavonoid metabolites in humans

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ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Science of the University of Helsinki, for public examination in the Small Auditorium, Haartman Institute, on 3 November 2006, at 12 noon.

Helsinki 2006

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ISBN 952-92-1054-X (pbk.)
ISBN 952-10-3430-0 (pdf)
http://ethesis.helsinki.fi/julkaisut/mat/kemia/vk/
Yliopistopaino
Helsinki 2006

To Harri, Oskari, Ilona, and Annika

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ABSTRACT

Epidemiological studies have associated high soy intake with a lowered risk for certain hormone-dependent diseases, such as breast and prostate cancers, osteoporosis, and cardiovascular disease. Soy and soy foods are rich sources of isoflavones, diphenolic plant compounds that have been shown to possess several biological activities. Soy is not part of the traditional Western diet, but many dietary supplements are commercially available in order to provide the proposed beneficial health effects of isoflavones without changing the original diet. These supplements are usually manufactured from extracts of soy or red clover, which is another important source of isoflavones.

For many years, the interest in isoflavonoids and their possible role in the prevention of diseases has mainly focused on research on daidzein and genistein, the principal isoflavones of soy and the main metabolites of formononetin and biochanin A, the principal isoflavones of red clover. Until recently, detailed studies of the metabolism of these compounds have been lacking. However, understanding the metabolic fate of dietary compounds shown to possess biological activities is important since metabolism affects the ingested compound in many ways, either enhancing or reducing its bioactivity, absorption, levels of active compounds in plasma or target tissues, and elimination of the compound from the human body.

The aim of this study was to identify urinary metabolites of isoflavones originating from soy or red clover using gas chromatography - mass spectrometry (GC-MS). To examine metabolism, two supplementation studies with human volunteers were carried out. In the first experiment, six volunteers included three soy bars per day in their normal Western diet for a two-week period, after which daily urine samples were collected. In the second experiment, seven volunteers ingested four dietary supplements manufactured from red clover extract, and urine, plasma and fecal samples were collected on five consecutive days after the isoflavone supplementation. In addition to soy and red clover supplementation studies, the metabolism of isoflavones was investigated *in vitro* by identification of metabolites formed during a 24-h fermentation of pure isoflavones with a human fecal inoculum. Qualitative methods for identification and analysis of isoflavone metabolites in urine and fecal fermentation samples by GC-MS were developed. Moreover, a detailed investigation of fragmentation of isoflavonoids in electron ionization - mass spectrometry (EI-MS) was carried out by means of synthetic reference compounds and deuterated trimethylsilyl derivatives.

After isoflavone supplementation, 18 new metabolites of isoflavones were identified in human urine samples. For most of the metabolites, synthetic reference compounds were available for structure characterization. Of the nine metabolites for which the authentic reference compounds were unavailable, identification of five was confirmed by comparison of their chromatographic and mass spectrometric data with those of metabolites identified in fecal fermentations of pure isoflavones. Four metabolites were

tentatively identified by interpretation of their mass spectrum using deuterated trimethylsilyl derivatives.

The most abundant urinary metabolites of soy isoflavones daidzein, genistein, and glycitein were found to be the reduced metabolites, i.e. analogous isoflavanones, α-methyldeoxybenzoins, and isoflavans. Metabolites having additional hydroxyl and/or methoxy substituents, or their reduced analogs, were also identified. The levels of these metabolites in urine samples were notably lower than those of the reduced isoflavone metabolites. The main metabolites of red clover isoflavones formononetin and biochanin A were identified as daidzein and genistein, which is consistent with the results of earlier studies. In addition, reduced and hydroxylated metabolites of formononetin and biochanin A were identified; however, they occurred at much lower levels in urine samples than daidzein or genistein or their reduced metabolites.

The results of this study show that the metabolism of isoflavones is diverse; many isoflavonoid metabolites are found in urine after isoflavone supplementation. Metabolism may thus play an important role in effects and hitherto unknown mechanisms of action of isoflavones. Recently, equol, an intestinal metabolite of daidzein with an isoflavan structure, has gained considerable interest. Evidence suggests that the benefits of soy may be related to the ability of intestinal microflora to convert daidzein to equol, which differs largely between individuals. More studies are needed to determine whether the new isoflavan metabolites and the other isoflavonoid metabolites identified here have biological activities that contribute to the proposed beneficial effects of isoflavones on human health. Another task is to develop validated quantitative methods to determine the actual levels of isoflavones and their metabolites in biological matrices in order to assess the role of isoflavones in prevention of chronic diseases.

ACKNOWLEDGMENTS

These studies were carried out at the Institute for Preventive Medicine, Nutrition, and Cancer, Folkhälsan Research Center, and at the Division of Clinical Chemistry, University of Helsinki. I am grateful to Professor Herman Adlercreutz, Head of the Institute for Preventive Medicine, Nutrition and Cancer, and Docent Per-Henrik Groop, former Research Director of the Folkhälsan Research Center, for setting the research facilities of the laboratory at my disposal. Financial support from the Sigrid Jusélius Foundation, the Finnish Cultural Foundation, and the European Commission is gratefully acknowledged.

My deepest gratitude is due to my main supervisor, Professor Herman Adlercreutz, who introduced me to the fascinating field of phytoestrogen research. Herman, thank you for your inspiring and supportive guidance. Your consistent enthusiasm and optimistic attitude towards research are qualities I most wish to emulate. I also sincerely thank my second supervisor, Professor Kristiina Wähälä, for constructive comments, advice, and continuous support throughout the studies. Professor Tapio Hase is warmly acknowledged for the interesting and instructive discussions we had on mass spectrometry of isoflavonoids.

Professors Pirjo Vainiotalo and Patricia Murphy are thanked for careful review of this thesis and for the constructive comments that led to its improvement. Carol Ann Pelli is acknowledged for revision of the English language of the manuscript.

I am grateful to all collaborators for their contributions. Professor Kaisa Poutanen, Docent Kirsi-Helena Liukkonen, and Dr. Anna-Marja Aura from VTT Biotechnology are thanked for carrying out the *in vitro* experiments and for fruitful cooperation in lignan research outside the focus of this thesis. I also thank Antti Hoikkala, Tuija Jokela, and Dr. Auli Salakka from Laboratory of Organic Chemistry for carrying out the syntheses of the reference compounds.

Over the years, I have been lucky to meet and work with many outstanding people. I thank Dr. Tarja Nurmi, my collegue, for expert comments, counsel, and comfort on any problem that I faced in analytical methods, research, or life in general. I am grateful for her friendship and enjoyable company, especially on congress trips abroad. I am also grateful to Dr. Katariina Stumpf, Dr. José Peñalvo, Anna-Maria Linko, and Pia Fyhrquist for sharing their thoughts, time, and work space with me. Thank you for the many long and deep scientific and nonscientific discussions that we had. Anna and Katariina, thank you for skillful advice whenever I needed a doctor's opinion on my work – or on my own health. I also thank all members of the other groups working at the Folkhälsan Research Center, especially Dr. Kati Donner, Riikka Hämäläinen, Kirsi Alakurtti, Dr. Tarja Joensuu, Docent Peter Hackman, and Ann-Liz Träskelin, for cheerful moments in different activities inside and outside the laboratory.

My thanks are also owed to Anja Koskela, Inga Wiik, Aila Heikkinen, Ritva Takkinen, Adile Samaletdin, and Sirkka Adlercreutz. Anja, thank you for teaching me all the tricks of working in the lab. Your knowledge, enthusiasm, and encouragement made my work much easier. Inga, Ritva, and Aila, thank you for skillful technical assistance and help in organizing the red clover study. Marjatta Valkama is acknowledged for valuable help in practical matters and Aila Koponen for librarian assistance.

I am very grateful to my parents Marja-Liisa and Mikko Raatikainen for their endless love, care, and support. Their help in taking care of me as well as my family has been invaluable.

Finally, with all of my heart I thank Harri, Oskari, Ilona and Annika, to whom I dedicate this work. Harri, thank you for your love, patience, and support during these trying years. You have made my dreams come true.

Vantaa, September 2006

Satu-Maarit Heinonen

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals:

I Heinonen, S., Wähälä, K., and Adlercreutz, H., Identification of isoflavone metabolites dihydrodaidzein, dihydrogenistein, 6'-OH-*O*-dma, and *cis*-4-OH-equol in human urine by gas chromatography-mass spectrometry using authentic reference compounds. *Anal Biochem* **1999**, *274*, 211-219.

II Heinonen, S.-M., Hoikkala, A., Wähälä, K., and Adlercreutz, H., Metabolism of the soy isoflavones daidzein, genistein and glycitein in human subjects. Identification of new metabolites having an intact isoflavonoid skeleton. *J Steroid Biochem Mol Biol* **2003**, *87*, 285-299.

III Heinonen, S.-M., Wähälä, K., Liukkonen, K.-H., Aura, A.-M., Poutanen, K., and Adlercreutz, H. Studies of the *in vitro* intestinal metabolism of isoflavones aid in the identification of their urinary metabolites. *J Agric Food Chem* **2004**, *52*, 2640-2646.

IV Heinonen, S.-M., Wähälä, K., and Adlercreutz, H. Identification of urinary metabolites of the red clover isoflavones formononetin and biochanin A in human subjects. *J Agric Food Chem* **2004**, *52*, 6802-6809.

V Heinonen, S.-M., Wähälä, K., and Adlercreutz, H. Metabolism of isoflavones in human subjects. *Phytochemistry Rev* **2002**, *1*, 175-182.

ABBREVIATIONS

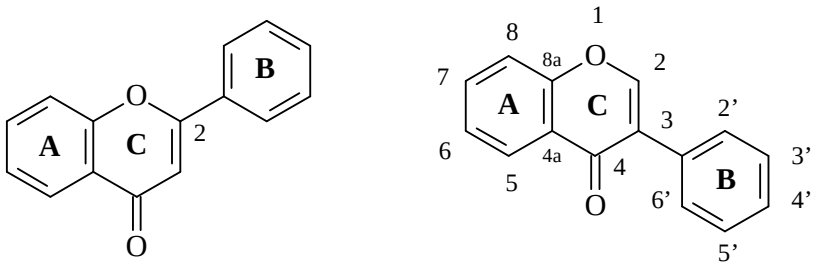
ACC	Acetyl-CoA carboxylase
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
AUC	Area under curve
BSTFA	Bis-(trimethylsilyl)-trifluoroacetamide
CI	Chalcone isomerase
CoA	Co-enzyme A
COMT	Catechol-*O*-methyltransferase
CS	Chalcone synthase
CV%	Coefficient of variation
DAD	Diode array detector
EI	Electron ionization
ER	Estrogen receptor
ESI	Electrospray ionization
GC	Gas chromatography
GI	Gastrointestinal
HMDS	Hexamethyldisilazane
HME	Human mammalian epithelial cell
HPLC	High-performance liquid chromatography
HRT	Hormone replacement therapy
IC_{50}	Half maximal inhibitory concentration
IFS	Isoflavone synthase
ISTD	Internal standard
LC	Liquid chromatography
LDL	Low-density lipoprotein
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NADPH	Nicotinamide diphosphate
NMR	Nuclear magnetic resonance spectroscopy
OMT	*O*-Methyltransferase
rDA	Retro Diels-Alder
SAM	*S*-Adenosyl-L-methionine
SCFA	Short-chain fatty acid
SD	Standard deviation
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SULT	Sulfotransferase
TBDMS	*t*-Butyldimethylsilyl
TMCS	Trimethylchlorosilane
TMS	Trimethylsilyl
UDP	Uridine diphosphate
UGT	UDP glucuronosyl transferase

1. INTRODUCTION

1.1 Structure, nomenclature, and occurrence of isoflavonoids

Isoflavonoids are a subclass of flavonoids, a large group of diphenolic plant compounds with a phenylchroman skeleton of 15 carbons (Figure 1). The three rings in isoflavonoids (and flavonoids) are labeled A, B, and C, and the numbering starts from oxygen of the heterocyclic ring C. Isoflavonoids differ from flavonoids by position of ring B, which is at C-2 in flavonoids and at C-3 in isoflavonoids. To aid systematic classification, isoflavonoids have been further divided, according to the oxidation level of the 3-phenylchroman skeleton (ring C), into subgroups of isoflavones, isoflavanones, isoflavans, and α-methyldeoxybenzoins. Table 1 lists the trivial names and structures of the isoflavonoids discussed in this work.

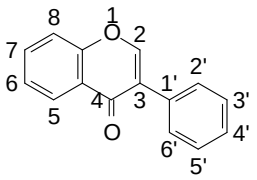
Isoflavones constitute the largest group of natural isoflavonoids, with some 360 known aglycones reported by the year 1994.[1] The most abundant isoflavones are daidzein **1** and genistein **2**. In plants the isoflavonoids occur mainly conjugated with sugars, most often with glucose at the 7-*O*-position. The principal isoflavones of soy are daidzein, genistein, and glycitein **3** and their glycoside conjugates, including 7-*O*-glucosides, i.e. daidzin **4**, genistin **5**, and glycitin **6**, 6''-*O*-acetyl-, and 6''-*O*-malonyl-7-*O*-glucosides.[2,3] In red clover, the principal isoflavones are formononetin **7** and biochanin A **8** and their 7-*O*-glucosides, ononin **9** and sissotrin **10**, respectively.[4,5] Other isoflavones identified in red clover include daidzein, genistein, calycosin **11**, prunetin **12**, pratensein **13**, pseudobaptigenin **14**, orobol **15**, 3'-methylorobol, texasin **16** and afrormosin **17**, and their glycoside conjugates.[4,5]



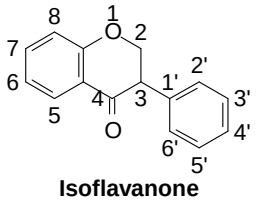
FLAVONE **ISOFLAVONE**

Figure 1. *Basic structures of flavonoids and isoflavonoids.*

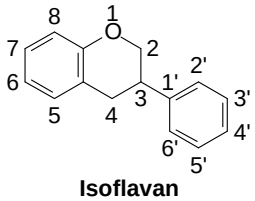
Table 1. *Structures, names, and numbering of the isoflavonoids discussed in this work.*



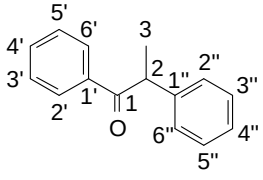
Isoflavone



Isoflavanone



Isoflavan



α-methyldeoxybenzoin

		Substitution	Trivial name
Isoflavone	**1**	7,4'-$(OH)_2$	Daidzein
	2	5,7,4'-$(OH)_3$	Genistein
	3	7,4'-$(OH)_2$, 6-OCH_3	Glycitein
	4	4'-OH, 7-OGlc	Daidzin
	5	5,4'-$(OH)_2$, 7-OGlc	Genistin
	6	4'-OH, 7-OGlc, 6-OCH_3	Glycitin
	7	7-OH, 4'-OCH_3	Formononetin
	8	5,7-$(OH)_2$, 4'-OCH_3	Biochanin A
	9	7-OGlc, 4'-OCH_3	Ononin
	10	5-OH, 7-OGlc, 4'-OCH_3	Sissotrin
	11	7,3'-$(OH)_2$, 4'-OCH_3	Calycosin
	12	5,4'-$(OH)_2$, 7-OCH_3	Prunetin
	13	5,7,3'-$(OH)_3$, 4'-(OCH_3)	Pratensein
	14	7-OH, 3'-O-CH_2-O-4'	Pseudobaptigenin
	15	5,7,3',4'-$(OH)_4$	Orobol
	16	6,7-$(OH)_2$, 4'-OCH_3	Texasin
	17	7-OH, 6,4'-$(OCH_3)_2$	Afrormosin
	18	4'-OH, 7-OCH_3	Isoformononetin
Isoflavanone	**19**	7,4'-$(OH)_2$	Dihydrodaidzein
	20	5,7,4'-$(OH)_3$	Dihydrogenistein
	21	7,4'-$(OH)_2$, 6-OCH_3	Dihydroglycitein
	22	7-OH, 4'-OCH_3	Dihydroformononetin
	23	5,7-$(OH)_2$, 4'-OCH_3	Dihydrobiochanin A
Isoflavan	**24**	7,4'-$(OH)_2$	Equol
	25	7-OH, 4'-OCH_3	4'-*O*-Methylequol
	26	4, 7,4'-$(OH)_3$	4-OH-equol
	27	5, 7,4'-$(OH)_3$	5-OH-equol
α-Methyldeoxybenzoin	**28**	2',4',4''-$(OH)_3$	O-desmethylangolensin
	29	2',4',6',4''-$(OH)_4$	6'-OH-*O*-desmethylangolensin
	30	2',4'-$(OH)_2$, 4''-OCH_3	Angolensin
	31	2',4',6'-$(OH)_3$, 4''-OCH_3	6'-OH-angolensin
	32	2',4',3'',4''-$(OH)_4$	3''-OH-*O*-desmethylangolensin
	33	2',4',5',4''-$(OH)_4$	5'-OH-*O*-desmethylangolensin
	34	2',4',4''-$(OH)_3$, 5'-OCH_3	5'-OMe-*O*-desmethylangolensin

1.2 Biosynthesis

Isoflavonoids are formed by a branch of the phenylpropanoid pathway of plant secondary metabolism.[6] Other branches of this pathway produce flavonoids, lignin, and anthocyanin pigments (Figure 2). Ring B and part of the heterocyclic ring C are formed from 4-coumaric acid co-enzyme A (CoA) ester via the shikimate pathway, starting from carbohydrate. Ring A is formed via the polyketide pathway from three units of malonyl CoA, derived from acetyl CoA and carbon dioxide. The reaction is catalyzed by acetyl-CoA carboxylase (ACC, Figure 2) in the presence of ATP and Mg^{2+}. Chalcone synthase (CS) catalyzes the stepwise condensation of these precursors to a C_{15} intermediate, 4,2',4',6'-tetrahydroxychalcone **35** (Figure 2), an intermediate of flavonoids that have oxygen substituents at the 5-position, including the isoflavones genistein **2** and biochanin A **8**. Isoliquiritigenin **36** (Figure 2), an intermediate of daidzein **1**, formononetin **7,** and probably also glycitein **3**, is formed by CS, but with coaction of NADPH-dependent reductase. Chalcone is converted to (2*S*)-flavanone by a stereospecific cyclization, which is catalyzed by chalcone isomerase (CI).[6]

The isoflavonoid pathway begins by the abstraction of a hydrogen radical at C-3, followed by ring B migration from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical. This reaction is catalyzed by isoflavone synthase (IFS) or 2-hydroxyisoflavanone synthase.[6] IFS is regioselective; (2*R*)-flavanones are not substrates. The resulting 2-hydroxyisoflavanone is unstable and undergoes dehydration to form isoflavone. The dehydration reaction can take place nonenzymatically. Daidzein is formed from liquiritigenin **37** and genistein from naringenin **38**, as shown in Figure 2. The biosynthesis of glycitein has not been fully established yet, but is likely to be derived from isoliquiritigenin **36** (Figure 2).[7]

Recent studies have shown that formononetin and biochanin A, both of which have a 4'-methoxy group, are not formed by simple methylation of daidzein and genistein, respectively. Methyl transfer from *S*-adenosyl-L-methionine (SAM) to 4'-hydroxyl of daidzein has not been detected in any plants that produce formononetin **7**. The *O*-methyltransferase (OMT) towards daidzein thus far has only been found to produce the 7-*O*-methylated isoflavone isoformononetin **18**.[8-10] These 7-*O*-methylated isoflavonoids are scarce in the plant kingdom. To form formononetin, the substrate is 2,7,4'-trihydroxyisoflavanone rather than daidzein,[11] and the resulting 4'-*O*-methylated 2-hydroxyisoflavanone subsequently undergoes dehydration to form formononetin (Figure 2). The enzyme catalyzing the methylation reaction is 2,7,4'-trihydroxyisoflavanone 4'-OMT.[11]

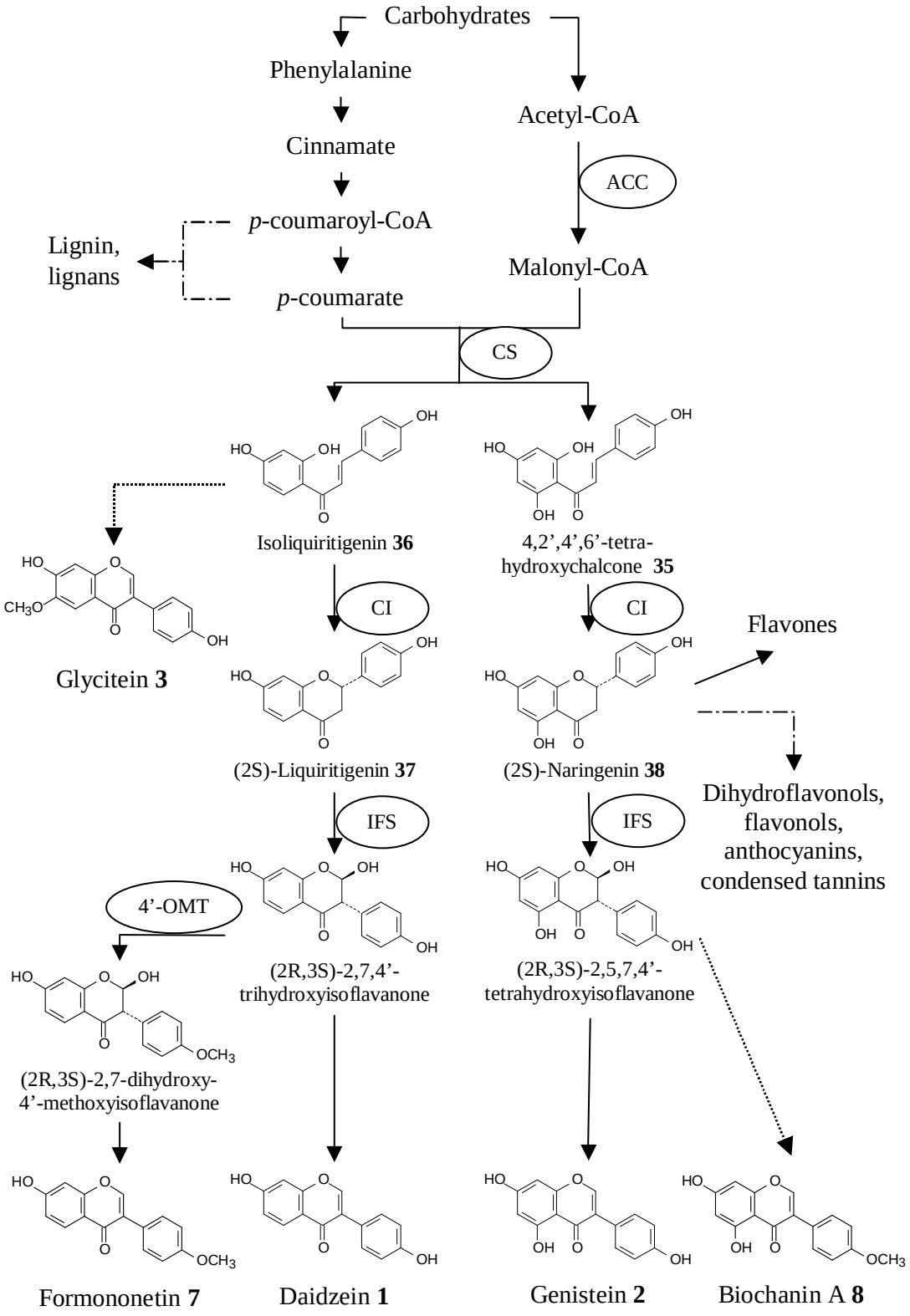


Figure 2. *A simplified diagram of the phenylpropanoid pathway showing intermediates and enzymes involved in isoflavone synthesis. Dashed arrows represent speculative steps.*

1.3 Sources of isoflavonoids in the human diet

Unlike flavonoids, which are widely spread in the plant kingdom, the distribution of isoflavonoids is very limited. The occurrence is almost entirely restricted to plants belonging to the subfamily of *Papilionoideae* of the *Leguminosae*.[1] Only these plants and a few other species have the unique enzymatic activity of isoflavone synthase (IFS), which carries out a 2,3 migration of the ring C, resulting in the production of isoflavones.

Because of the restricted distribution of isoflavones, the legumes, including beans and peas, are the most important sources of isoflavones. The concentration of isoflavones in soybeans is much higher than in other legumes; soy and soy-based foods are thus considered the main sources of isoflavones in the human diet.[12-14] In Asian cultures, whole soybeans or soy flour are widely used in food preparation, whereas in Western cultures the consumption of soy foods is very low. The intake of isoflavones is limited in Western diets, typically <1 mg/d, compared with consumption in Asian countries, where the levels range from 20 to 50 mg/d.[15] In Finland, the average intake of total isoflavones was estimated to be 668 (SD 963) μg/d in women and 902 (SD 368) μg/d in men.[16]

In addition to isoflavone levels in diets, the type of soy food eaten differs in these two populations. In Eastern countries, soybeans are eaten whole, either cooked or raw, as sprouted beans. Traditionally prepared foods include tofu or miso, soy sauce or tempeh, made by fermentation. The isoflavone levels in traditional soy foods reflect the composition of the raw material beans, which may differ vastly due to differences in genetics, cultivar, climate, location, and agricultural practices.[3,12,13,17]

In Western countries, especially in Europe, second-generation soy foods are more popular than traditional soy foods. These products are produced from whole soybeans, flour, or tofu, but may also contain soy protein concentrates or isolates. The products largely resemble analogs of Western foods, particularly dairy or meat products, and are commonly used by consumers who have an intolerance for dairy products or follow a vegetarian diet. The soy derivatives are also widely used in foods at varying concentrations for nutritional, technological, and economic reasons. For example, textured soy protein is used as a meat extender in some commercially prepared meat products, and low levels of soy ingredients are added to bread to improve loaf texture and quality. In addition, a number of different kinds of pills and capsules, manufactured from soy or red clover, are commercially available for health-conscious customers who want to achieve the suggested beneficial properties of isoflavones without changing the original diet.

The existing information on isoflavone levels in Eastern and Western foods has been evaluated[18] and compiled in databases available online at www.venus-ca.org and www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html. Table 2, which is based on these two databases, lists the levels of isoflavones in selected traditional and second-generation soy foods.

Table 2. *Concentrations of isoflavones in selected traditional and second-generation foods. Values given are from two online databases at* www.nal.usda.gov/fnic/foodcomp/Data/isoflav/ *and* www.venus-ca.org.

Food	Tot (mg/100 g)
Bread	0.02-0.83
Infant formulas	2.63-26
Miso[1]	13-43
Natto[1]	22-59
Soy bacon	12
Soy cheese[1]	6.4-31
Soy chicken	14
Soy flour	132-265
Soy hot dog	12-15
Soy milk	8-10
Soy sauce[1]	0.1-1.6
Soybean butter	0.57
Soybeans (cooked)	14-55
Soybeans (raw)	60-145
Soybeans (sprouted)	41
Tempeh[1]	44-53
Tofu yoghurt	16
Tofu[1]	11–24

[1] *Fermented during preparation.*

1.4 Metabolism and bioavailability of isoflavones in humans

The biological activities observed in laboratory or human studies have been assumed to originate from the isoflavones investigated, although these may have been biotransformed into one or more structurally different compounds. Metabolism affects ingested isoflavones in two ways: 1) absorption of the compound may be enhanced or reduced relative to that of the ingested isoflavone or 2) catalyzed conversions may lead to deactivation of bioactive compounds or activation of inactive compounds. Thus, the metabolism of isoflavones may play an important role in the effect and mechanism of action of isoflavones. Beside limited knowledge concerning the metabolism of isoflavones, investigations of bioavailability and disposition are also scarce. These aspects are of the utmost importance when evaluating the biological and physiological effects of isoflavones. Isoflavonoid metabolites that have been identified in humans have been reviewed in Study V and summarized in Table 3. A schematic drawing of the metabolic fate of isoflavones in humans is shown in Figure 3.

Table 3. *Isoflavones and their metabolites identified in human urine.*

Compound	Year	Identification	Reference
Equol	1982	Reference compound	Axelson et al. (1982)[19] Adlercreutz et al. (1982)[20]
Daidzein	1984	Reference compound	Bannwart et al. (1984)[21]
O-dma	1984	Reference compound	Bannwart et al. (1984)[21]
Formononetin	1987	Reference compound[1]	Bannwart et al. (1987)[22]
Isoflav-2-ene (Intermediate E)	1987	Tentative	Adlercreutz et al. (1987)[23]
Dihydrodaidzein (Intermediate O)	1987	Tentative	Bannwart et al. (1987)[22] Adlercreutz et al. (1987)[23]
	1993	Reference compound	Kelly et al. (1993)[24] Joannou et al. (1995)[25]
4'-*O*-methylequol	1987	Reference compound[1]	Bannwart et al. (1987) [22] Joannou et al. (1995)[25]
3',7-dihydroxyisoflavan	1988	Reference compound	Bannwart et al. (1988)[26]
Genistein	1991	Reference compound	Adlercreutz et al. (1991)[27]
Glycitein	1993	Reference compound	Kelly et al. (1993)[24] Joannou et al. (1995)[25]
2-dehydro-*O*-dma	1993	Tentative	Kelly et al. (1993)[24] Joannou et al. (1995)[25]
4-OH-equol	1993	Tentative	Kelly et al. (1993)[24] Joannou et al. (1995)[25]
Dihydrogenistein	1993	Tentative	Kelly et al. (1993)[24] Joannou et al. (1995)[25]
6'-OH-*O*-dma	1993	Tentative	Kelly et al. (1993)[24] Joannou et al. (1995)[25]
Biochanin A	1994	Reference compound	Franke et al. (1994)[28]
Several hydroxylated metabolites of daidzein and genistein	2000	Both tentative and with reference compound	Kulling et al. (2000)[29]
2-(4-hydroxyphenyl)-propionic acid, HPPA	2001	Reference compound	Coldham et al. (2001)[30] Boersma et al. (2001)[31]

[1]*Spectral data not reported*

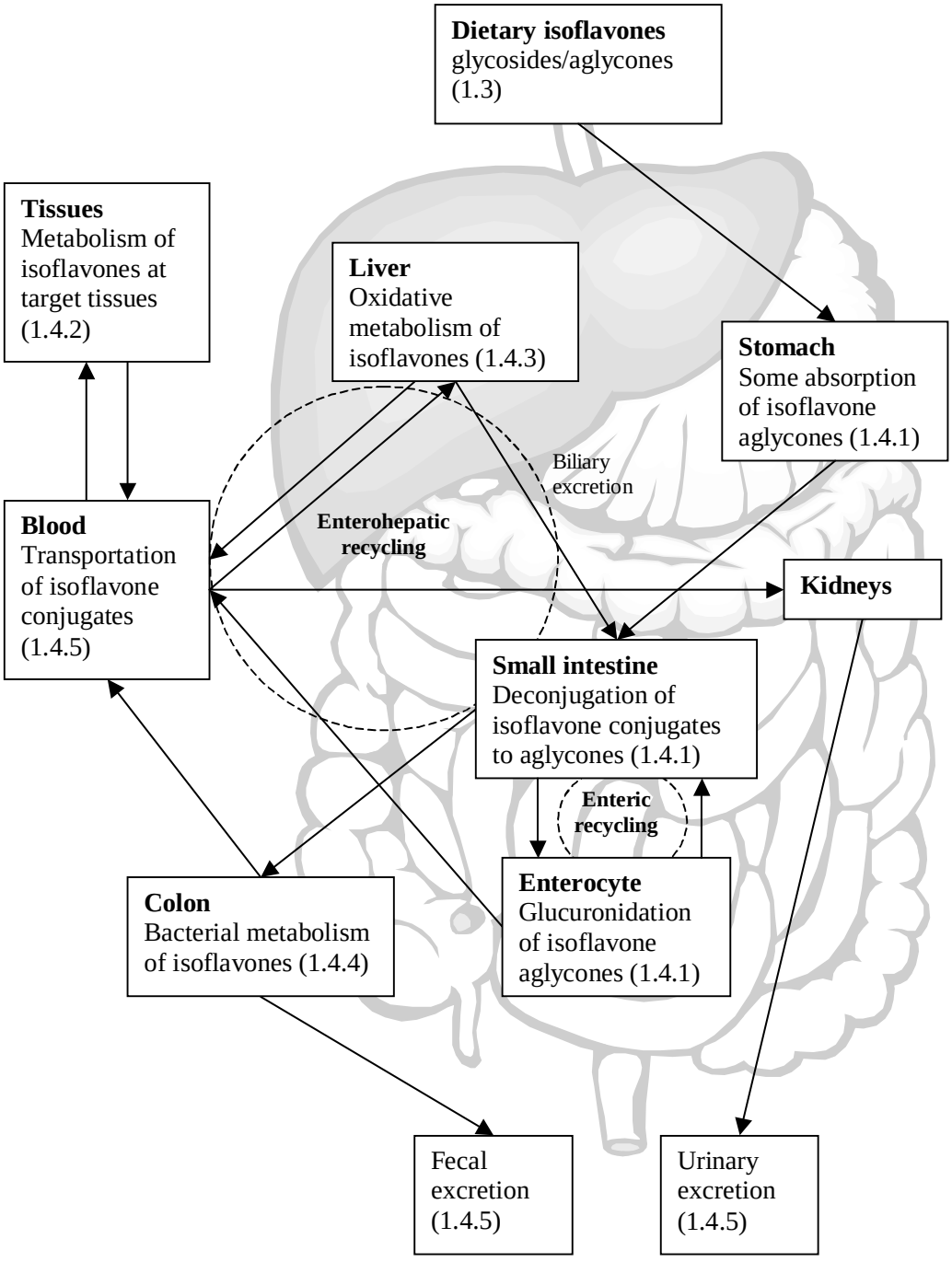


Figure 3. *The metabolic fate of isoflavones in humans. Numbers in parentheses refer to the sections of this thesis, in which the issue is discussed in more detail.*

1.4.1 Deglycosylation and absorption

After ingestion of isoflavone-rich foods, the isoflavone glycosides, the predominant form of isoflavones in nonfermented soy products, undergo deglycosylation. Nonenzymatic deglycosylation of polyphenols in the human body, such as in the acidic conditions of the stomach, does not occur.[32] The absorption of isoflavones would thus seem to be controlled by enzyme specificity and distribution. Enzymes capable of carrying out the deglycosylation step are found at the small intestinal brush border (lactase phloridzin hydrolase)[33-37] and in enterocytes (cytosolic β-glucosidases).[33] In addition, several major groups of colonic bacteria possess β-glucosidase activity, including *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacterium* spp.[38] Recent studies have demonstrated that deglycosylation of isoflavones may also occur to some extent already in the mouth.[39]

Considerable discussion has centered around whether isoflavone conjugates could be absorbed intact. A study of absorption of pure genistin **5** in an isolated rat intestine perfusion model showed that small amounts of genistin (1.3% of added amount) pass into the mucosal cells, where hydrolysis takes place. The total intestinal absorption of genistin was found to be 14.9% (±2.3), the majority of which as genistein **2** glucuronide (11.6%) and a smaller amount (1.9%) as the genistein aglycone. Genistein, but not genistin, can be readily absorbed through the wall of the stomach.[40] This may explain the faster absorption rates of aglycones compared with glycosides.[41] Setchell et al.[42] were unable to detect any isoflavone glycosides in plasma samples collected 1, 2, and 8 h after the ingestion of either pure compounds or a soyfood matrix; thus, current *in vivo* evidence supports the notion that isoflavonoid glycosides are not absorbed intact in humans.

Free aglycones are readily reconjugated to glucuronic or sulfonic conjugates by intestinal cytochrome P450 enzymes.[43] The isoflavone aglycones and their conjugates have three possible fates (Figure 3): 1) they are transported to target tissues in blood and end up in the liver, where they are subjected to further metabolism, 2) they enter the enterohepatic circulation, i.e. are first transported through the portal vein to the liver and then excreted back to the gut through bile, or 3) they are excreted back to the intestinal lumen in a process called enteric recycling.[44] The fate of isoflavones is thus very similar to that of endogenous estrogens.[45,46]

1.4.2 Metabolism in extrahepatic tissues

Studies of tissue metabolism of isoflavones are very scarce. Petersen et al.[47,48] have investigated the ability of human mammalian epithelial (HME) cells and four different breast cancer cell lines (MCF-7, ZR-75-1, BT20, and T47D) to metabolize [4-^{14}C] -labeled genistein **2** and biochanin A **8**. Breast cancer cells metabolized both isoflavones, while significant metabolism of genistein or biochanin A was not observed in HME cells. Using radio-HPLC mass spectrometry, two metabolites of genistein, i.e. genistein 7-sulfate, and a hydroxylated and methylated metabolite of genistein, the structure of which was not elucidated, and four metabolites of biochanin A, including genistein, genistein 7-

sulfate, biochanin A 7-sulfate, and a hydroxylated and methylated metabolite of biochanin A, were identified in cell culture media.[47,48] Since the metabolites were found primarily in cell media, not in cell lysate fractions, it was suggested that once formed these polar metabolites are excreted from the cells.[48]

A marked difference in the ability of different cell lines to metabolize genistein and biochanin A was observed. In contrast to MCF-7 and T47D cell lines, which have similar IC_{50} (the concentration at which the growth rate is halved) values around 19 μmol/L, hydroxylated and methylated metabolites of genistein and biochanin A were not formed with less sensitive ZR-75-1 and BT-20 cell lines, which have IC_{50} values of 46-74 μmol/L.[47] The correlation of IC_{50} values with the production of the unresolved hydroxylated and methylated metabolite led the authors to speculate that the hydroxylated and methylated metabolites could be the active forms of genistein in human breast cancer cell lines.[47]

Boersma et al.[31,49,50] have examined whether metabolism of genistein and daidzein occurs by interaction of isoflavones with oxidants produced by inflammatory cells such as peroxynitrite ($ONOO^-$), hypochlorous (HOCl), or hypobromous (HOBr) acid. Using freshly isolated human neurophils and differentiated human leukemia cells (HL-60) stimulated with phorbol ester to elicit a respiratory burst, they showed that monochlorinated, dichlorinated, and nitrated isoflavones are formed through a myeloperoxidase-dependent mechanism and can be detected in cell media.[50] However, *in vivo* evidence of the presence of these metabolites in human fluids is lacking.

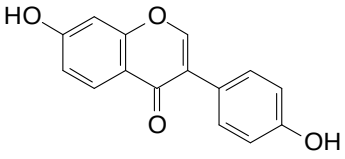
1.4.3 Metabolism in the liver

In the liver, isoflavones are subjected to oxidative reactions catalyzed by liver cytochrome P450 enzymes. Roberts-Kirchhoff et al.[51] conducted a study in which genistein **2** was incubated with rat and human liver microsomes or recombinant human cytochrome P450 enzymes in the presence of NADPH. They reported the formation of five different metabolites of genistein; three of these were hydroxylated metabolites, one of which was identified as orobol **15**. Using microsomes from rats treated with P450-inducing agents, they found out that the formation of metabolites was NADPH- and time-dependent. Three different human microsomal samples investigated exhibited different product profile suggesting that the genistein metabolite profile is dependent on the P450 profile of the microsomes.[51]

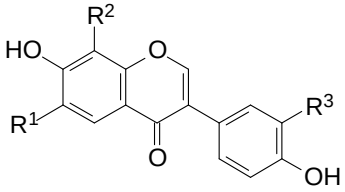
A more detailed study on the identification of oxidized metabolites of isoflavones has been carried out by Kulling et al.[29,52,53] Using liver microsomes from Aroclor-treated male Wistar rats, daidzein **1** was found to be metabolized to nine metabolites: four monohydroxylated, four dihydroxylated, and one trihydroxylated metabolite. Genistein **2** was converted to four monohydroxylated and two dihydroxylated products. The identity of the metabolites was elucidated with GC-MS and HPLC-ESI-MS using reference compounds to aid the interpretation of chromatographic and mass spectrometric data.[29]

The additional hydroxyl groups were introduced into the ortho positions of existing phenolic hydroxy groups. One of the monohydroxylated metabolites of both daidzein and genistein has a hydroxy group at aliphatic position C-2 of ring C.

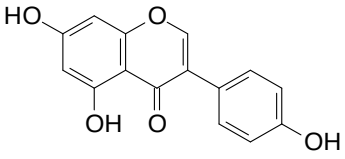
The oxidative *in vitro* metabolism of soy isoflavones was investigated further with human liver microsomes.[52] In this study, daidzein was metabolized to three monohydroxylated and three dihydroxylated metabolites, whereas genistein was converted to four monohydroxylated and two dihydroxylated metabolites. Five major human microsomal metabolites were tentatively identified in human urine after soy intake on the basis of their retention time and UV and mass spectra. In addition, using a more sensitive selected ion mode with GC-MS, five oxidized metabolites of daidzein (**38**-**42**) and five oxidized metabolites of genistein (**43**-**47**) could be detected in urine samples.



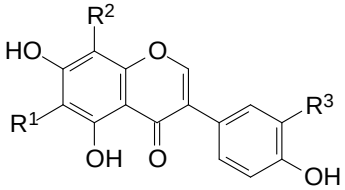
Daidzein **1**



38 $R^2=R^3=H$, $R^1=OH$
39 $R^1=R^3=H$, $R^2=OH$
40 $R^1=R^2=H$, $R^3=OH$
41 $R^2=H$, $R^1= R^3=OH$
42 $R^1=H$, $R^2= R^3=OH$



Genistein **2**

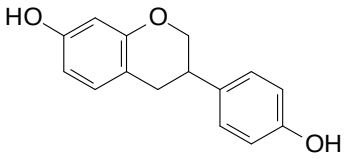


43 $R^2=R^3=H$, $R^1=OH$
44 $R^1=R^3=H$, $R^2=OH$
45 $R^1=R^2=H$, $R^3=OH$
46 $R^2=H$, $R^1= R^3=OH$
47 $R^1=H$, $R^2= R^3=OH$

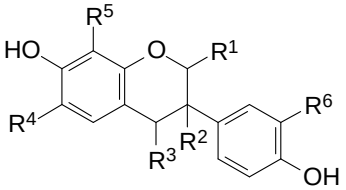
In addition to daidzein and genistein, Kulling et al.[53] have evaluated the oxidative metabolism of such isoflavonoids as the clover isoflavones formononetin **7** and biochanin A **8**, soy isoflavone glycitein **3**, and equol **24** – an important intestinal metabolite of daidzein. Formononetin and biochanin A, both of which have methoxy substituents at the 4'-position, are demethylated rather than hydroxylated when incubated with Aroclor-treated rat liver microsomes. The main metabolites of formononetin were identified as daidzein **1**, 6,7,4'-trihydroxyisoflavone**,** and 7,8,4'-trihydroxyisoflavone **39**, whereas hydroxylated formononetin metabolites were formed only in small amounts. Similarly, genistein and hydroxylated genistein metabolites are the major compounds formed when

biochanin A is incubated with rat liver microsomes. By contrast, preliminary studies with glycitein, which has a methoxy substituent at the 6-position, have suggested that aromatic hydroxylation is preferred over demethylation.[53]

When equol **24**, the intestinal metabolite of daidzein, was incubated with Aroclor-treated rat liver microsomes, several hydroxylated metabolites were detected by HPLC-DAD and HPLC-MS analysis. The main metabolite has been tentatively identified as 7,3',4'-trihydroxy-isoflavan **48**, and, in addition 6,7,4'-trihydroxyisoflavan **49**, 7,8,4'-trihydroxy-isoflavan **50**, 2,7,4'-trihydroxyisoflavan **51**, 3,7,4'-trihydroxyisoflavan **52,** and 4,7,4'-trihydroxyisoflavan **26** have been detected in smaller amounts.[53]



Equol **24**



48 $R^1=R^2=R^3=R^4=R^5=H, R^6=OH$
49 $R^1=R^2=R^3=R^5=R^6=H, R^4=OH$
50 $R^1=R^2=R^3=R^4=R^6=H, R^5=OH$
51 $R^2=R^3=R^4=R^5=R^6=H, R^1=OH$
52 $R^1=R^3=R^4=R^5=R^6=H, R^2=OH$
26 $R^1=R^2=R^4=R^5=R^6=H, R^3=OH$

Preliminary studies by Kulling et al.[52] suggest that vicinal hydroxyl groups of oxidized isoflavone metabolites are methylated by the action of catechol *O*-methyltransferase (COMT). Incubation of the hydroxylated metabolites with COMT and SAM as a methyl group donor yielded methylated catechol products that were identified with the aid of GC-MS. By using the *in vitro* formed methylated products as reference compounds, Kulling et al. were able to tentatively identify four methylated metabolites (two monomethylated and two dimethylated metabolites) in human urine samples collected after soy supplementation. The methylated catechol metabolites were found in urine in trace amounts, thus, it was suggested that isoflavones are poor substrates for COMT and the methylation of hydroxylated metabolites does not play a major role in metabolism of isoflavones *in vivo*.[52]

1.4.4 Metabolism in the gut

Isoflavonoids that are not absorbed in the stomach or small intestine will be carried to the colon (Figure 3), where they are subjected to reactions catalyzed by enzymes of the gut microflora. In addition, isoflavonoids that are absorbed, metabolized in the liver, and excreted in the bile or directly from the enterocyte back to the small intestine may also reach the colon, but in a different chemical form, e.g. as glucuronide or sulfate conjugates

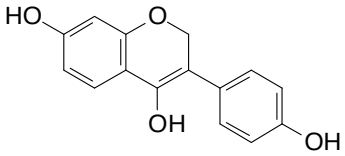
(Figure 3).[44] Bacterial deglycosylation and desulfonation liberates the aglycones, which can be further metabolized by enzymes of the gut microflora.

Metabolism by gut microflora and mucosal cells plays an important role in isoflavone metabolism and determines for the most part the levels of circulating isoflavonoids. Infants with undeveloped gut microflora are unable to metabolize daidzein **1** or genistein **2,** resulting in high isoflavone plasma levels if they are fed soy-based infant formulae.[54,55] Studies with germ-free animals have also demonstrated the importance of gut microflora in metabolism of isoflavones; urinary excretion of daidzein and genistein was found to be significantly greater in germ-free animals than in animals with human microflora, suggesting a reduced metabolism of isoflavones from a soy-containing diet.[56] The use of antibiotics greatly affects the microfloral populations, and thus, the metabolism of isoflavones. Increased urinary excretion of intact ingested isoflavones during antibiotic treatment in monkeys[57] and inhibition of bacterial metabolism of daidzein *in vitro* by certain antibiotics have been reported.[58] One study investigated the effect of prebiotics on the bacterial metabolism of genistein.[59] The results showed that the addition of fructooligosaccharides preserved genistein *in vitro* by reducing the number of metabolizing bacteria and increased the numbers of such beneficial bacteria as bifidobacteria and lactobacilli.

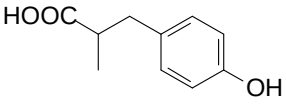
Bacterial metabolism of flavonoids and isoflavonoids has been investigated *in vitro* by incubating compounds of interest with human fecal inoculum and following their degradation.[59-61] The structure of a flavonoid has a remarkable effect on determining the extent and rate of metabolism of the compound by intestinal bacteria. For example, the degradation rates of flavonoids possessing hydroxyl substituents at 5-, 7-, or 4'-positions are significantly faster than flavonoids lacking any of these hydroxyls.[60,61] Genistein with the hydroxyl substituents at 5-,7-, and 4'-positions has been found to have a half-life of 3.3 h, whereas the estimated half-life for daidzein, which has hydroxyl substituents at 7- and 4'-positions, is considerably longer, 7.5 h.[62] Presence of the methoxy group at ring A or B has been observed to render the isoflavonoid resistant to microbial degradation. Preliminary studies have shown glycitein **3** to degrade at a slower rate than genistein.[63]

The metabolites formed in *in vitro* incubations have been identified by means of GC-MS, LC-MS, or NMR.[30,64-68] Kim et al.[64] have examined the metabolism of daidzin by human intestinal bacteria and identified daidzein and calycosin **11** in 24-h fermentation extracts. In a study by Chang et al.[65] daidzein was incubated for 72 h with human fecal flora after which the three metabolites present in fermentation extracts were identified as dihydrodaidzein **19**, 7,4,4'-trihydroxyisoflav-3-ene **53,** and equol **24**. The *in vitro* metabolism of genistein was simultaneously examined. Only one metabolite, dihydrogenistein **20**, was identified in the fermentation extract. Because the amount of this metabolite did not increase as the amount of genistein declined, the authors suggested that genistein was converted to other metabolites that could not be detected.[65] The metabolism of genistein was later investigated by Coldham et al.[30,66] who incubated genistein, [2',3',5',6'-^{3}H] and [4-^{14}C]-labeled genistein with both human and rat gut microflora. Using LC-MS/MS and radio-LC-MS^{n} techniques, they identified dihydrogenistein and 6'-

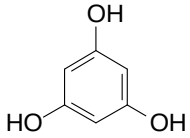
OH-*O*-dma **29** as intermediate metabolites and 2-(4-hydroxyphenyl)-propionic acid **54** and 1,3,5-trihydroxybenzene **55** as end-products of genistein metabolism.



7,4,4'-trihydroxy-isoflav-3-ene **53**



2-(4-hydroxyphenyl)-propionic acid **54**

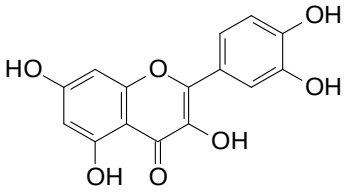


1,3,5-trihydroxybenzene **55**

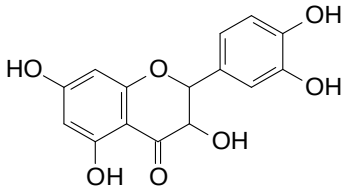
There is a marked interindividual variation in the bacterial metabolism of isoflavones regarding the capability to metabolize daidzein. Only about one-third of humans are capable of converting daidzein to equol **24**, a metabolite with an isoflavan structure.[69-75] For genistein, the corresponding isoflavan metabolite, 5-OH-equol **27**, has not been identified, which is probably due to a protective effect of hydrogen bonding between the C-5 hydroxyl group and the carbonyl oxygen in the genistein structure. Interindividual differences in the ability to produce equol are most likely due to variations in the composition of intestinal microflora.

The results from both animal and human studies assessing the role of diet in equol production are conflicting and have failed to provide an explanation for why different daidzein-metabolizing phenotypes exist.[74-77] In some studies, a diet rich in carbohydrates has been associated with higher equol production, suggested to be due to improved gastrointestinal (GI) conditions.[74,77] However, other studies have found no correlation between carbohydrate and dietary fiber intake with equol production.[73,75,78] The type of dietary fiber and the carbohydrate source have been demonstrated to have differening influences on plasma isoflavone levels and may thus change the physiology or metabolism and composition of intestinal microflora.[79] A recent familial correlation and segregation study has suggested that genetic factors have some contribution to equol production.[80]

Very little is known about the bacteria responsible for isoflavone metabolism. The metabolism likely involves many different bacteria. So far, only a few species of bacteria have been isolated, characterized, and studied for their ability to metabolize isoflavones. An interesting finding is that bacteria associated with the metabolism of flavonoids, such as quercetin **56** and taxifolin **57**, are different from isoflavone-metabolizing bacteria.[81]



Quercetin **56**



Taxifolin **57**

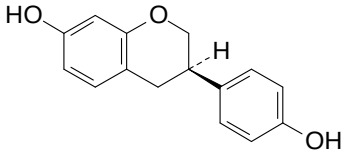
One of the most investigated bacterial species is *Eubacterium ramulus*, a bacterium accounting approximately for 0.16% of total bacterial cell counts in the human gastrointestinal tract, which is comparable in number to *Escherichia coli*.[82-84] *E. ramulus* has been found to hydrolyze isoflavone 7-*O*-glycosides daidzin **4** and genistin **5** to their aglycones, daidzein **1** and genistein **2**, respectively.[82,83] In addition, this bacterium catalyzes the ring C cleavage of daidzein to *O*-dma **28** and genistein to 2-(4-hydroxyphenyl)-propionic acid **54,** forming 6'-OH-*O*-dma **29** as an intermediate metabolite.[82] Two other strains of bacteria capable of metabolizing glycosides of daidzein and genistein have been isolated and designated as *E. coli* HGH21 and *Clostrium* sp. HGH6. Both strains catalyzed conversion of isoflavone glucosides to their aglycones. In addition, under anoxic conditions, HGH6 reduced the double bond of isoflavone ring C to yield dihydrodaidzein **19** from daidzein and dihydrogenistein **20** from genistein. This reductive reaction was specific for isoflavones since with flavonoids a similar reduction did not occur. No further metabolism of isoflavanones by this bacterial strain was observed.[85] *Eubacterium limosum* has been shown to be associated with the demethylation process of isoflavones with methyl groups, including formononetin **7**, biochanin A **8**, and glycitein **3**.[86]

Recently, the interest in bacterial *in vitro* metabolism of isoflavones has concentrated on the characterization of bacteria involved in equol production. Equol **24** has been found in soymilk fermented with some strains of bifidobacteria,[87,88] suggesting that bifidobacteria may be associated with equol production also in the human gut. However, supplementing the diet with soy and probiotic capsules containing *Lactobacillus acidophilus* and *Bifidobacterium longum* did not improve equol excretion or change the equol producer/non-producer status in human subjects.[89,90] Furthermore, animal feeding studies with ingestible short-chain fructooligosaccharides that increase the number of *Bifidobacterium* and *Lactobacillus* in the gut[91] have provided conflicting data, with both reductions and enhancements of equol production being observed.[92,93]

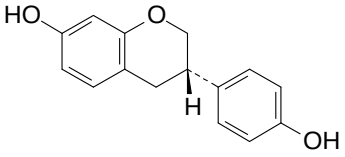
Decroos et al.[94] investigated the *in vitro* metabolism of daidzein by human fecal samples. In one equol-producing fecal sample, they isolated a mixed microbial culture in which four bacterial species were detected. The three strains of the mixed culture could be identified as *Lactobacillus mucosae* EPI1, *Enterococcus faecium* EPI2, and *Finegoldia magna* EPI3. The fourth species could not be brought into a pure culture; it was tentatively identified as *Veillonella* sp strain EP. None of the three isolated and well-characterized strains were able to produce equol, so it was suggested that the fourth strain, uncharacterized *Veillonella* sp is responsible for equol production. Based on the results of *in vitro* studies carried out with the mixed culture, a diet rich in carbohydrates appears to create beneficial conditions for equol production in the gut by providing H_2 and short-chain fatty acids (SCFA), the former probably acting as an electron donor in biotransformation and the latter preventing the consumption of H_2 in SCFA production by a feedback mechanism.[94]

Wang et al.[95] recently isolated a rod-shaped, Gram-negative anaerobic bacterium from human feces that enantioselectively produces (*S*)-equol **55** from dihydrodaidzein. The 16S

rRNA gene sequence of the bacterium, designated Julong 732, has a 92.8% similarity with *Eggerthella hongkongenis*, suggesting that the strain could be a new species of *Eggerthella*. By incubation of the isolated strain with compounds of interest, they made the following observations: the strain 1) is able to convert racemic dihydrodaidzein or 4-OH-equol **26** (also known as tetrahydodaidzein) enantioselectively to pure (*S*)-equol, 2) is not involved in the reduction of daidzein in dihydrodaidzein or in the cleavage of ring C to yield *O*-dma, and 3) does not have racemase activity to transform (*S*)-equol **58** to (*R*)-equol **59**, or vice versa. No data on the abundance and occurrence of this newly isolated bacterial strain in the human GI tract are available. Nor is it known whether there are other bacteria capable of producing equol. Thus, further studies are needed to fully identify the bacteria associated with equol production.



(*S*)-equol **58**



(*R*)-equol **59**

1.4.5 Conjugation, distribution, and excretion

In blood and urine, the isoflavones exist as glucuronide and sulfate conjugates.[96,97] Conjugation takes place in enterocytes of the intestinal wall during first-pass uptake.[44,98] This is supported by animal studies with rats, which have shown that portal venous blood contains almost exclusively glucuronide conjugates.[99] In addition, liver microsomes are sites for conjugate formation, but according to current views intestinal conjugation is more important in isoflavone metabolism.

The formation of glucuronide and sulfate conjugates of daidzein **1** and genistein **2** has been examined *in vitro* using purified bovine UDP glucuronosyl transferase (UGT), recombinant human UGT, and sulfotransferase (SULT) isoforms or microsomes isolated from several human tissues.[43] With all UGTs studied, the glucuronide group was preferentially attached to the hydroxyl group at C-7 of ring A, 4'-*O*-glucuronides being formed only to a small extent. By comparing the ability of human tissue microsomes to catalyze the glucuronidation, Doerge et al.[43] suggested that genistein is readily glucuronidated already in the gut, whereas daidzein glucuronidation predominantly occurs in liver or kidney microsomes. Sulfation of isoflavones by SULT isoforms *in vitro* succeeded for genistein only; no activity was seen with daidzein under the same conditions. Unfortunately, an insufficient amount of genistein sulfate was formed, so further structural characterization was not possible.[43]

The levels of circulating aglycones and glucuronide and sulfate conjugates in blood have been estimated by using the selective hydrolysis of a sample with pure glucuronide and/or

sulfatase preparations[43,100,101]or by fractionating the different types of isoflavonoid conjugates by ion chromatographic techniques, after which the conjugates are hydrolyzed and analyzed as aglycones.[97] Moderate amounts of isoflavones occurs as free (18-27%) or sulfated (20-21%) forms in blood, the predominant conjugates being isoflavone glucuronides (63-62%).[100] The distribution of isoflavonoids in tissues has not been fully established. Isoflavones have been identified and measured in human breast secretions (nipple aspirate and breast milk)[102-104] and prostatic fluids.[105] In rats, concentrations of isoflavones have been determined in various tissues, including the heart, brain, epididymis, fat, lung, testis, liver, pituitary gland, prostate gland, mammary glands, uterus, and kidney.[106-110]

The main route of excretion is via the kidneys. Urinary excretion of daidzein and genistein has been found to account for approximately 30-61% and 10-30% of administered doses, respectively.[111-114] The average urinary recovery of ^{13}C-labeled daidzein and genistein was 30% and 9%, respectively; remaining ingested compounds being converted to metabolites that could not be identified by the methods used in the study.[115] In urine, isoflavones exist mainly (70-90%) as glucuronides, 7-*O*-monoglucuronides being the most abundant metabolites.[100,116-118] Free isoflavonoids account for 1-10% and sulfates 10-25% of total isoflavonoids.[116,117]

Fecal excretion of isoflavones has not been investigated extensively. From data of the few studies available, the fecal excretion of daidzein and genistein seems to be between 1% and 4% of the ingested dose,[111,114] suggesting that excretion in feces occurs only to a minor degree. In feces, isoflavones occur mainly in a free aglycone form, conjugated isoflavones accounting for less than 10% of all isoflavones.[119]

1.4.6 Pharmacokinetics and bioavailability

Limited studies have investigated the pharmacokinetics and bioavailability of isoflavones in humans. These studies have been carried out with pure isoflavone preparations containing only one isoflavone[115,120] or with mixtures of isoflavones obtained from dietary supplements or soy food.[111,113,114,116,117,121-123] In general, plasma and urine samples are collected before and after the isoflavone supplementation at certain time intervals. The samples are analyzed for their isoflavone levels which are plotted (concentration vs. time) to yield a pharmacokinetics graph. From this graph, the relative bioavailability of a compound is calculated by integrating the area under the curve (AUC).

Isoflavones have been shown to be present in blood samples already 15-30 min after ingestion, reaching their peak concentrations between 2 and 12 h. Intake of approximately 50 mg of isoflavones/d yields plasma concentrations that range from 50 ng/mL to 800 ng/mL (0.2 – 3.2 µmol/L) in human adult.[124] After a single oral bolus dose of isoflavones, plasma levels return to basal level within 72 h.[115] The major portion of the ingested isoflavones is excreted in urine during the first 24 h.[115] Limited data on the excretion of

isoflavonoid metabolites are available. Equol, the metabolite of daidzein, appears in urine several hours after ingestion and is typically excreted within 12-48 h.[103, 115]

Bioavailability of a compound refers to the proportion of molecules that enter the blood circulation intact after ingestion of a compound of interest from a food source. Factors contributing to the bioavailability include absorption, distribution, metabolism, and elimination. According to current knowledge, isoflavone glycosides, the predominant form of isoflavones in foods, are not absorbed intact and are thus not bioavailable.[42] The deglycosylation of isoflavones by gut microflora enables the absorption of isoflavone aglycones, but the extensive conjugation with glucuronic acid lowers the levels of circulating free isoflavones. The metabolism of isoflavones in the gut also affects the levels of circulating isoflavones; if an individual possesses bacteria that effectively metabolize and degrade the isoflavones, less ingested isoflavones and more metabolites are absorbed. Given that isoflavones are present in blood mainly as glucuronide conjugates (>62%)[100,125] and 10-60% of ingested isoflavones are recovered in urine,[111,114,123,126] one may anticipate that, in general, isoflavones are rather poorly bioavailable.

Many studies have investigated the bioavailability of isoflavones by using methods that allow determination of isoflavone levels both in ingested food sources and in biological samples as aglycones. With pure isoflavones, three studies have been carried out. In these studies, genistein has been shown to be more bioavailable than daidzein, plasma or serum concentrations of genistein being consistently higher than those of daidzein.[120,121,127] When comparing the bioavailability of isoflavones as aglycones or from glucoside conjugates, either no apparent difference in bioavailability or pharmacokinetics of isoflavones[121] or a slight increase in bioavailability from isoflavone glycosides has been observed.[120,122]

The effect of food matrix on isoflavone bioavailability has also been evaluated. Regular soy consumption along with pure isoflavone preparation supplementation does not change the bioavailability and pharmacokinetics of ingested isoflavones.[115] In young females, regular soy ingestion (as soymilk) has been reported to increase equol **24** production.[128] In males, by contrast, regular soy intake did not affect isoflavone metabolism, but altered the time-courses of excretion.[129] No differences in the pharmacokinetics of isoflavones were observed in a study where isoflavone glycosides of a soy drink were hydrolyzed to aglycones.[122] The urinary recovery of isoflavones during a diet containing fermented soy (tempeh) was significantly greater than during a diet containing nonfermented soy, suggesting greater bioavailability of isoflavone aglycones.[130] The absorption of isoflavones from fermented foods has also been shown to be enhanced.[41]

1.5 Mass spectrometry in identification and quantification of isoflavones and their metabolites in biological fluids

1.5.1 Gas chromatography - mass spectrometry (GC-MS)

By definition, gas chromatography – mass spectrometry is suitable for volatile compounds that are easy to get into gas phase. Polar compounds with a low vapor pressure may be analyzed after derivatization, for example, the attachment of trimethylsilyl groups to free hydroxyl groups of isoflavonoids. The sample containing a mixture of compounds to be analyzed is initially injected into the gas chromatograph, where it is vaporized in a heated injection port. The vapor mixture then travels with the aid of a carrier gas, normally helium, through a capillary GC column, in which the compounds are separated as they interact with the column material. These interactions depend on the structure of the compound and the stationary phase used in the capillary column, and therefore, some compounds are retained in the column for a longer time than others. The end of the capillary column leads to an ionization chamber of a mass spectrometer, held under vacuum, so as soon as the separated compounds exit the column they enter the mass spectrometer and are ionized for mass spectrometric analysis.

Electron impact (EI) ionization is the most commonly used ionization technique in GC-MS. Gaseous molecules are bombarded with high-energy electrons that are obtained from a heated filament and accelerated across the ionization chamber by a voltage, usually 70 eV. The collision reaction of an electron with the molecule may lead to electronic excitation in the molecule or, preferably, to ejection of an electron from the molecule to yield a radical cation called a molecular ion. The energy used in the ionization is so high that, in addition to ionization, fragmentation of the molecule to smaller ionic and/or neutral species occurs. Normally positive ions are analyzed in EIMS, so any neutral or negatively charged species are pumped away and will not reach the mass analyzer and the detector. A mass analyzer separates the ions formed during the ionization process according to their mass-to-charge (*m/z*) ratio for detection and recording. There are many types of different mass analyzers (quadrupole, magnetic sector, time-of-flight, ion trap, etc.) used in GC-MS, but these will not be discussed here in detail. As in other applications of GC-MS, in flavonoid and isoflavonoid analyses, the quadrupole mass analyzer is the one most commonly used.
An advantage of using GC-EIMS is that this technique has been available for many decades. The ionization process, formation of ions, and mechanisms of reactions occurring during ionization have been well studied.[131] By using a constant energy of 70 eV in ionization, the mass spectra of the compounds are reproducible and are not dependent on the instrument used, thus enabling the creation and use of large mass spectral libraries, some available on-line (http://www.aist.go.jp/RIODB/SDBS and http://webbook.nist.gov/chemistry). However, as mentioned earlier, the GC-MS technique is only suitable for volatile compounds, i.e. it is not applicable for very polar compounds or compounds with high molecular weights. In addition, thermally unstable compounds may decompose during evaporation of a sample for GC. Also the high energy used for the ionization may

cause extensive fragmentation so that the molecular ion cannot be determined or no structural information for a compound is obtained.

1.5.2 Liquid chromatography - mass spectrometry (LC-MS)

Liquid chromatography – mass spectrometry has become more important in recent years, since unlike GC-MS it can be used for polar compounds and compounds having high molecular weights, such as isoflavonoid conjugates. Furthermore, derivatization is not necessary and laborious sample pretreatment of biological samples needed for GC-MS analyses can be obviated. In LC-MS, the sample containing the compounds of interest dissolved in the mobile phase is injected into a high-performance liquid chromatograph (HPLC). When passing through the column, the compounds become separated owing to their interactions with the stationary phase. In isoflavonoid analysis, most commonly a C18 stationary phase is used, while the mobile phase consists of mixtures of water/methanol or water/acetonitrile containing a small percentage of formic acid or ammonium acetate as a modifier.

Because of the large volume of solvent emanating from HPLC, the ionization is commonly carried out under atmospheric pressure. The prevailing atmospheric pressure ionization (API) techniques include electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). In ESI, the solvent passes through a capillary tube to an ion source, where application of a large electric field to the end of the tube disrupts the emerging liquid surface and provides a spray of highly charged droplets. The charge of the droplets has the same polarity as the applied field, either positive or negative, depending on the structure of the compound of interest. The formation of charged droplets is generally aided by adding a small percentage of an organic modifier (formic acid, acetic acid, ammonium acetate) in mobile phase. There are two theories of how gas phase ions are formed from charged initial droplets. According the ion evaporation theory, the evaporation of the solvent shrinks the droplets, increasing the charge density, and at a certain point, when the repulsive forces between the charges become equal to surface tension (Raleygh limit), the ions are evaporated.[132] The charge residue theory suggests that ions are formed through consecutive steps of coulombic fission, which eventually lead to the formation of droplets containing only one ion.[133]

In the APCI, the solution is sprayed from the capillary tube and converted to a fine mist by heated nebulizing gas. Following desolvation, the gas is carried by a flow of nitrogen past a corona discharge needle, where the ionization occurs. The vaporized solvent molecules are ionized and act as a secondary reactant gas ions, transferring the charge to the sample molecules. As the gas flow and the ions are expanded through a sampling orifice into a region of vacuum, the solvent molecules attached to protonated $(M+H)^+$ or deprotonated $(M-H)^-$ molecules are stripped off and the $(M+H)^+$ or $(M-H)^-$ ions enter the mass analyzer.

Both ESI and APCI are so-called soft ionization techniques, and very little fragmentation of molecules occurs during the ionization process. Therefore, the mass spectra of the

compounds usually contain the $(M+H)^+$ or $(M-H)^-$ ions only. To increase the specificity and to obtain structural information on the compounds, tandem mass spectrometry (MS/MS) is often used in combination with LC techniques. A triple quadrupole is commonly used as a mass analyzer, the first and last quadrupoles acting as mass analyzers, while the second quadrupole in the middle serves as a collision chamber. The ions filtered through the first quadrupole are collided with gas (typically nitrogen or argon), resulting in the fragmentation of an ion to smaller charged or neutral species that are separated by their *m/z* in the third quadrupole.

The use of LC-MS or LC-MS/MS, as well as other methods applying liquid chromatography, has many advantages in the analysis of isoflavonoids in biological samples. Compared with GC-MS, the laborious and time-consuming sample pretreatment may be avoided and the analysis of intact biomolecules, such as isoflavone glycosides in food items or glucuronides and sulfates in biological fluids, is possible.[43] However, for identification and structure characterization purposes, this technique is not as suitable as GC-MS, since the fragmentation reactions of compounds in LC-$(MS)^n$ systems are not fully established and understood.[131]

1.5.3 Analysis of isoflavonoids in plasma samples

The mass spectrometric methods for analyzing isoflavonoids in plasma samples are summarized in Table 1 (Appendix 1). In general, internal standards (ISTD) are added to 0.1-4.0 mL of plasma, and the sample is hydrolyzed with glucuronidase/sulfatase (commonly from *Helix pomatia*) at 37ºC overnight. Commercially available *H. pomatia* preparations may contain small levels of isoflavones, so in some studies the enzyme has been purified.[69,115,125,134] Hydrolysate is extracted, either by solid-phase extraction (SPE) with C_{18} cartridges[115,125,135-137] or by liquid-liquid extraction (LLE) with diethyl ether.[125,134,138,139] Some methods use additional purification of the sample with ion exchange chromatography[115,125,134,138] or remove fat-soluble compounds by extraction with hexane[69,140] before analysis. For GC-MS analyses, the samples have been derivatized to trimethylsilyl (TMS)[69,125] or *t*-butyldimethylsilyl (TBDMS)[115] derivatives. Selected ion monitoring (SIM) has been used to increase the sensitivity of the method. The ions selected in GC-MS analyses are commonly the molecular ions, except for genistein and dihydrogenistein, for which the $(M-15)^+$ ion has been used for quantification. In LC-MS analyses, usually the deprotonated molecules have been monitored.

1.5.4 Analysis of isoflavonoids in tissue samples

Mass spectrometric analyses of tissue samples have thus far been carried out for samples from laboratory animals only. Using LC-MS or LC-MS/MS techniques, the isoflavonoid distribution has been examined in mammary gland, uterus, ovary, testes, prostate, thyroid, liver, and brain of rats.[107, 108, 109] The pretreatment methods of tissue samples are summarized in Table 2 (Appendix 1). In study by Chang et al.[107] LC-ESI/MS has been

used in positive ion mode to monitor protonated molecule ions for genistein and d_4-genistein, the internal standard. Samples containing isoflavone levels close to the limit of detection have been analyzed using LC-ESI/MS/MS to add specificity to the method.[107]

A recent study by Gu et al.[106] suggested that using enzymatic hydrolysis in sample pretreatment of tissue samples underestimates the levels of isoflavone conjugates, and the authors recommend the use of acid hydrolysis in analysis of total isoflavones. The tested sample pretreatment methods, including enzymatic hydrolysis with *H. pomatia*, sequential hydrolysis with β–glucuronidase and sulfatase enzyme preparations, and acid hydrolysis, are summarized in Table 2 (Appendix 1). In these methods, biochanin A was used as an internal standard, added at the end of the sample pretreatment methods. LC-APCI/MS instrumentation was used in negative mode to monitor $(M-H)^-$ for daidzein and genistein. LC-APCI/MS conditions were the same as those applied for analysis of isoflavone and their sulfate and glucuronide conjugates in urine.[141]

1.5.5 Analysis of isoflavonoids in urine samples

Several methods using GC-MS or LC-MS techniques have been developed for the quantitative analysis of isoflavonoids in human urine samples. The methods, summarized in Table 3 (Appendix 1), employ three different approaches to measure isoflavonoids. The first is the measurement of total isoflavones as aglycones using enzymatic hydrolysis, typically with β-glucuronidase and arylsulfatase from *H. pomatia*.[139,142] The second approach is the estimation of isoflavone aglycones and conjugates using selective hydrolyses with glucuronidase and/or sulfatase enzymes,[141,143] or by separating the aglycones and different conjugates by ion exchange chromatography followed by the hydrolysis of the conjugates to aglycone analytes.[27,138] The third and most straightforward approach is the direct measurement of aglycones and conjugates from urine samples. However, the lack of suitable conjugate standards has limited the direct analysis of these compounds, and thus far, only semiquantitative methods are available.[118]

In all methods, the selected ion monitoring (SIM) or multiple reactions monitoring (MRM) has been used to increase sensitivity. In GC-MS methods, ions selected for SIM are commonly the molecular ions of derivatized isoflavonoids, except for genistein, for which the more abundant $(M-15)^+$ ion has been used. In LC-MS methods, the negative ionization mode has been applied, therefore the monitored ions are deprotonated molecule ions. In one method, equol was measured separately using the positive mode, and thus, the protonated molecule ion was used for analysis.[118]

The analysis of isoflavonoids has been carried out from either spot urine samples[143] or urine samples collected over 24 h.[27] The volumes of the samples to be analyzed may vary greatly, from 200 µL to 20 mL, the biggest sample volumes being from older studies using either GC-MS[27,143] or LC-MS.[141] The most recent GC-MS method requires only 200 µL of urine, and compared with other analytical methods, very little sample pretreatment is

needed for total isoflavone analysis, making the method an effective tool in the analysis of large sample batches with a limited volume of urine sample available.[142]

The methods determining the levels of isoflavonoid aglycones and conjugates by sequential hydrolysis seem to provide only estimates of excreted conjugates due to the presence of mixed sulfate and glucuronide conjugates.[141] The method available for direct measurement of isoflavone aglycones and conjugates is considered semiquantitative owing to the lack of appropriate reference standards.[118] So far, the most reliable method for conjugate analysis is separation of the aglycones and different conjugates by ion exchange chromatography, followed by the hydrolysis of the conjugates and analysis as aglycones.[27,138] However, due to the laborious sample pretreatment method, including several extraction and ion exchange chromatographic steps, this method is not suitable for routine analysis of large sample series. The quantitative sample pretreatment methods have also been applied in the identification of urinary isoflavonoids,[21,22,26,144,145] but some studies have supplemented these by using their own sample pretreatment methods.[24,25,71]

1.5.6 Analysis of isoflavonoids in fecal samples

To date, only one mass spectrometric method for quantitative analysis of isoflavones in fecal samples has been reported.[119] The conjugated isoflavonoids occur in very low amounts in feces (<10%), and thus have not been included in the method. ISTDs (deuterated analogues of daidzein **1**, genistein **2**, equol **24,** and *O*-dma **28**) are mixed with 0.3–0.6 g of homogenized sample. After addition of acetone and ethanol, the sample is further homogenized, and the extract is filtered and further purified with ion exchange chromatography prior to derivatization to TMS ethers. Quantitative analysis is carried out using GC-MS with selected ion monitoring (SIM) of molecular ions, or in the case of genistein, the more abundant $(M-15)^+$ ion. Qualitative methods for the identification of isoflavones and their metabolites in fecal samples or *in vitro* fecal fermentation samples have also been reported.[30,62,66]

1.6 Biological activities of isoflavones and their metabolites

1.6.1 In plants

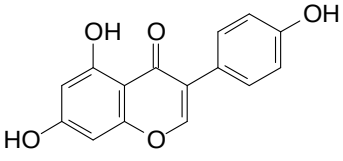
Isoflavonoids occur mainly in plants belonging to *Leguminosae*, i.e. plants capable of fixing atmospheric N_2 by symbiotic interaction with bacteria (*Rhizobia*) internalized within root nodules. The seeds of the legumes are abundant reservoirs of isoflavonoids, some of which can be released into soil during germination.[146,147] The isoflavonoids play an important role in mediating the multiple plant-microbial interactions, acting as signal substances to bacteria.[148] They serve as chemoattractants for bacteria, influence bacterial growth, and selectively activate the expression of the nodulation (*nod*) genes of symbiotic bacteria.[149,150]

In addition to acting as signal molecules, isoflavones exhibit antimicrobial or antifungal activities and are therefore believed to help the plant fight various diseases. Genistein, for example, is toxic to several classes of fungal and oomyetic pathogens,[151] while daidzein is suggested to be a precursor for the synthesis of glycollin, a phytoalexin.[152,153] The levels of isoflavones in plants are to a great extent dependent on growing conditions. In general, the levels of isoflavones increase when the plant grows under conditions that induce a stress response. For instance, low growing temperatures, waterlogging, and mineral deficiency have been reported to increase isoflavone levels.[154]

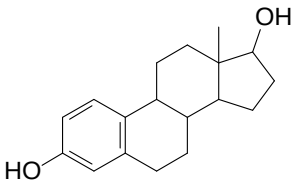
1.6.2 In humans

The interest in isoflavones, their biological activities, and possible beneficial effects on human health started in the 1980s when human urine was found to contain high levels of isoflavonoids originating from soy, which in animals had been demonstrated to exert some hormone-like effects.[19,155,156] Epidemiological and immigrant studies associated a diet rich in isoflavones with a lowered risk for certain hormone-dependent cancers,[27,76,129] including breast and prostate cancers, and later studies have shown that isoflavones may play a role in the prevention of osteoporosis and cardiovascular disease.[157-159]

In general, high levels of biologically active hormones, androgens or estrogens, are associated with an increased prostate or breast cancer risk. Structurally, isoflavonoids share similarities with endogenous estrogens, especially with 17β-estradiol **60**, and they bind to estrogen receptors.[160,161] Binding of isoflavonoids to estrogen receptors may exert both estrogenic and antiestrogenic effects, isoflavonoids acting as weak agonists or antagonists. As weak agonists, isoflavonoids occupy the estrogen receptor in the presence of a more potent ligand, such as 17β-estradiol, but the estrogenic response they produce is low. In the case of an antagonist compound, the binding of the compound to the receptor produces no response.[161]



Genistein **2**

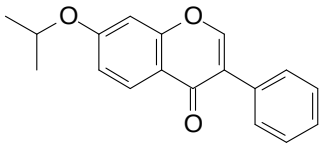


17β-Estradiol **60**

Isoflavones interfere with the biosynthesis of endogenous estrogens and androgens by inhibiting the key enzymes (steroid dehydrogenase, aromatase, or 5α-reductase). They reduce the concentrations of free estrogens by stimulating the production of sex hormone binding protein (SHBG)[23,162] or by activating enzymes forming inactive conjugates of the estrogens.[163] Besides affecting the production, metabolism, and actions of hormones, isoflavones, especially genistein, have an effect on tumor cell growth by suppressing angiogenesis and inhibiting protein tyrosine kinase, topoisomerase II, and protein histidine

kinase, which play important roles in cell proliferation, transformation, growth, and differentiation.[48,164,165]

During and after menopause, endogenous estrogen secretion decreases, which has been associated with a higher incidence of osteoporotic fractures in postmenopausal women. Hormone replacement therapy (HRT) has been shown to reduce postmenopausal osteoporosis.[166] Given the estrogenic nature of isoflavonoids without the harmful effects of estrogens, there has been a growing interest in studying isoflavones both for the treatment of osteoporosis and as an alternative to HRT. The results from human clinical studies on the relationship between isoflavonoid intake and osteoporosis are inconsistent.[167,168] Many of the studies have been too small and short to assess possible benefits of isoflavonoids on bone quality. Longer-term studies suggest that isoflavones may have an impact on bone health, although the effect observed is only moderate.[167,169] Most of the data regarding the benefits of isoflavonoids on bone health derives from studies of ipriflavone **61**, (7-*O*-isopropylisoflavone) a synthetic drug candidate, which has been reported to reduce bone loss in postmenopausal women by inhibiting osteoclast activity and stimulating osteoblast activity.[169-173] Several mechanisms by which genistein improves bone health have been suggested, many of which are supported by *in vitro* laboratory experiments. These include genistein's estrogenic and inhibitory effects on protein tyrosine kinase,[174] inhibitory effect on osteoclast-like cell formation,[175] modulation of production of nitric oxide, and stimulation of protein synthesis in osteoblast cells.[175]



Ipriflavone **61**

Many studies have investigated the efficacy of isoflavonoids in treatment of menopausal symptoms. No clear positive effects of soy or isoflavones on menopausal symptoms have been found. However, some studies indicate that the use of isoflavones to address vasomotor symptoms may provide small benefits beyond a placebo effect, but no benefit for genital atrophy.[176] There are also studies showing that soy and soy isoflavones given to postmenopausal women improve their cognitive functions, memory performance, and frontal lobe functions of mental flexibility and planning.[177-179] Furthermore, the use of soy in alleviating menopausal symptoms may also reduce the risk for coronary heart disease. A soy diet has been shown to decrease low-density lipoprotein (LDL) and total plasma cholesterol levels, possibly by upregulation of LDL receptors.[180] In addition, genistein has been found to possess antithrombolic effects and to decrease platelet activation, deposition, and aggregation, thus decreasing the progression of atherosclerosis.[181-184]
Most of the results obtained from human clinical studies are inconclusive and the mechanisms by which isoflavones act remain obscure. The most recent studies suggest that the metabolites of isoflavones may play a role in the prevention of the diseases

mentioned above.[185] For example, in a two-year intervention study using soy milk, increases in both bone mineral density and bone mineral content were observed in women who were equol producers.[186] In laboratory trials, equol has been found to possess estrogenic activity, having an affinity for both estrogen receptors ER-α and ER-β of a similar magnitude to genistein.[187] Only a few sporadic studies have examined the biological activities of other isoflavonoid metabolites, but there are some indications that they might be involved in the mechanisms of action of isoflavones.[49,188-195]

2. AIMS OF THE STUDY

For several years, the interest in isoflavonoids and their potential role in the prevention of certain diseases has focused on research on daidzein and genistein, the principal isoflavones of soy and soy-based foods, and the main metabolites of formononetin and biochanin A, the principal isoflavones of red clover. Until recently, comprehensive studies on the metabolism of these compounds have been lacking. The aim of this study was to investigate the metabolism of isoflavones in humans – to identify all metabolites excreted in urine after soy or red clover supplementation, which have an intact isoflavonoid structure, and thus might possess biological activity. To achieve this aim, the following studies were conducted:

- Investigation of fragmentation of isoflavonoids in electron ionization mass spectrometry (I-IV).

- Evaluation of *in vitro* bacterial metabolism of isoflavones (III).

- Identification of isoflavonoid metabolites in human urine after soy supplementation (I-III, V).

- Identification of isoflavonoid metabolites in human urine after red clover supplementation (IV, V).

3. EXPERIMENTAL

3.1 Materials

3.1.1 Standards, chemicals, and reagents

Daidzein, genistein, glycitein, 7,8,4'-trihydroxyisoflavone, pseudobaptigenin, formononetin, biochanin A, dihydrodaidzein, dihydrogenistein, dihydroglycitein, dihydroformononetin, dihydrobiochanin A, equol, *cis* and *trans* 4-hydroxyequol, 7,3'-dihydroxyisoflavan, 4'-*O*-methylequol, 7,3',4'-trihydroxyisoflavan, 7,4'-dihydroxy-6-methoxyisoflavan, 7,4'-dihydroxy-3'-methoxyisoflavan, *O*-dma, 6'-hydroxy-*O*-dma, angolensin, and 6'-hydroxyangolensin were synthesized at the Laboratory of Organic Chemistry, Department of Chemistry, University of Helsinki, Finland.[196-200] Calycosin, prunetin, 7,3',4'- and 6,7,4'-trihydroxyisoflavones, and orobol were obtained from Apin Chemicals Ltd., United Kingdom.

All reagents were pro-analysis (pa) grade or higher. Acetic acid glacial 100%, L(+)-ascorbic acid and, diethyl ether were obtained from Merck, Germany. Ethyl acetate, heptane, hexane, and methanol were from Rathburn Chemicals Ltd., Scotland. *Helix pomatia* juice was purchased from BioSepra SA, France. Hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were obtained from Pierce, USA, and pyridine, Super Purity Solvent, from Romil Ltd., Great Britain. Lipidex-5000 was purchased from Packard Bioscience B.V., the Netherlands, and QAE Sephadex ^{R} A-25 and Sephadex ^{R} LH-20 from Pharmacia Biotech AB, Sweden.

3.1.2 Dietary supplements

Soy bar (Soya confectionery bar, L 1073/01/2) was provided by SHS International Ltd., United Kingdom. The red clover dietary supplement (Red Clover™) was obtained from Novogen, Australia.

3.1.3 Human urine samples

Urine collection

Daily (24-h) urine samples were collected in plastic containers, and 1% of ascorbic acid and 0.1% of sodium azide were added as preservatives. After collection, the volumes of the samples were measured and aliquots of 50-100 mL were stored in a refrigerator (-20°C).

Soy supplementation study (I-III)

Six volunteers (three men and three women) were recruited for this study among students of medicine and they included three soy bars per day into their normal Western diet for a two-week period. The 24-h urine samples were collected one day before and on two consecutive days after the soy supplementation period.

Red clover supplementation study (IV)

Seven healthy Finnish women, aged 20-60 years, were recruited for this study among the staff of the Folkhälsan Research Center. Subjects were asked to abstain from foods containing high levels of isoflavones, e.g. soy or foods derived from soy, for one week (days 1-7) before and during the study (days 8-12). On the morning of the eighth day, subjects took four red clover -based dietary supplements with a glass of water. A baseline 24-h urine sample was collected one day (day 7) before isoflavone supplementation. After the supplementation a 24-h urine sample collection was continued for five consecutive days (days 8-12). The study protocol was approved by the Ethics Committee for Research in Epidemiology and Public Health, Hospital District of Helsinki and Uusimaa, Finland.

3.1.4 *In vitro* fecal fermentation samples

The incubation method of Karppinen et al.[201] was modified as follows: a carbonate-phosphate buffer solution with trace elements was held in an anaerobic chamber for two days prior to the fermentation. Feces were collected from three healthy human volunteers, who had ingested a Western diet, presented no digestive disease, and had not received antibiotics for at least three months. Freshly passed feces were immediately taken in an anaerobic chamber, pooled, and homogenized at the same time with an equal weight of culture medium using a Waring blender. The slurry was diluted to 16.7% (w/w) with culture medium, filtered through a 1-mm sieve, and used immediately as inoculum.

A 1- to 2-mg quantity of each substrate (daidzein **1**, genistein **2**, 7,3',4'-trihydroxyisoflavone, 6,7,4'-trihydroxyisoflavone, 7,8,4'-trihydroxyisoflavone, and orobol **15**), corresponding to 2-3.5 μmol, was weighed into 50-mL glass vials and 10 mL of fecal suspension was added in an anaerobic chamber at 30°C. The vials were sealed with rubber stoppers and shaken in a water bath at 37°C for 24 h. Duplicate incubations were carried out for each substrate. Also duplicate blanks, containing only culture medium and inoculum, were incubated for 0 and 24 h. The fermentation was stopped by plunging the vials into iced water, after which the vial contents were freeze-dried and weighed.

3.2 Methods

3.2.1 Analysis of soy bar and red clover dietary supplements

The isoflavone content of soy bar and red clover dietary supplements was determined by a quantitative method for food samples with minor modifications.[202] Because of the high levels of isoflavones in both samples, purification with ion exchange chromatography was unnecessary. The combined ether extracts from *H. pomatia* hydrolysates were evaporated to dryness, and the sample were dissolved in 5 mL of methanol, from which an appropriate aliquot (100-500 μL) was pipetted for analysis.

3.2.2 Sample pretreatment method for urine samples

The sample pretreatment method was an application of the method used by Kelly et al.[24] An additional purification step with SPE Sep-Pak C_{18} cartridges (Waters, USA) was introduced at the beginning of the method to remove salts and compounds inhibiting enzymatic hydrolysis and to yield samples of equal sizes before hydrolysis.[203] From daily urine samples, duplicate samples of a 1/300 fraction were analyzed each time. The pH of the sample was adjusted to 3.0 by adding 1/10 of sample volume of 1.5 M acetate buffer pH 3.0. Urine samples were extracted with pre-washed (6 mL of MeOH followed by 10 mL of water) Sep-Pak C_{18} cartridges. The sample was then washed with 5 mL of 0.15 M acetate buffer pH 3.0. The analytes were eluted with 3 mL of methanol and evaporated to dryness, after which the dry samples were hydrolyzed with *H. pomatia*, extracted twice with diethyl ether (2 x 6 mL), and chromatographed on a Sephadex LH-20.

Neutral steroids and other urinary compounds interfered with the analysis of isoflavonoids that had eluted in the first fraction of the Sephadex LH-20 run. This was a problem when identifying the reduced isoflavonoid metabolites of red clover isoflavones formononetin and biochanin A, which had eluted in this fraction. Further purification was performed with chromatography on QAE acetate column, used in the method for quantitative analysis of isoflavones in human urine. The first fraction of the Sephadex LH-20 run was evaporated to dryness under nitrogen flow and the samples were dissolved in 0.5 mL of methanol and applied to a column (0.5x5.0 cm) of QAE acetate. The first fraction was eluted with 5 mL of methanol and the second fraction with 7 mL of 0.2 M acetic acid in methanol. Both fractions were evaporated to dryness. The method and the distribution of soy and red clover isoflavones and their metabolites in the different chromatographic fractions are presented in the flow chart in Figure 4.

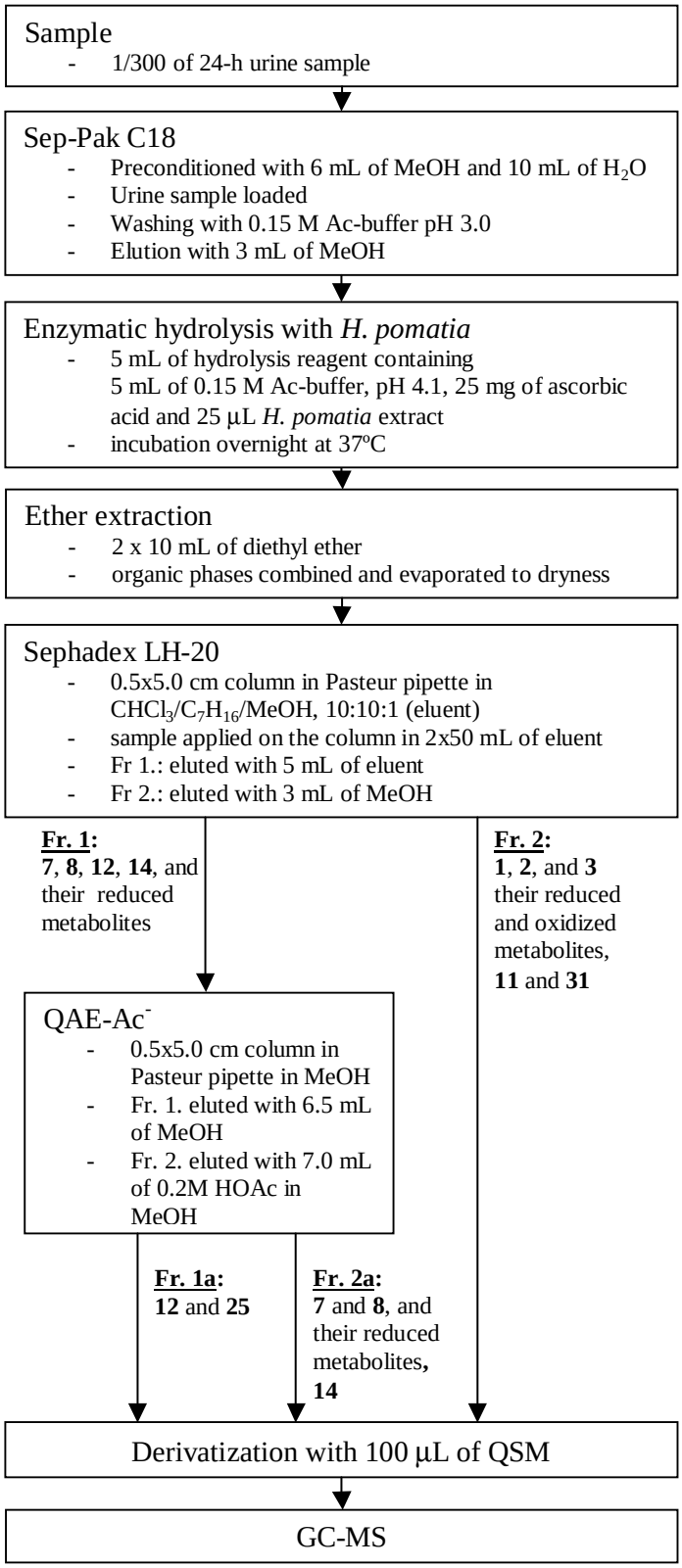


Figure 4. *Sample pretreatment method.*

Table 4. *Isoflavonoids added to control urine and fecal fermentation samples, the internal standards (ISTD), and the ions that were monitored for determination of specificity, recovery, and precision of the analytical method developed for identification of isoflavones and their metabolites in urine and fecal fermentation samples.*

Analyte	Added (μg)	MW	Monitored ion (m/z)	ISTD	MW	Monitored ion (m/z)
Daidzein	2.35	398	398	d_4-Daidzein	402	402
Genistein	1.58	486	471	d_4-Genistein	500	475
Equol	1.89	386	386	d_4-Equol	400	400
O-dma	2.02	474	459	d_5-*O*-dma	479	464
Dihydrodaidzein	1.59	400	400	d_4-Dihydrodaidzein	404	404
Dihydrogenistein	1.42	488	473	d_4-Dihydrogenistein	502	477
Formononetin	4.84	340	340	d_3-Formononetin	343	343
Biochanin A	4.68	428	413	d_4-Biochanin A	432	417
4'-*O*-Methylequol	2.03	328	328	d_4-Equol	400	400
Angolensin	1.98	504	489	d_4-*O*-dma	479	464
Dihydroformonetin	3.22	342	342	d_4-Dihydrodaidzein	404	404
Dihydrobiochanin A	2.39	430	417	d_4-Dihydrogenistein	502	477
7,3',4'-tri-OH-isoflavone	2.15	486	471	d_4-Daidzein	402	402
6,7,4'-tri-OH-isoflavone	1.98	486	471	d_4-Daidzein	402	402
8,7,4'-tri-OH-isoflavone	2.32	486	471	d_4-Daidzein	402	402
5,7,3',4'-tetra-OH-isoflavone	2.21	574	559	d_4-Genistein	500	475

Specificity, recovery, and precision of the sample pretreatment method were evaluated with six replicate analyses of baseline (low control) and soy- or red clover-supplemented (high control) urine samples spiked with reference compounds (1.42-4.84 μg/10 mL of urine) (Table 4). In addition, duplicate samples of both baseline urine and reagent blank samples were included in the sample batches. For evaluation of specificity, additional fractions from both chromatographic runs (Sephadex LH-20 and QAE-acetate) were collected after the elution of the fraction containing the compounds of interest and analyzed for their isoflavone content. Deuterated internal standards (Table 4), added just before analysis by GC-MS, were used as internal standards in calculations of concentrations. Recoveries of the analytes were determined by comparing the peak area ratios of selected ions of each compound vs. internal standard. The selected ions for each analyte and internal standard are shown in Table 4.

3.2.3 Sample pretreatment method for fecal fermentation samples

The qualitative method for identification of isoflavone metabolites in fecal fermentation samples was based on a previous study in which the *in vitro* metabolism of plant lignans was investigated.[204] Freeze-dried fecal fermentation sample (20 mg) was weighed and 500 µL of distilled water and 10 µL of 6 M HCl were added. The slurry was extracted twice with 6 mL of diethyl ether, and the combined organic phases were evaporated to dryness under nitrogen flow. The sample was applied to a Lipidex 5000 column (0.5 x 5 cm) in 2 x 200 µL of MeOH:$CHCl_3$:H_2O (4:1:1). Compounds of interest were eluted and collected with 4 mL of the same eluent. The collected fraction was evaporated to dryness, derivatized, and analyzed by GC-MS.

The sample pretreatment was tested for recovery, specificity, and precision by six replicate analyses of spiked blank fermentation samples. Standards (1.42-2.35 µg, Table 4) were added to the blank fecal fermentation sample (20 mg) at the beginning of the sample pretreatment. After the sample pretreatment, the deuterated internal standards were added to the sample, and the samples were derivatized and analyzed by GC-MS. Recoveries, precision, and specificity of the sample pretreatment method were evaluated as described above in the sample pretreatment method for urine samples.

3.2.4 Derivatization

The dry samples were silylated with 100 µL of pyridine:HMDS:TMCS (9:3:1), i.e. quick silylation mixture (QSM), for 30 min at room temperature. The mixture was evaporated to dryness and the samples were dissolved in 200-400 µL of hexane. Later, the evaporation of the sample and dissolving in hexane was omitted, and the samples were silylated in an appropriate amount (200-400 µL) of silylation reagent and injected into GC-MS directly in this solution. Deuterated TMS ethers were used for the structural elucidation of unknown metabolites for which reference compounds were unavailable. The deuterated TMS derivatives were obtained by the derivatization procedure described above, except that d_9-HMDS and d_3-TMCS were used in the silylation mixture.

3.2.5 Instrumentation and analysis

The analysis of urinary samples was carried out using a Fisons GC 8000 gas chromatograph (Milan, Italy) coupled to a Fisons Instrument MD 1000 quadrupole mass spectrometer (Cambridge, United Kingdom). The column used in the gas chromatograph was a BP-1 (12 m x 0.22 mm x 0.25 µm) from SGE (Scientific Glass Engineering, Australia). The flow rate of helium carrier gas was 1 mL/min. The oven temperature was programmed as follows: 150°C for 1 min, then 50°C/min to 250°C, 250°C for 15 min, 50°C/min to 290°C, and 290°C for 5 min. The temperatures of the injection port, interface, and ion source were 280°C, 250°C and 200°C, respectively. Electron-impact ionization with 70 eV electron energy was used, and mass range from 100 to 900 mass units was

scanned. The operation of the GC-MS instrument and data analysis were performed by XCalibur software (version 1.2, Finnigan, Cambridge, United Kingdom).

Isoflavones and their metabolites in urine or fecal fermentation samples were distinguished from other compounds present in the sample by comparing the mass spectrometric chromatograms obtained from blank samples (baseline urine samples or fecal blank) with chromatograms obtained after isoflavone supplementation. The isoflavones and their metabolites were identified using synthetic reference compounds by comparing the retention times and the mass spectra of the metabolite and the authentic reference compound. The criterion used to positively identify a metabolite in a urine sample was that the eight most abundant fragment ions of the mass spectrum of the TMS derivative of the metabolite had the correct relative ion ratios compared with the mass spectrum of a TMS derivative of the reference compound. Compounds for which no authentic reference compounds were available were tentatively identified by interpretation of their mass spectrum. Deuterated TMS derivatives were used to aid the interpretation of the spectrum and to confirm the proposed structures of fragment ions. A computer library search using the library created from mass spectra of reference and tentatively identified compounds was utilized to compare the distribution of the identified compounds in urine samples of different volunteers. The criterion of >95% match was used to positively identify a compound in urine samples.

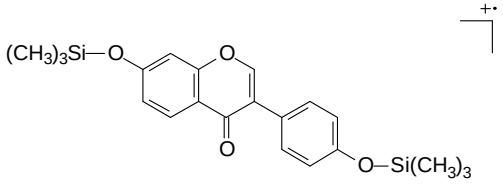
4. RESULTS AND DISCUSSION

4.1 Electron ionization - mass spectra of trimethylsilyl derivatives of isoflavonoids (I-IV)

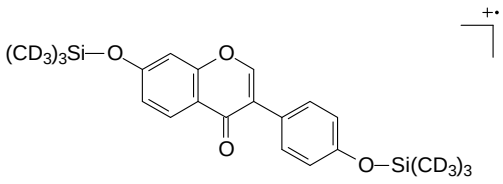
The mass spectra of all TMS derivatives of isoflavonoids discussed in this thesis are summarized in Appendix 2. Most of the spectra are from authentic reference compounds; all other cases are clearly indicated. The mass spectra of deuterated TMS derivatives are also presented. Representative mass spectra of each isoflavonoid group are provided in Figures 5-8, with TMS derivatives of 7,4'-dihydroxy-6-methoxy substituted isoflavonoids serving as model compounds.

4.1.1 Use of deuterated isoflavones in interpretation of mass spectra

The use of deuterated TMS derivatives aids the interpretation of mass spectra, allowing confirmation of the proposed structures of molecular and, especially, fragment ions. As a result of the derivatization process, every free hydroxy group in the molecule is converted to a TMS ether. In deuterated TMS derivatives, every hydrogen atom in the methyl groups of the silylating reagent is replaced by a deuterium. This means that the mass difference between deuterated and nondeuterated derivatives is nine mass units for each TMS group present in a molecule. By comparing the mass spectra of deuterated and nondeuterated compounds, it is easy to calculate the number of TMS groups present in the molecule, and thus establish the number of original hydroxy groups in the compound. For example, the molecular ion of TMS ether of daidzein **1a** is at *m/z* 398, whereas the molecular ion of its deuterated TMS derivative **1b** is 18 mass units higher, at *m/z* 416, from which one can deduce that daidzein has two hydroxyl groups (2x9=18) in the molecular ion.

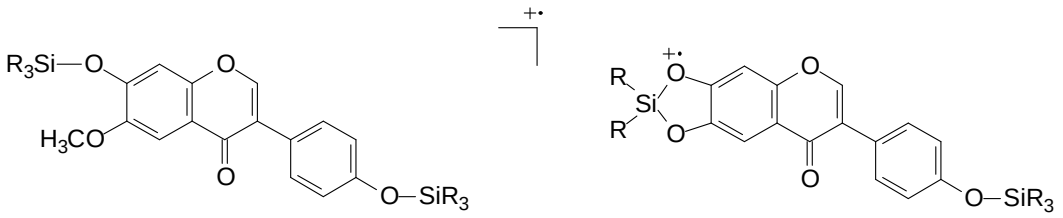


1a
m/z 398



1b
m/z 416

The intensive fragment ion at *m/z* 398 in the mass spectrum of the TMS ether of glycitein **3** (Figure 5) contains five methyl groups originating from TMS groups, which is reflected in the 15 mass unit difference of this ion when comparing the mass spectrum of TMS ether of glycitein **3** with the mass spectrum of its deuterated TMS derivative (Table 1, Appendix 2) as shown below.



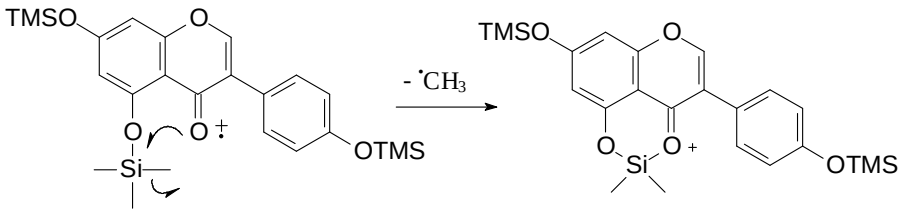
3

R= CH_3: *m/z* 428
R= CD_3: *m/z* 446

R= CH_3: *m/z* 398
R= CD_3: *m/z* 413

4.1.2 Mass spectra of isoflavones

Typical of the mass spectra of TMS derivatives of isoflavones is that they contain intensive peaks at *m/z* values close to the molecular ion (Table 1, Appendix 2). The base peak of the mass spectrum is commonly the molecular ion $M^{+\bullet}$ or a fragment ion $(M\text{-}CH_3)^+$ formed from cleavage of one methyl radical from the molecular ion. $(M\text{-}CH_3)^+$ is the base peak of mass spectra of compounds with a hydroxyl substituent at 5-position, such as genistein **2**, biochanin A **8**, orobol **15**, and prunetin **12**. For example, the loss of a methyl radical from the molecular ion of the TMS ether of genistein **2** yields a more stable ion at *m/z* 471, the structure of which is shown in Scheme 1.



2

m/z 486 (<1)

m/z 471 (100)

Scheme 1. *Loss of a methyl radical from the molecular ion of the TMS ether of genistein **2**.*

In addition to intensive molecular and $(M\text{-}CH_3)^+$ ions, the compounds with a methoxy group ortho to trimethylsilylated hydroxyl group, such as glycitein **3**, calycosin **11**, and 7,4'-dihydroxy-3'-methoxyisoflavone, show intensive peaks formed by subsequent losses of two methyl radicals from the molecular ion (Table 1, Appendix 2). The peaks of these radical cations occur at even *m/z* values. The first methyl radical is cleaved from the TMS group, which is verified by the presence of the fragment ion at *m/z* $(M\text{-}CD_3)^+$ in the mass spectrum of a deuterated TMS derivative of the compound (Scheme 2 and Table 1 in Appendix 2). The second methyl radical is lost from the methoxy group, yielding fragment ions at *m/z* $(M\text{-}30)^{+\bullet}$ and $(M\text{-}33)^{+\bullet}$ in the mass spectra of TMS and deuterated TMS derivatives, respectively. From these radical cations, α-cleavage of one additional methyl radical yields ions of moderate abundance occurring at *m/z* $(M\text{-}45)^+$ and $(M\text{-}48)^+$. For example, in the mass spectrum of the TMS derivative of glycitein **3** (Figure 5), the intensive peaks at *m/z* 383, 398, 413, and 428 have structures shown in Scheme 2.

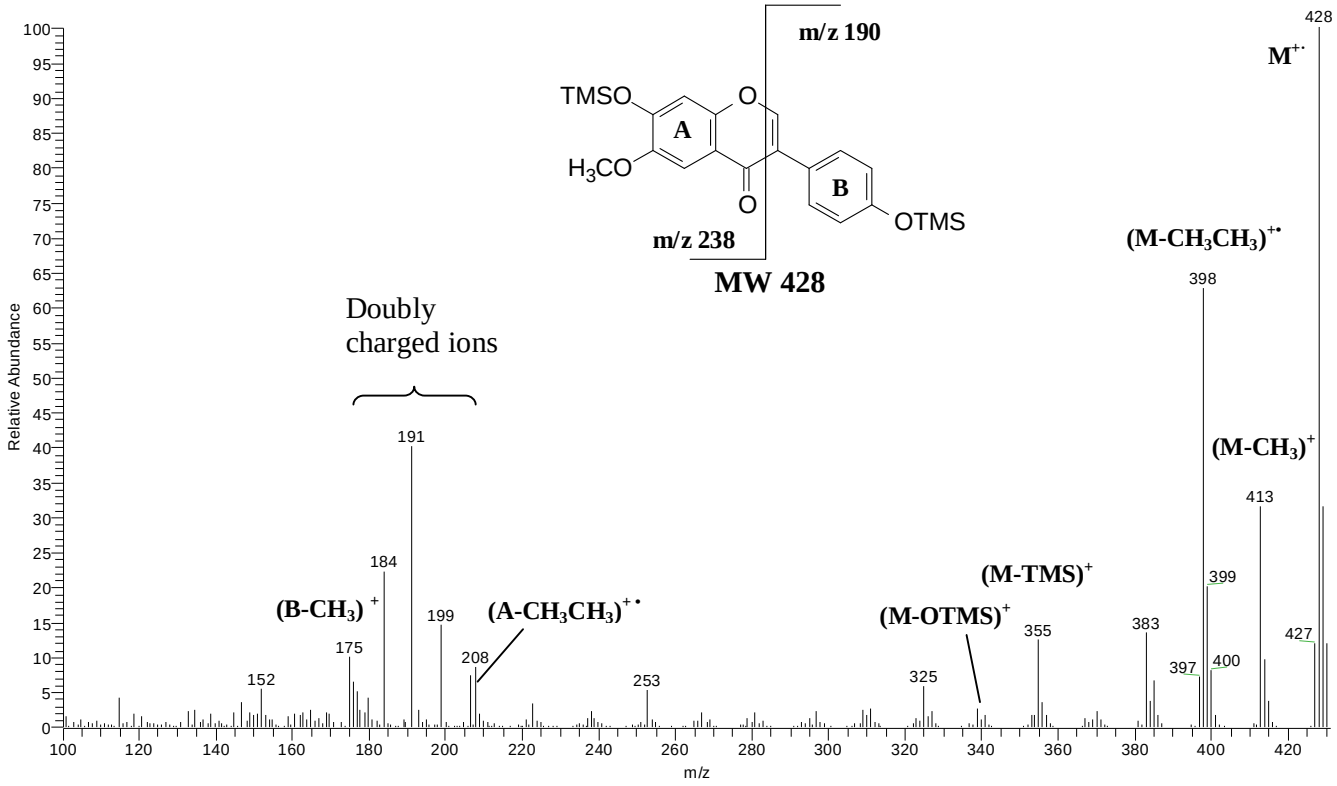
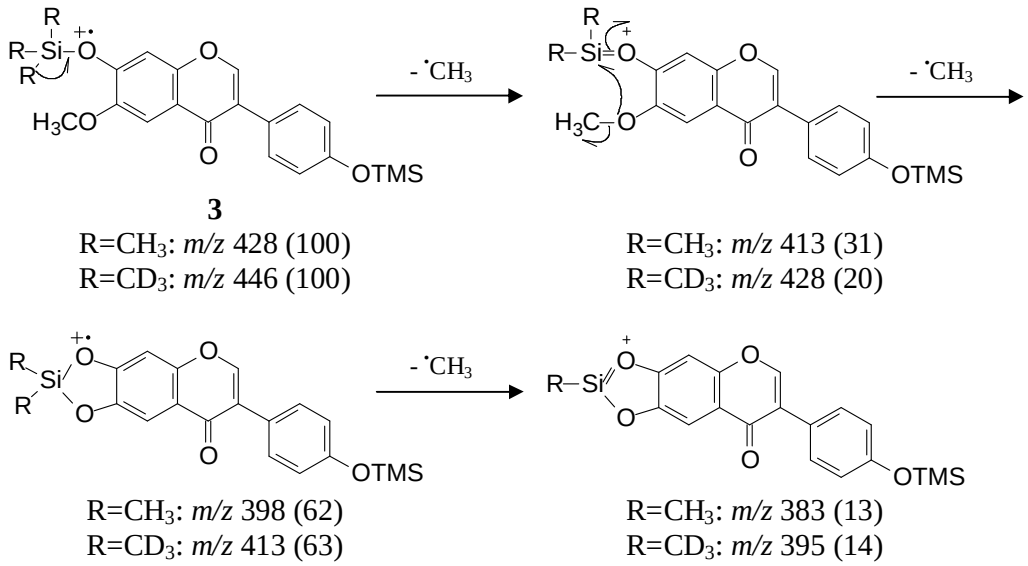


Figure 5. *Mass spctrum of trimethylsilylether of glycitein **3**.*



3

R=CH_3: *m/z* 428 (100)
R=CD_3: *m/z* 446 (100)

R=CH_3: *m/z* 413 (31)
R=CD_3: *m/z* 428 (20)

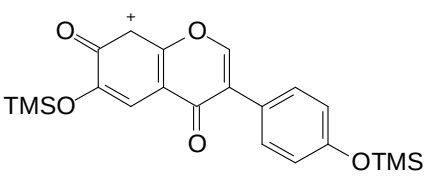
R=CH_3: *m/z* 398 (62)
R=CD_3: *m/z* 413 (63)

R=CH_3: *m/z* 383 (13)
R=CD_3: *m/z* 395 (14)

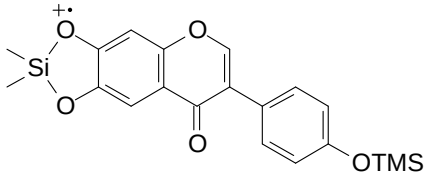
Scheme 2. *Fragmentation of the TMS ether of glycitein 3.*

Another possibility is that two methyl radicals are lost from the molecular ion in a form of an ethane molecule, CH_3CH_3. Small stable neutrals can be eliminated from cations or radical cations, particularly, when the new ion shows a substantial increase in stability over the precursor ion (ref. 130, pp. 218-219).

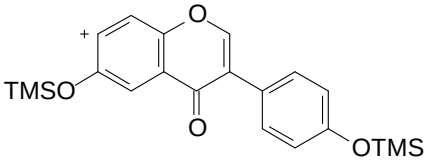
Loss of TMS radical from the molecular ion is common for all isoflavones with trimethylsilylated hydroxyl groups, yielding low or moderate intensity peak at *m/z* value $(M-73)^+$. Compounds with vicinal hydroxyl groups attached to either ring A or ring B have low intensity peaks at *m/z* values $(M-88)^{+\bullet}$, $(M-89)^+$ and $(M-103)^+$, corresponding to cleavages of neutral $Si(CH_3)_4$, OTMS radical, and $Si(CH_3)_4$ followed by loss of a methyl radical, respectively. For example, in the mass spectrum of the TMS ether of 6,7,4'-trihydroxyisoflavone **38** (Table 1, Appendix 2), peaks at masses $(M-73)^+$, $(M-88)^{+\bullet}$, $(M-89)^+$, and $(M-103)^+$, i.e. at *m/z* 413, 398, 397, and 383, respectively, are fragment ions with the structures shown below.



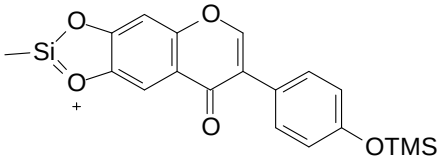
m/z 413 (2)



m/z 398 (3)

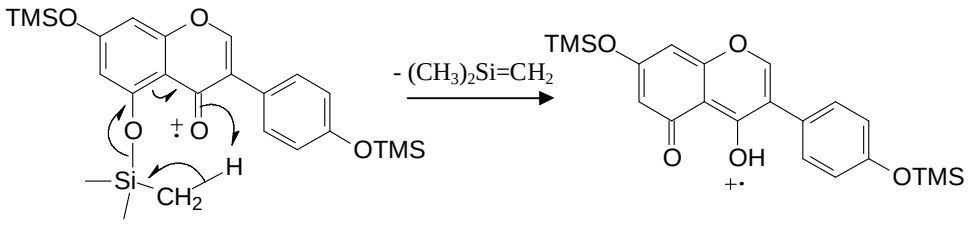


m/z 397 (2)



m/z 383 (8)

TMS ethers of genistein **2**, biochanin A **8**, orobol **15**, and prunetin **12**, all of which have hydroxyl groups at C-5, show an elimination of $(CH_3)_2Si=CH_2$. This could happen by the 8-centered vinylous McLafferty-type elimination presented in Scheme 3. Ions formed by this kind of loss appear in the mass spectrum as low intensity peaks at *m/z* values $(M-72)^{+\bullet}$. The α-cleavage of a methyl radical from the $(M-72)^{+\bullet}$ ion yields $(M-87)^{+}$ and $(M-98)^{+}$ ions in the mass spectra of nondeuterated and deuterated TMS ethers, respectively.

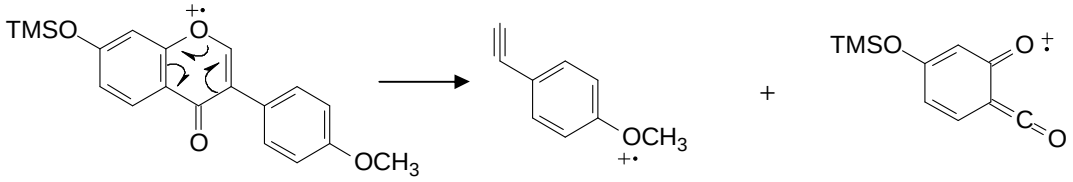


2
m/z 486 (1)

m/z 416 (4)

Scheme 3. *8-Centered vinylous McLafferty type elimination of $(CH_3)_2Si=CH_2$ from molecular ion of genistein **2**.*

Fragment ions formed by a retro Diels-Alder (rDA) reaction (Scheme 4) appear at low or moderate intensity peaks at even *m/z* values. The reaction favors the delocalized π-electron system of the ring A rather than the double bond of ring C, and the charge may be retained at either of the forming radical cation fragments. The most intensive rDA peaks are in the mass spectra of the TMS derivative of formononetin **7**, where the ring A rDA fragment ($A^{+\bullet}$) occurs at *m/z* 208 (14%), and the ring B rDA fragment ($B^{+\bullet}$) at *m/z* 132 (57%). In the mass spectrum of its deuterated TMS derivative, the fragments are at *m/z* 217 and 132, respectively.



7
m/z 340 (100)

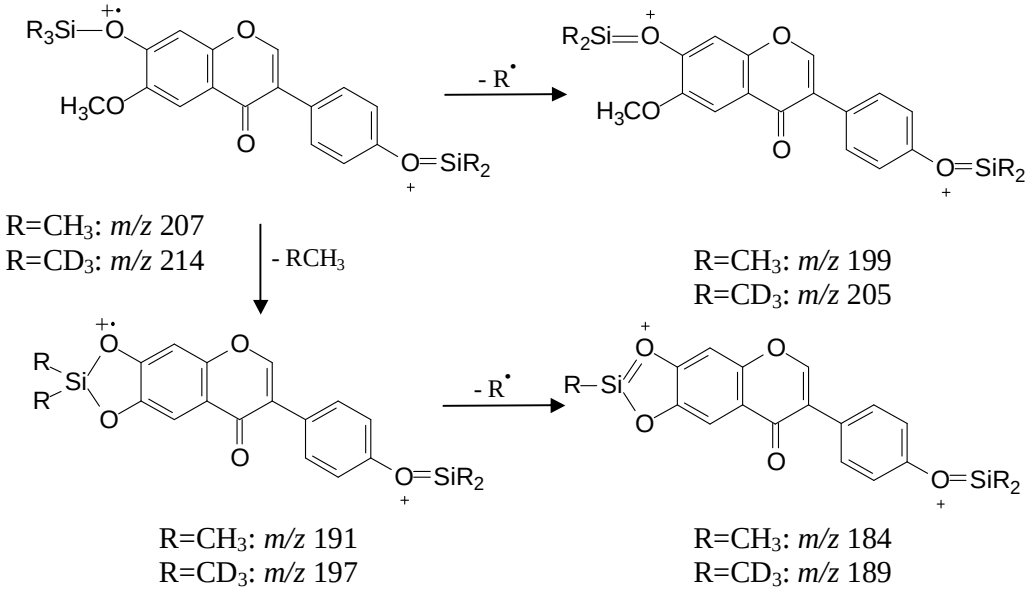
B$^{+\cdot}$
m/z 132 (57)

A$^{+\cdot}$
m/z 208 (14)

Scheme 4. *Retro Diels-Alder reaction of the TMS ether of formononetin **7**.*

An interesting feature in the mass spectrum of TMS derivatives of some isoflavones are the rather abundant ions that show no isotope peaks (footnote 1 in Table 1, Appendix 2). When using a quadrupole mass analyzer, these kinds of peaks are common with doubly charged ions, which, in general, are rare in EI-MS. The isotope peaks of doubly charged ions occur at distance of half mass units and cannot be separated from each other by the

unit resolution of the quadrupole mass analyzer. In the mass spectrum of the TMS derivative of glycitein **3** (Figure 5), three ions at *m/z* values 184, 191, and 199 show no isotopic peaks and probably originate from the doubly charged molecular ion. Their structures are shown in Scheme 5. The analysis of the mass spectra of the deuterated TMS derivative (Table 1, Appendix 2) supports the presence of doubly charged ions; the fragment at *m/z* 205 in the mass spectrum of the deuterated TMS derivative of glycitein can be formed by the loss of two d_3-methyl radicals from a doubly charged molecular ion. The formation of two other ions at *m/z* 197 and 189 involves the loss of d_3-ethane and one or two d_3-methyl radicals, respectively, from a doubly charged molecular ion.



Scheme 5. *Formation and proposed structures of ions at m/z 184, 191, and 199 in the mass spectrum of the TMS ether of glycitein **3**.*

4.1.3 Mass spectra of isoflavanones

The mass spectrum of the TMS ether of isoflavanone dihydroglycitein **21** is shown in Figure 6. Due to the absence of a double bond in the ring C, isoflavanones have a less rigid ring structure than isoflavones, and thus, more fragment ions are seen in the mass spectra of these compounds (Table 2 in Appendix 2). The molecular ion and the primary fragments formed by losses of one or two methyl radicals, TMS radicals, or $Si(CH_3)_4$ molecules are low in intensity. The predominant peaks in the mass spectra originate from ions formed by the retro Diels-Alder reaction. The rDA fragments occur at even mass-to-charge values, are easily recognized among other ions of the spectrum, and provide valuable information on the degree of substitution at each phenolic ring. For example, both dihydrodaidzein **19** and dihydroglycitein give the same ring B fragment at *m/z* 192, but the $A^{+\bullet}$ ion of dihydrodaidzein is 30 mass units lower than the $A^{+\bullet}$ ion of dihydroglycitein, i.e. at *m/z* 208 instead of at *m/z* 238, indicating one additional methoxy group in ring A (Scheme 6).

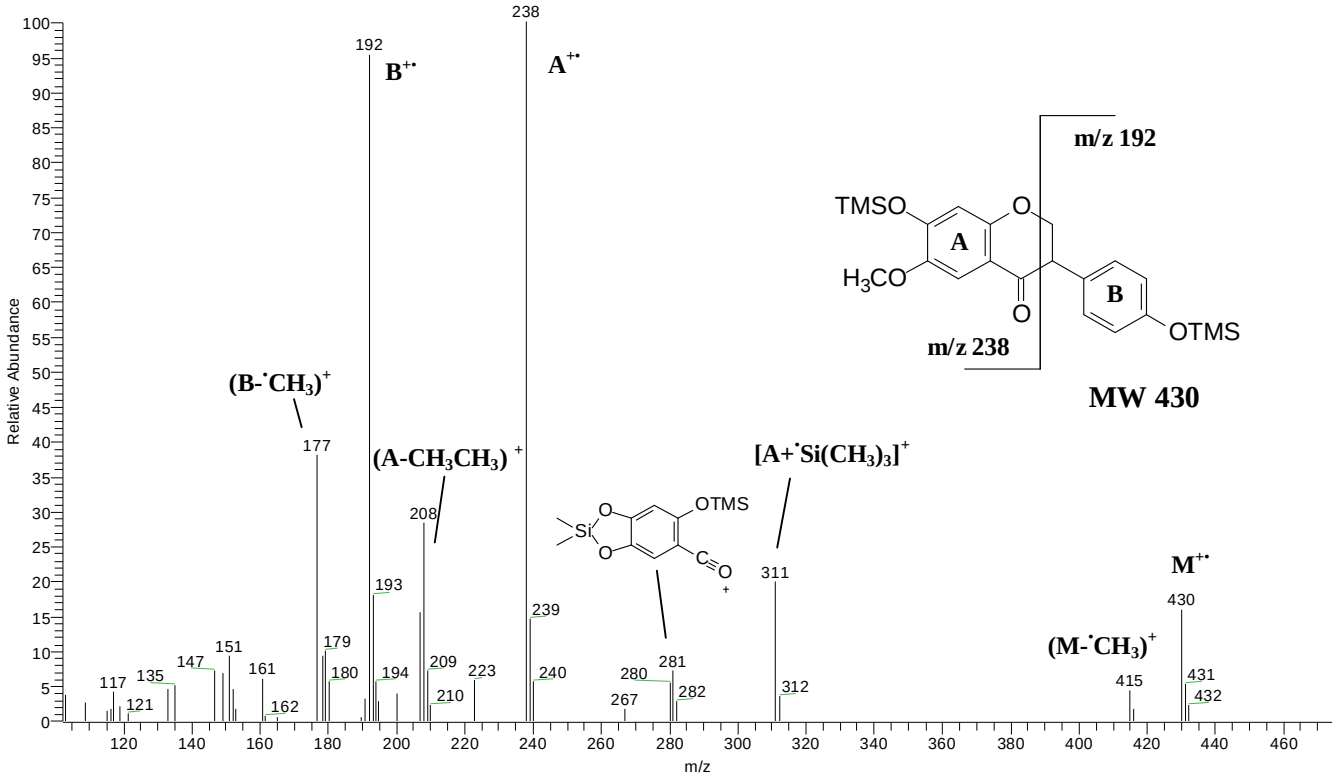
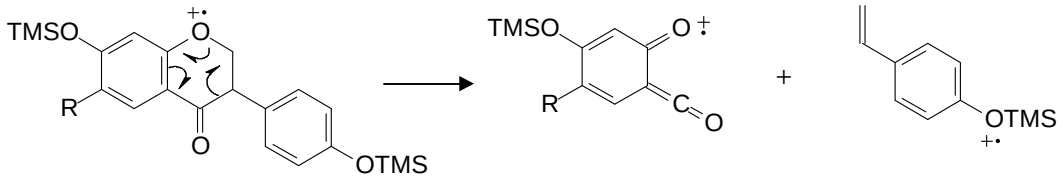


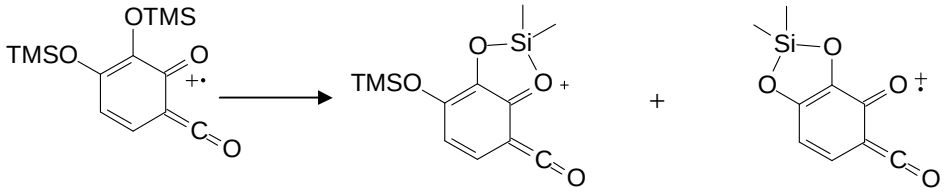
Figure 6. *Mass spectrum of trimethylsilylether of dihydroglycitein **21***



		$A^{+\bullet}$	$B^{+\bullet}$
19	R=H: *m/z* 400	R=H: *m/z* 208	*m/z* 192
21	R=OCH_3: *m/z* 430	R=OCH_3: *m/z* 238	

Scheme 6. *Retro Diels-Alder fragmentation of TMS derivatives of dihydrodaidzein **19** and dihydroglycitein **21**.*

The ratios of the rDA peak intensities depend on the structure of isoflavanones. In general, the $B^{+\bullet}$ rDA fragments are more abundant, but the abundance decreases as more substituents, hydroxyl or methoxy groups, are attached to ring A. For example, the abundances of $B^{+\bullet}$ fragments of TMS ethers of dihydrodaidzein, dihydroglycitein, and 6,7,4'-trihydroxyisoflavanone at *m/z* 192 are 100%, 95%, and 18% (Table 2, Appendix 2), respectively, showing that introduction of charge-stabilizing substituents on ring A decreases the abundance of the ring B rDA fragment. Elimination of $Si(CH_3)_4$, and one or two methyl radicals from rDA fragments is common in the mass spectra of isoflavanones. For example, in the mass spectrum of the TMS ether of 7,8,4'-trihydroxyisoflavanone **39**, ions at *m/z* 281 (base peak of the spectrum) and 208 can be formed by losses of the methyl radical and $Si(CH_3)_4$ from the ring A rDA fragment ($A^{+\bullet}$, *m/z* 296), respectively (Scheme 7).

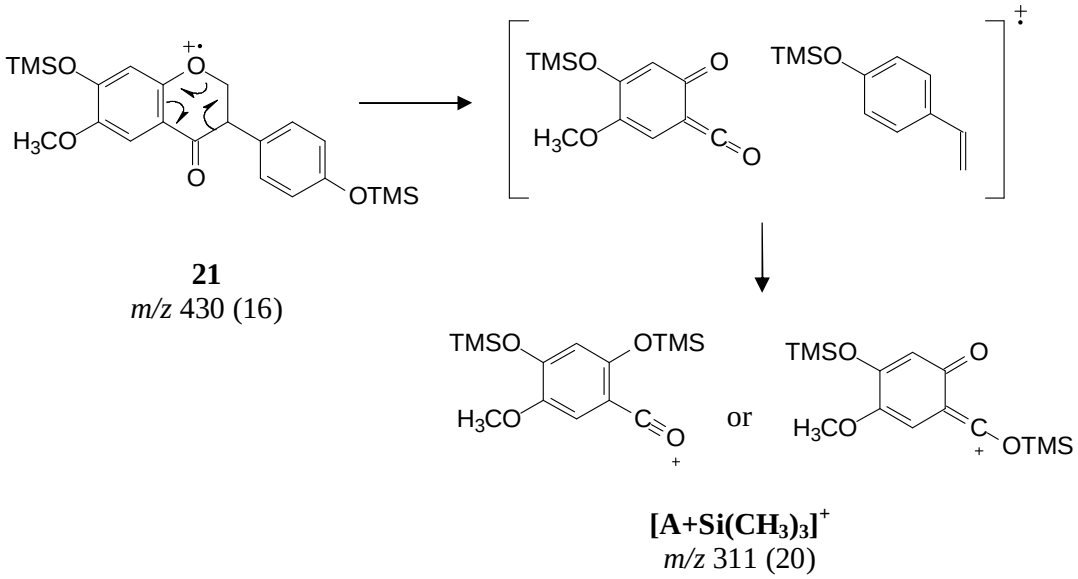


$A^{+\bullet}$	$(A\text{-}^{\bullet}CH_3)^{+}$	$[A\text{-}Si(CH_3)_4]^{+\bullet}$
m/z 296 (7)	*m/z* 281 (100)	*m/z* 208 (3)

Scheme 7. *Elimination of the methyl radical and $Si(CH_3)_4$ from the ring A fragment of the TMS ether of 7,8,4'-trihydroxyisoflavanone.*

Peaks formed by the losses of one or two methyl radicals or $Si(CH_3)_4$ from rDA fragments are common in the mass spectra of isoflavanones. For example, the loss of the methyl radical from the $B^{+\bullet}$ fragment of the TMS ether of dihydrodaidzein **19** or dihydroglycitein **21** at *m/z* 192 yields an ion at *m/z* 177 (Table 2, Appendix 2). In the mass spectrum of a deuterated derivative, the ion is six mass units higher, at *m/z* 183, indicating the presence of two deuterated methyl groups in the fragment. Peaks due to doubly charged ions are also present in the mass spectra of some isoflavanones (Table 2, Appendix 2), however, at considerably lower intensities than in the mass spectra of isoflavones.

One of the rather abundant ions in the mass spectra of TMS ethers of isoflavanones is formed by the migration of a TMS group from the ring B to the ring A rDA fragment (Scheme 8). This reaction only occurs with isoflavanones with a TMS group attached to the ring B. The reaction probably involves a long-lived ion – molecule complex.[205,206] For example, in the mass spectrum of dihydroglycitein **21** (Figure 6), the ion at *m/z* 311 refers to the ring A rDA fragment (**$A^{+\bullet}$**, *m/z* 238) plus one additional TMS group, which is supported by the presence of an ion at *m/z* 329 in the mass spectrum of the deuterated TMS derivative. Two possible structures of the ion, for which reasonable mechanisms of formation can be written, are presented in Scheme 8.



Scheme 8. *Retro Diels-Alder fragmentation of the TMS ether of dihydroglycitein **21**.*

4.1.4 Mass spectra of isoflavans

In the mass spectra of isoflavans (Table 3 in Appendix 2 and Figure 7), the molecular ion is more abundant than in the analogous isoflavanones. For example, the abundance of the molecular ion of equol **24** is 16%, whereas the abundance of that of dihydrodaidzein **19** is 3% (Tables 2 and 3, Appendix 2). The absence of the carbonyl group thus seems to stabilize the ring C of isoflavans. Depending on the structure, similar losses from molecular ions as those reported for isoflavones, i.e. losses of methyl or TMS radicals and of $Si(CH_3)_4$ or C_2H_6 (as subsequent losses of two methyl radicals), commonly occur. The abundances of the ions are, however, much lower. As in the mass spectra of isoflavanones, the most intensive peaks in the spectrum are rDA-fragments of ring A or ring B, depending on the substitution of each phenolic ring, and subsequent fragments. Losses of methyl radical or TMS groups, or C_2H_6 or $Si(CH_3)_4$ molecules from rDA fragments are also common. Moreover, an $A^{+\bullet}$ fragment with an additional TMS group is formed. A proposed mechanism of formation and the structure of the fragment in the mass spectrum of the TMS ether of 7,4'-dihydroxy-6-methoxyisoflavan **62** are shown in Scheme 9.

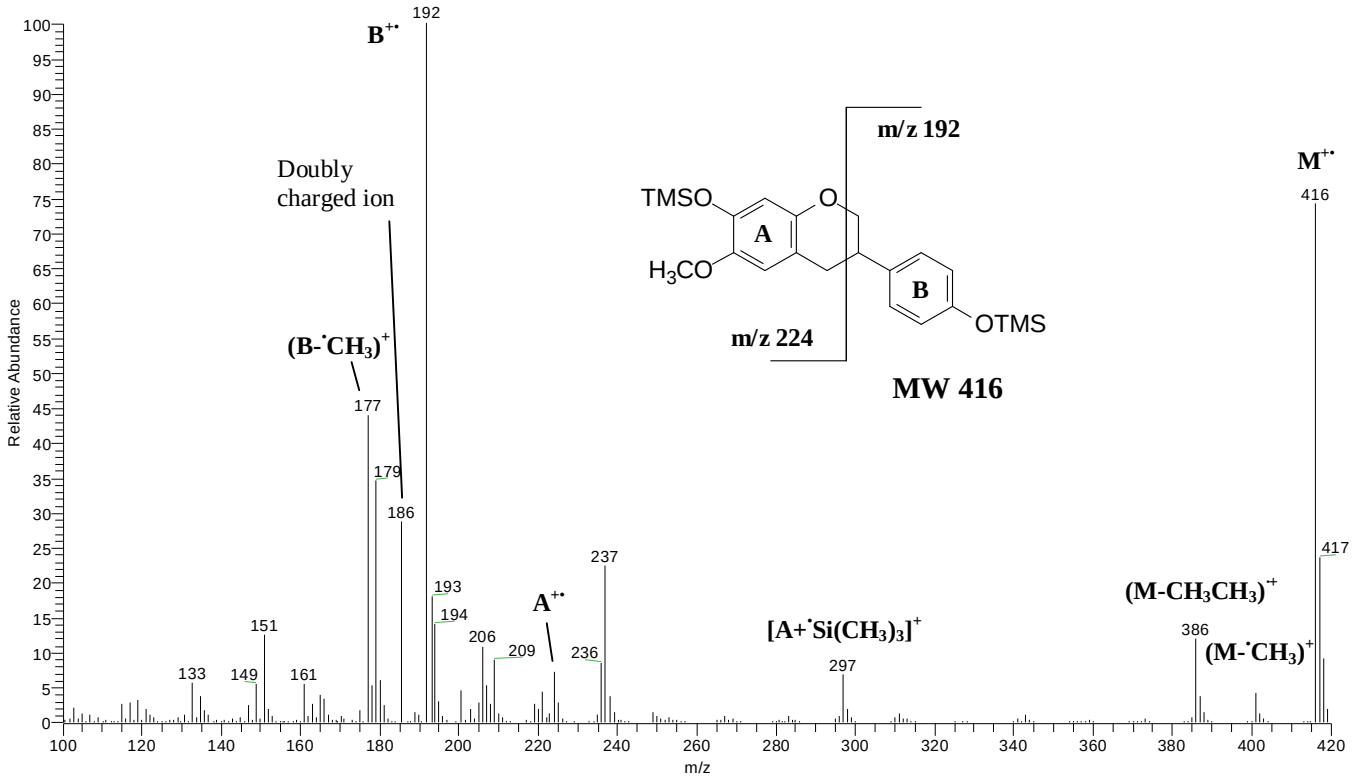
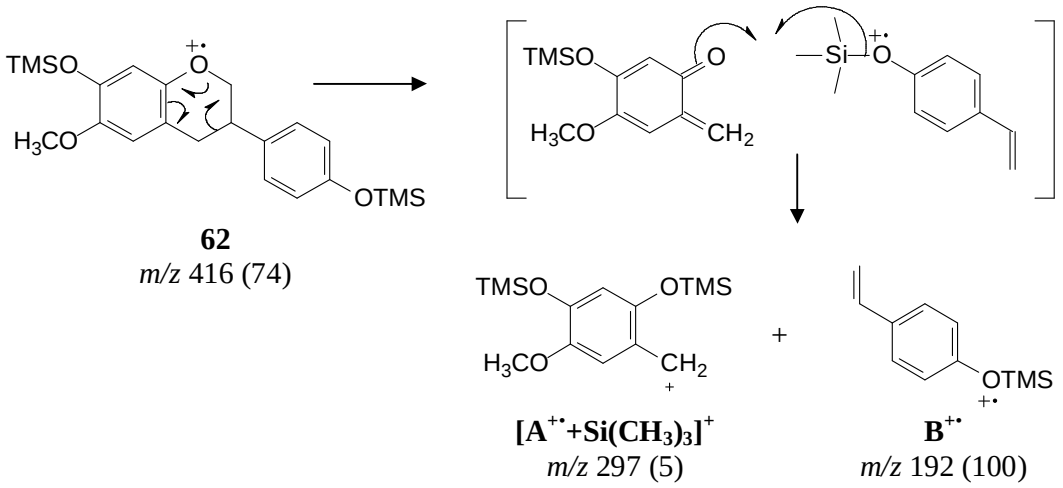


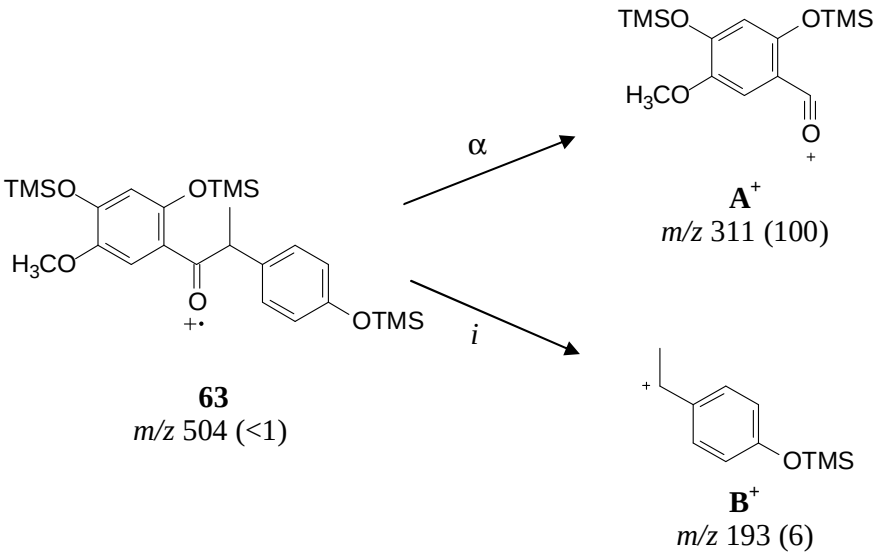
Figure 7. *Mass spectrum of trimethylsilylether of 7,4'-dihydroxy-6-methoxyisoflavan***.**



Scheme 9. *Proposed mechanism for formation of rDA fragments of 7,4'-dihydroxy-6-methoxy-isoflavan* ***62****.*

4.1.5 Mass spectra of α-methyldeoxybenzoins

The mass spectra of TMS derivatives of α-methyldeoxybenzoins (Table 4 in Appendix 2, and Figure 8) typically have only one intensive fragment ion, the base peak of the spectrum, which originates from the α-cleavage of the bond between carbon atoms 1 and 2 yielding the ring A fragment shown in Scheme 10.



Scheme 10. *Fragmentation of α-methyldeoxybenzoins.*

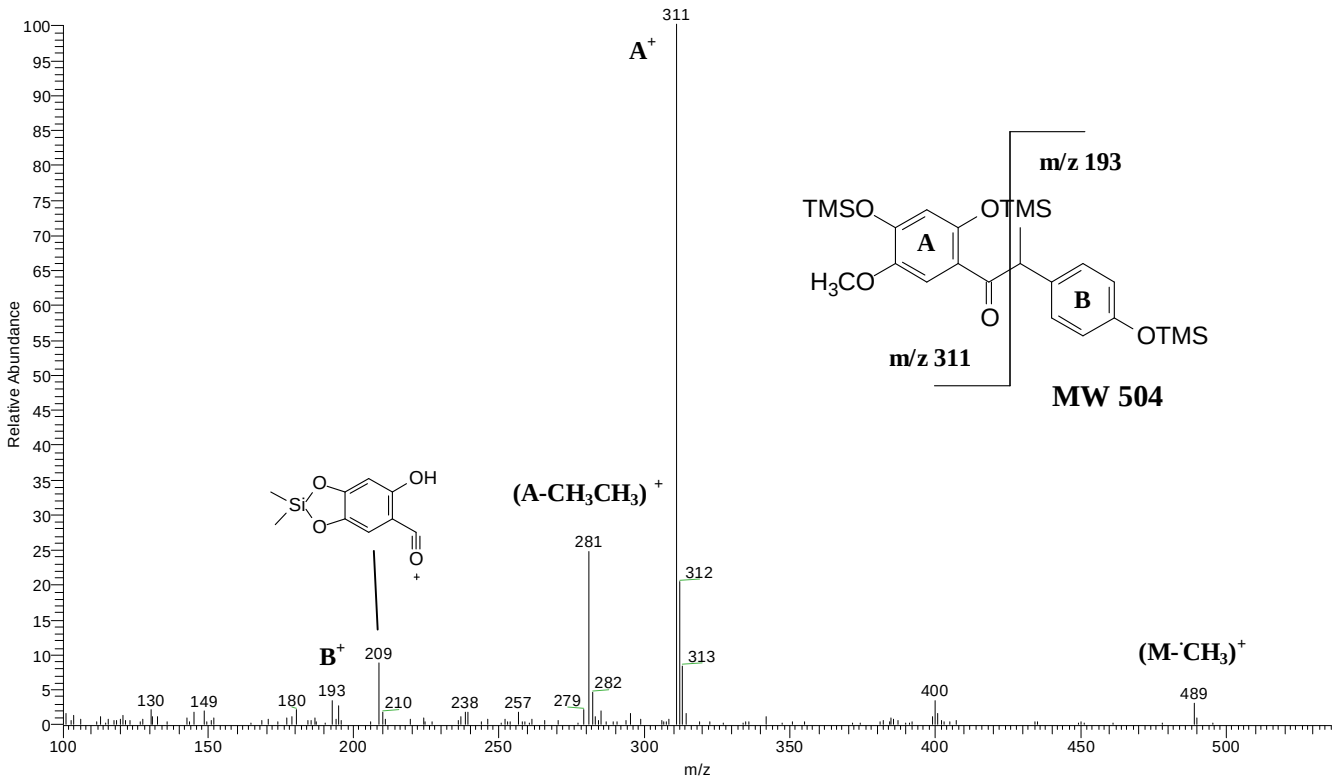


Figure 8. *Mass spectrum of trimethylsilylether of 7,4'-dihydroxy-6-methoxyisoflavan.*

The molecular ion peak, as well as other peaks of the mass spectra of TMS ethers of α-methyldeoxybenzoins, are of very low intensity. Inductive cleavage of the bond between the carbonyl and the benzylic carbon yields the ring B fragment. The degree of substitution at both phenolic rings can be elucidated by examining the *m/z* values of these fragments. For example, the base peak of the 5'-OMe-*O*-dma **63** (Scheme 10) is at *m/z* 311, which is 30 mass units higher than the base peak of *O*-dma **28** (Table 4, Appendix 2), indicating one additional methoxy group in ring A. The loss of $(CH_3)_2Si{=}CH_2$ from the ring A fragment is common for mass spectra of α-methyldeoxybenzoins.[21] For example, in the mass spectrum of TMS ether of *O*-dma **28**, the fragment at *m/z* 209 is formed from the ion *m/z* 281 possibly by a similar mechanism as shown in Scheme 3 for isoflavones. The rearrangement of a deuterium to an α- methyldeoxybenzoin nucleus is reflected in the peak at 10 mass units higher, at *m/z* 219, in the mass spectrum of the TMS ether of a deuterated derivative (Table 4, Appendix 2). An analogous peak, originating from a similar fragmentation reaction followed by the loss of C_2H_6 (as subsequent losses of two methyl radicals), is found in the mass spectrum of 5'-methoxy-*O*-dma **28** at *m/z* 209 (Figure 8).

4.2 *In vitro* bacterial metabolites of isoflavones (III)

4.2.1 Sample pretreatment method of freeze-dried fecal fermentation samples

The sample pretreatment method developed for fecal fermentation samples showed good recoveries for analytes, 69-93% of the spiked amount (Table 5). Chromatography with Lipidex-5000 efficiently removed the nonpolar compounds interfering with the analysis of the samples and with identification of the compounds,[204] and also improving the sensitivity and precision of the method. Variation of the retention times was minimal, ± 2 s (CV% < 0.3). Intra-assay variation for analytes, determined by repetitive analysis of six replicate samples, was <10%.

4.2.2 Identification of isoflavonoids in fecal fermentation samples

Isoflavonoids that were identified in fecal fermentation samples are summarized in Table 6. All were reduced metabolites of investigated isoflavones, mainly the corresponding isoflavanones and α-methyldeoxybenzoins. In general, the extent of metabolism of isoflavones by fecal bacteria was low. The main compounds in fermentation extracts were found to be substrates added for fermentation. Exceptions to this were the isoflavones with hydroxyl groups at the 5-position, i.e. genistein **2** and orobol **15**, which were completely converted to metabolites that could not be detected by the methods used in the study. Coldham et al.[66,68] have reported that the end-products of metabolism of genistein are 2-(4-hydroxyphenyl)-propionic acid **54** and 1,3,5-trihydroxybenzene **55**; thus, it is

reasonable to assume that both genistein and orobol were converted to these smaller molecular weight metabolites during the 24-h fecal fermentation. Since the aim of this thesis was to identify metabolites with an intact isoflavonoid structure, no attempts were made to identify these degraded compounds in fermentation extracts.

Table 5. *Limit of detection (LOD), retention time (t_R) variation, recovery, and intra-assay variation determined for the method developed for identification of isoflavones and their metabolites in fecal fermentation samples.*

Compound	LOD[1] (ng on column)	t_R variation (s)	Recovery (%)	Intra-assay variation (CV%)
Daidzein	2.35	0.68	81	5.2
Genistein	1.58	1.18	86	3.1
Equol	0.38	0.57	69	6.7
O-dma	0.40	1.68	71	6.7
Dihydrodaidzein	0.32	0.82	80	8.6
Dihydrogenistein	1.42	1.05	93	5.7
6,7,4'-trihydroxyisoflavone	2.15	1.91	82	4.3
7,8,4'-trihydroxyisoflavone	1.98	1.52	75	6.7
7,3',4'-trihydroxyisoflavone	2.32	1.48	78	5.8
5,7,3',4'-tetrahydroxyisoflavone	2.21	1.18	86	6.6

[1]*LOD of the method was set at the point at which reliable mass spectrum fullfilling the criteria presented in section 4.2.5 was obtained from standard solution.*

Table 6. *Isoflavonoids identified in fecal in vitro fermentation samples (III).*

Substrate	Isoflavonoids identified in fecal fermentation samples		
	Isoflavones	Isoflavanones	α-methyl-deoxybenzoins
Daidzein	Daidzein	Dihydrodaidzein	*O*-dma
Genistein	-	-	-
7,3',4'-trihydroxyisoflavone	7,3',4'-trihydroxy-isoflavone	7,3',4'-t rihydroxy-isoflavanone[1]	3''-OH-*O*-dma[1]
6,7,4'- trihydroxy isoflavone	6,7,4'-trihydroxy-isoflavone	6,7,4'-trihydroxy-isoflavanone[1]	5'-OH-*O*-dma[1]
7,8,4'- trihydroxy isoflavone	7,8,4'-trihydroxy-isoflavone	7,8,4'-trihydroxy-isoflavanone[1]	-
5,7,3',4'-tetrahydroxyisoflavone	-	-	-

[1]*Authentic reference compound not available.*

4.2.3 Utilization of GC-MS data obtained from analysis of fecal fermentation samples

The retention times and mass spectra of metabolites formed during *in vitro* fecal fermentation were compared with those of unknown isoflavonoid metabolites, for which authentic reference compounds were unavailable. These metabolites were the reduced metabolites of 7,3',4'-trihydroxyisoflavone, 6,7,4'-trihydroxyisoflavone, and 7,8,4'-trihydroxyisoflavone, i.e. 3''-OH-*O*-DMA, 7,3',4'-trihydroxyisoflavanone, 6,7,4'-trihydroxyisoflavanone, and 7,8,4'-trihydroxyisoflavanone (III).

4.3 Identification of isoflavone metabolites in human urine after soy supplementation (I, II, IV-V)

4.3.1 Isoflavones of the soy bar

The only isoflavones identified in the soy bar were daidzein **1**, genistein **2**, and glycitein **3** (Figure 9). The isoflavones were determined as aglycones; thus no data on isoflavone conjugation or conjugate types are available. From the results of quantitative analysis of isoflavones, the daily intake was calculated to be 48.4, 40.2, and 4.1 mg of daidzein, genistein, and glycitein, respectively.

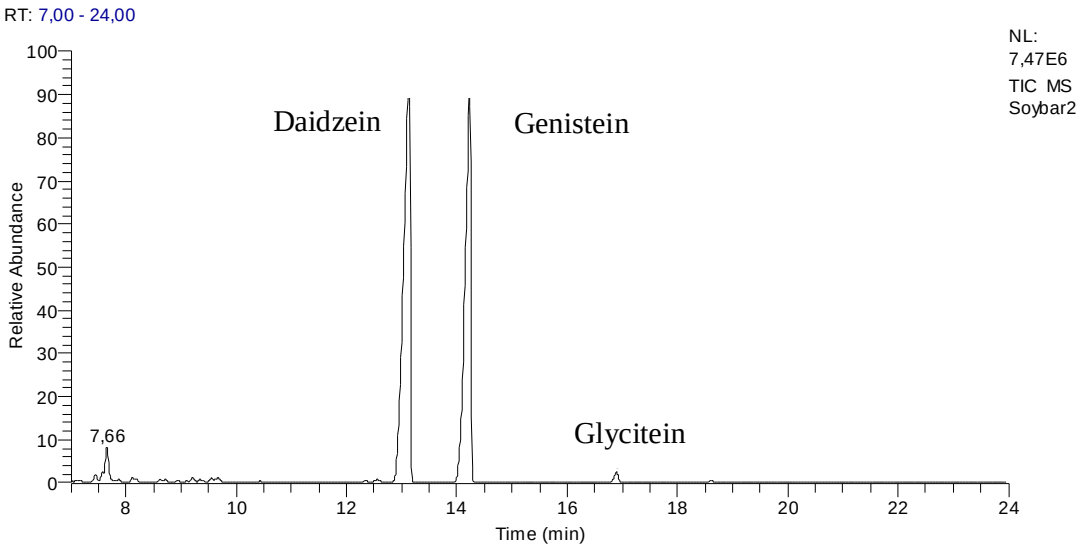


Figure 9. *Isoflavones identified in soy bar extract.*

4.3.2 Sample pretreatment method for urine samples

The qualitative method for identification of isoflavonoid metabolites in human urine samples was validated for specificity, recovery and precision. Analysis of the spiked urine samples showed that the compounds of interest (metabolites of daidzein, genistein, and glycitein) all eluted in the second fraction of Sephadex LH-20 chromatography. No traces were found either in the first fraction or in an additional 5-mL fraction. The recoveries of the analytes, listed in Table 7, were 69-90%. Variation in retention times in the analytes was ± 1…2 s with a spiked low control sample, whereas in the high control urine sample more variation in retention times, ± 2…7 s, was observed when total ion chromatograms of standards and spiked urine samples were compared (Table 7). The retention time was used as one criterion for identification of an unknown urinary compound with an authentic reference compound. The problems caused by variation in retention times of urine samples containing high levels of isoflavonoids were overcome by spiking the urine samples with reference compounds to confirm that the unknown urinary compound eluted at the same retention time as the spiked reference compound.

Table 7. *Limit of detection (LOD), retention time variation, recovery and intra-assay variation determined for the method developed for identification of soy (**A**) and red isoflavones(**B**) and their metabolites in human urine samples.*

	Compound	LOD[1] (ng on column)	Retention time variation (s)		Recovery (%)	Intra-assay variation (CV%)
			Low control	High control		
A	Daidzein	2.35	0.68	7.15	78	6
	Genistein	1.58	1.79	2.76	72	4
	Equol	0.38	0.57	1.67	69	4
	O-dma	0.40	2.15	2.30	71	8
	Dihydrodaidzein	0.32	0.82	3.40	88	6
	Dihydrogenistein	1.42	1.05	2.35	90	4
B	Formononetin	4.84	2.32	2.54	83	7
	Biochanin A	4.68	2.86	2.98	71	6
	4'-*O*-methylequol	2.03	1.98	2.35	98	17
	Angolensin	1.98	2.34	2.56	85	21
	Dihydroformononetin	3.22	2.40	2.89	72	14
	Dihydrobiochanin A	2.39	2.42	3.20	99	17

[1]*LOD of the method was set at the point at which reliable mass spectrum fullfilling the criteria presented in section 4.2.5 was obtained from standard solution.*

The detection limits, i.e. the lowest amounts of analytes to yield a reliable mass spectrum, ranged from 0.32 to 2.35 ng on column (Table 7). If a good mass spectrum was not obtained from the compound of interest, a bigger injection volume (up to 3 μL) or a more concentrated sample was used. In some cases, unknown urinary compounds co-eluted with the analytes. These compounds commonly were present in both pre- and post-supplementation samples, and thus, did not originate from the soy or red clover supplement. In some samples, the levels of these compounds were higher than the levels of metabolites, thus hindering the identification of the metabolites. In these cases, the mass spectra of the analytes were refined by subtracting the background, i.e. the ions of the co-eluting compounds. The refined mass spectra were then compared with those of reference compounds, and the criteria of identification for the metabolite described above were applied.

A problem with formation of silylation artifacts was encountered with isoflavanones. In the method from which the sample pretreatment was adopted, BSTFA was used as a derivatization reagent,[24,25] resulting in the formation of TMS ethers of enol tautomer and dehydro-*O*-dma in addition to the desired TMS ether of the keto tautomer (I). Formation of silylation artifacts interferes with identifcation of isoflavonoid metabolites and may lead to misinterpretations of the results.[197,207] Using QSM reduced the levels of unwanted byproducts of silylation; however, the formation of these could not be completely avoided (I). The sample pretreatment method was also checked with blank and spiked (with daidzein, genistein, and glycitein) urine samples to ensure that the metabolites identified did not originate from reagents or artifacts formed during sample pretreatment.

4.3.3 Isoflavonoid metabolites identified in urine samples

The principal isoflavones of soy, daidzein, genistein, and glycitein were detected in all urine samples that were collected from the seven participants after the soy supplementation. In addition to these ingested isoflavones, several other compounds with an isoflavonoid structure were identified. All isoflavonoids identified in urine samples are summarized in Table 8, with rough estimates of their abundances in samples.

Isoflavones. In addition to ingested isoflavones, several isoflavones with three or more hydroxyl substituents were identified in urine samples. With authentic reference compounds, the structures of four of the most abundant isoflavones were characterized as 7,3',4'-trihydroxyisoflavone, 6,7,4'-trihydroxyisoflavone, 7,8,4'-trihydroxyisoflavone, and 5,7,3',4'-tetrahydroxyisoflavone (II). Kulling et al.[52] have previously reported the identification of oxidized metabolites of daidzein and genistein in human urine after soy supplementation, including the four isoflavones identified here. On the basis of their findings, two metabolites eluting at the same retention time as 7,3',4'-trihydroxyisoflavone and 7,8,4'-trihydroxyisoflavone (Figure 10) could be identified as hydroxylated metabolites of genistein, 5,7,3',4'-tetrahydroxyisoflavone and 5,6,7,4'-tetrahydroxyisoflavone, respectively. No authentic reference compounds were available for these tetrahydroxyisoflavones.

Isoflavanones. Analogous isoflavanone metabolites for each isoflavone identified in soy supplementation samples were detected. Dihydrodaidzein and dihydrogenistein were characterized with authentic reference compounds (I). The tentative identification of 7,3',4'-trihydroxyisoflavanone, 6,7,4'-trihydroxyisoflavanone, and 7,8,4'-trihydroxy-isoflavanone was confirmed with chromatographic and mass spectrometric data obtained from analysis of *in vitro* fermentation samples (III). The tentative identification of dihydroglycitein **13** has now been confirmed using an authentic reference compound (IV, unpublished data).

The mass spectrum of one of the metabolites appearing in urine after ingestion of soy could be assigned as belonging to an analogous isoflavanone metabolite of orobol, 5,7,3',4'-tetrahydroxyisoflavanone (unpublished data). The mass spectrum is reported in Table 2, in Appendix 2. Flavonoid analogs were excluded by comparison of the retention time and the mass spectrum with those of analogous flavanone and flavan-3-ol, i.e. 5,7,3',4'-tetrahydroxyflavanone and 5,7,4'-trihydroxyflavan-3-ol. Conclusive identification of this metabolite requires an authentic reference standard. Identification of dihydrodaidzein and dihydrogenistein in human urine has been reported earlier,[22,24,25] but thus far there are no reports of the presence of other isoflavanone metabolites in human urine after soy supplementation.

Isoflavans. Equol, the further reduced metabolite of daidzein, was found in the urine samples of three volunteers (II), supporting earlier findings that only one third of humans are capable of producing equol.[71] In addition, several other metabolites with an isoflavan structure were identified in the urine samples of the three equol producers. These metabolites were not detected in the urine samples of other participants suggesting, that equol producing bacteria are involved in the formation of these metabolites. The structures of 3'-hydroxyequol, 3'-methoxyequol, and 6-methoxyequol were characterized using authentic reference compounds (II). 6-Hydroxyequol and 8-hydroxyequol were later tentatively identified by the interpretation of the mass spectra (Table 3, Appendix 2) of the compounds and their deuterated analogs (unpublished data). The levels of the newly identified isoflavan metabolites were low compared with the levels of equol, and thus, must be considered as minor metabolites.

In addition to equol, identification of 4'-*O*-methylequol **25** and 7,3'-dihydroxyisoflavan in human urine has been reported earlier.[25,26,145] No traces of 4'- *O*-methylequol were found in urine samples of seven participants after soy supplementation. In contrast, 7,3'-dihydroxyisoflavan was identified as a minor metabolite in the urine samples of equol producers (unpublished data). The metabolite eluted at the same retention time as *O*-dma and could not be detected until additional chromatography with a QAE-acetate column was carried out for the second fraction collected in Sephadex LH-20 chromatography.

Table 8. *Summary of isoflavones and their metabolites identified in human urine after soy (I-III) or red clover (IV) supplementation. The '+' signs represent rough estimations of the relative amounts of isoflavonoids in urine samples based on the intensity of the base peaks of the mass spectrum of each compound; (+) trace amounts, n.d. not detected.*

Compound	Soy	Red clover
Isoflavones		
Daidzein	+++++ +++++	+++++ +++++
Genistein	+++++ +++++	+++++ +++++
Glycitein	++++	(+)
Formononetin	n.d.	++
Biochanin A	n.d.	++
7,3',4'-tri-OH-isoflavone	++	++
6,7,4'-tri-OH-isoflavone	++	++
7,8,4'-tri-OH-isoflavone	++	++
5,7,3',4'-tetra-OH-isoflavone	+	+
Calycosin	n.d.	+
Pseudobabtigenin	n.d.	+
Prunetin	n.d.	+
3'-OMe-daidzein	+	+
Isoflavanones		
Dihydrodaidzein	++...+++[2]	++...+++[2]
Dihydrogenistein	+...+++[2]	+...+++[2]
Dihydroglycitein	+	+
Dihydroformononetin	+	+
Dihydrobiochanin A	+	+
7,3',4'-tri-OH-isoflavanone	+	+
6,7,4'-tri-OH- isoflavanone	+	+
7,8,4'-tri-OH- isoflavanone	+	+
5,7,3',4'-tetra-OH-isoflavanone	+	+
Isoflavans[1]		
Equol	+++…+++++ +++++[2]	+++…+++++ +++++[2]
3',7-dihydroxyisoflavan	+	+
6-OH-equol	+	(+)
8-OH-equol	+	(+)
3'-OMe-equol	+	+
6-OMe-equol	+	(+)
Cis-4-OH-equol	(+)	(+)
α-Methyldeoxybenzoin		
O-dma	++…+++++ +++[2]	+…+++++ +++[2]
6'-OH-*O*-dma	++	+
3''-OH-*O*-dma	+	++...+++[2]
5'-OH-*O*-dma	+…++[2]	+
5'-OMe-*O*-dma	(+)	(+)
Angolensin	n.d.	+…++[2]
6'-OH-angolensin	n.d.	++

[1] *Metabolites with an isoflavan structure were present in urine sample of equol producers only.*
[2] *Differences due to individual variation; range between the lowest and the highest amounts presented.*

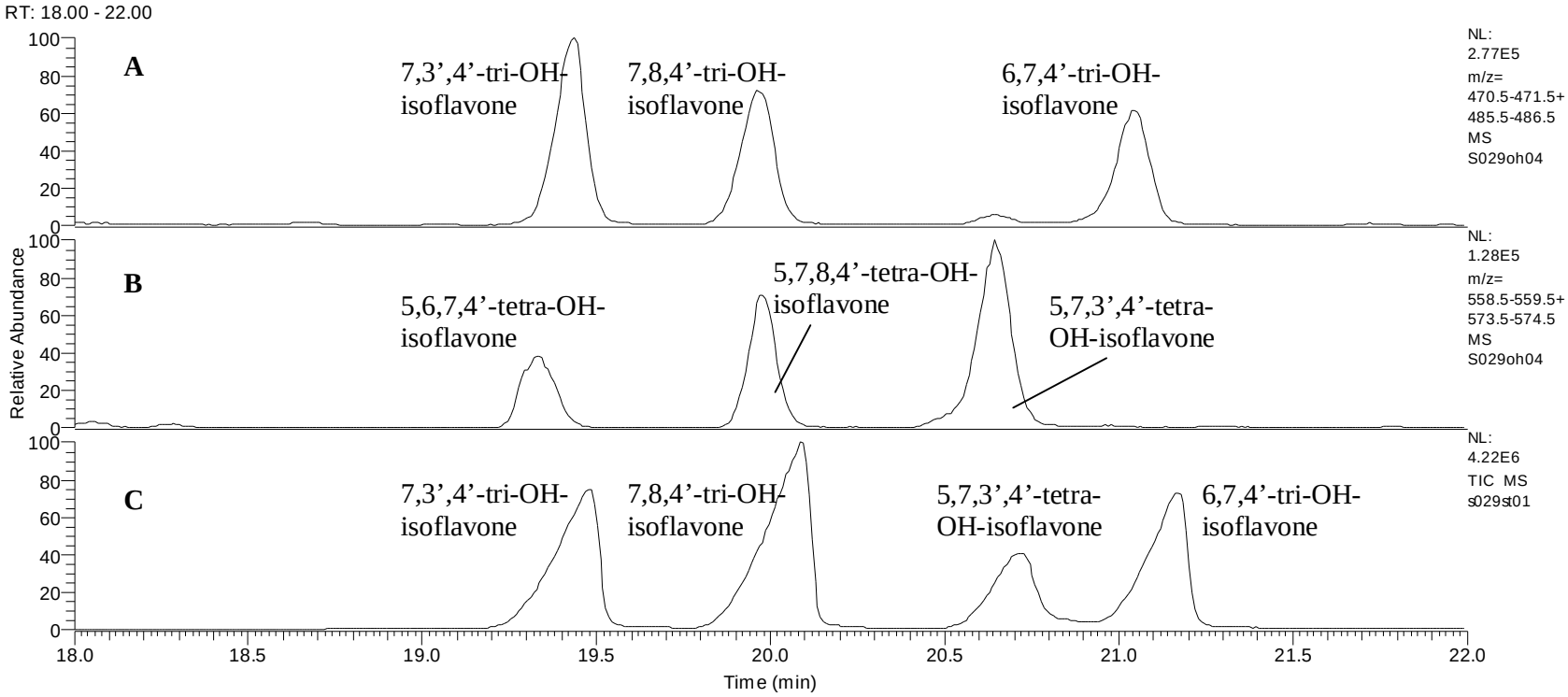


Figure 10. *Identification of hydroxylated metabolites of daidzein and genistein in urine samples collected after soy supplementation***.** *Selected ion chromatograms of a soy urine sample monitoring ions of hydroxylated metabolites of daidzein at m/z 471 and 486 (**A**) and genistein at m/z 559 and 574 (**B**), and total ion chromatogram of standard mixture (**C**).*

α-Methyldeoxybenzoins. Previously reported metabolites, *O*-dma and 6'-hydroxy-*O*-dma,[21,24,25] were identified in all urine samples collected after soy supplementation. Moreover, additional metabolites with the α-methyldeoxybenzoin structure were identified for the first time. The mass spectra of these metabolites are listed in Table 4 (Appendix 2). The previously reported tentative identification of 6'- hydroxy -*O*-dma **29**[24,25] was confirmed using an authentic reference compound (I), and 3''- hydroxy -*O*-dma (2',4',3''-trihydroxy-α-methyldeoxybenzoin) was identified by comparing its retention time and mass spectrum with those of the metabolite formed *in vitro* by the fecal fermentation of 7,3',4'-trihydroxyisoflavone (III). Identification of 5'- hydroxy -*O*-dma (2',4',5'- trihydoxy-α-methyldeoxybenzoin) and 5'-methoxy-*O*-dma **62** was carried out by interpretation of their nondeuterated and deuterated mass spectra (II).

4.4 Identification of isoflavonoid metabolites in human urine after red clover supplementation (IV)

4.4.1 Isoflavones in red clover dietary supplement

Figure 11 presents the mass spectrometric total ion chromatogram from the extract of a red clover dietary supplement showing the isoflavones identified using authentic reference compounds. The main isoflavones in the tablet were formononetin **7** and biochanin A **8**. The tablet also contained small amounts of daidzein **1**, genistein **2**, calycosin **11**, prunetin **12**, and pseudobaptigenin **14**. From the results of quantitative isoflavone analysis of dietary supplement, the ingested amounts of isoflavones were calculated to be 51.7 mg of formononetin, 84.0 mg of biochanin A, 3.2 mg of daidzein, and 5.2 mg of genistein.

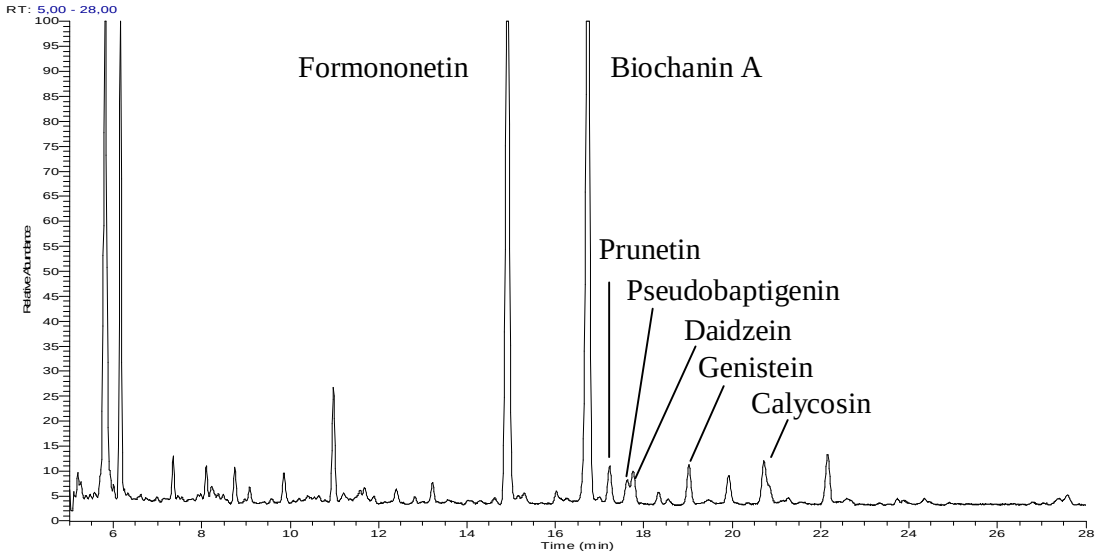


Figure 11. *Mass spectrometric total ion chromatogram of the extract of a red clover dietary supplement.*

4.4.2 Sample pretreatment method for urine samples

The neutral steroids and other urinary compounds interfered with the identification of the reduced metabolites of red clover isoflavones formononetin and biochanin A which were eluted in the first fraction in Sephadex LH-20 chromatography (IV). Additional purification of the fraction was performed by chromatography with a QAE-acetate column. In this technique, the nonpolar compounds were eluted in the first fraction, and the more polar compounds in the second fraction. The additional chromatography improved the sensitivity of the method by decreasing the background caused by other urinary compounds. During sample pretreatment, the ingested isoflavones and their urinary metabolites were distributed in three different chromatographic fractions (Figure 4), which were separately analyzed. The recovery of 4'-*O*-methylequol **25**, the analyte eluting in the first fraction of QAE-acetate column (Fr. 1a, Figure 4), was 98% (Table 7). The recoveries of analytes eluting in the second fraction of QAE-acetate column (Fr. 1b), i.e. angolensin **30**, dihydroformononetin **22**, dihydrobiochanin A **23**, formononetin **7**, and biochanin A **8**, were 71-99%; the lowest recoveries were determined for angolensin, formononetin, and biochanin A (Table 7). The recoveries of analytes eluting in the second fraction of Sephadex LH-20 (Fr. 2, Figure 4) were 69-90%, as reported above. The retention time variation was ± 2…3 s and the intra-assay variation 7-21%.

4.4.3 Isoflavonoid metabolites identified in urine samples

The isoflavonoids identified in urine samples of seven participants after red clover supplementation are summarized in Table 8 and discussed in detail below.

Isoflavones. The isoflavones of red clover, formononetin **7**, biochanin A **8**, daidzein **1**, genistein **2**, prunetin **12**, calycosin **11**, and pseudobabtigenin **14** were identified by comparison with authentic reference compounds in all urine samples collected after red clover supplementation (IV). Daidzein and genistein were the major compounds in urine samples of all participants, supporting the earlier reports of extensive demethylation of formononetin and biochanin A to daidzein and genistein, respectively.[53,120,208] The levels of ingested formononetin, biochanin A, and newly identified prunetin, calycosin, and pseudobabtigenin were low in urine. An interesting observation was that the levels of biochanin A and prunetin in human urine were almost equal, even though the red clover tablet contained higher biochanin A levels than prunetin levels (IV). Thus, demethylation of the methoxy group seems to occur more easily at ring B than at ring A. Similar reports have been made for flavonoids, where the demethylation was observed to occur more easily at C-4' than at C-6.[209]

Metabolites with methoxy substituents, including glycitein **3**, calycosin and 3'-methoxydaidzein, and four hydroxylated metabolites, 7,3',4'-trihydroxyisoflavone, 6,7,4'-trihydroxyisoflavone, 7,8,4'-trihydroxyisoflavone, and 5,7,3',4'-tetrahydroxyisoflavone, were identified as minor metabolites by comparison with authentic reference compounds (IV) (Figures 12 and 13).

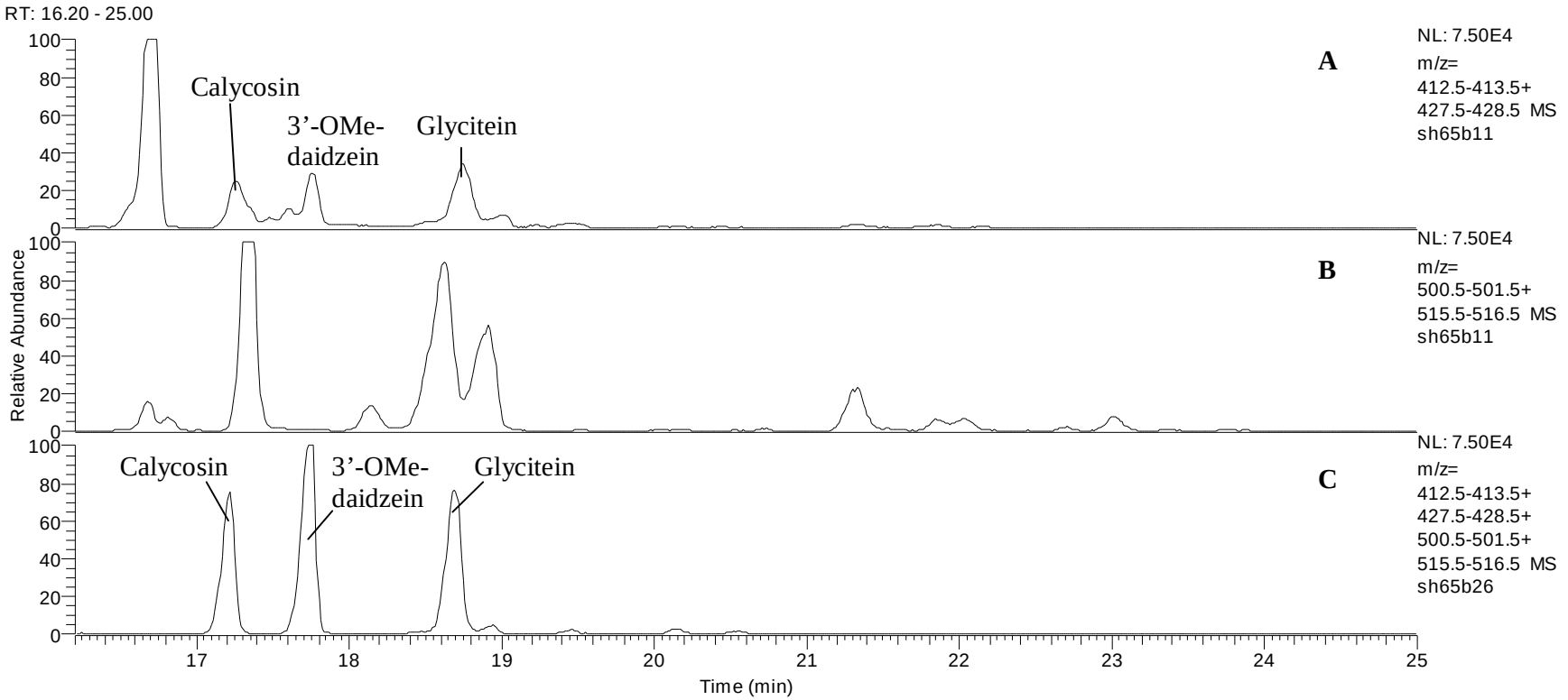


Figure 12. *Selected ion chromatograms of a urine sample after red clover intake monitoring ions of hydroxylated metabolites of formononetin at m/z 413 and 428 (**A**) and biochanin A at m/z 501 and 516 (**B**), and a total ion chromatogram of a standard mixture (**C**).*

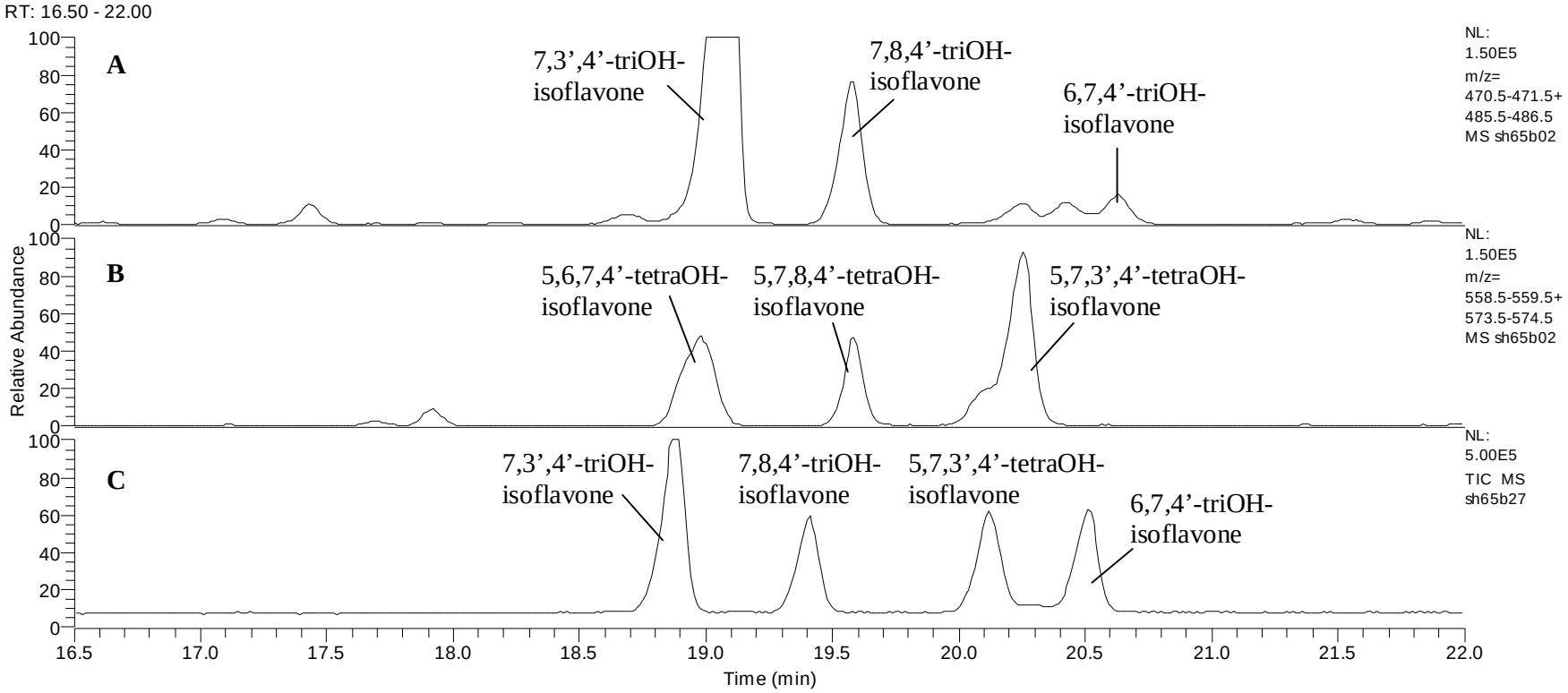


Figure 13. *Selected ion chromatograms of red clover urine sample monitoring ions of hydroxylated metabolites of daidzein at m/z 471 and 486 (**A**) and genistein at m/z 559 and 574 (**B**), and total ion chromatogram of standard mixture (**C**) at retention time window from 16.5 to 22.0 min.*

In addition, a number of other TMS ethers of isoflavones with molecular weights of 428, 516, 486, and 574 mass units were detected (Figures 12 and 13) (unpublished data). The first two are likely isoflavones with one methoxy and two or three hydroxy substituents, while the latter two are indicative of isoflavones with two or three hydroxy substituents. Full identification of these other metabolites requires access to suitable reference compounds.

Isoflavanones. The previously reported dihydrodaidzein and dihydrogenistein were the major isoflavanones identified in urine samples. These two and the new isoflavanones dihydroformononetin **22** and dihydrobiochanin A **23** were identified using authentic reference compounds in all urine samples after red clover supplementation (IV). The tentative identification of 7,3',4'-trihydroxyisoflavanone, 6,7,4'-trihydroxyisoflavanone, and 7,8,4'- trihydroxyisoflavanone was confirmed by chromatographic and mass spectrometric data obtained from the analysis of *in vitro* fermentation samples (III). The tentatively identified dihydro-orobol was also detected in all urine samples collected after the red clover supplementation (unpublished data).

Isoflavans. Three of seven participants in the study were so-called equol producers. The analogous isoflavan metabolite of formononetin, 4'-*O*-methylequol, was identified using an authentic reference compound in the urine of these persons only. Identification of the metabolite has been reported earlier.[22,25] Other isoflavan metabolites in urine samples were 7,3'-dihydroxyisoflavan, (unpublished data) 3'-hydroxyequol, 3'-methoxyequol, and 6-methoxyequol, identified using authentic reference compounds (IV), and the tentatively identified 6-hydroxyequol and 8-hydroxyequol (unpublished data).

α-Methyldeoxybenzoins. Metabolites with the α-methyldeoxybenzoin structure were identified using authentic reference compounds as *O*-dma and 6'-hydroxy-*O*-dma and 6'-hydroxyangolensin, the latter being reported for the first time in the literature (IV). The structure of 3''-hydroxy-*O*-dma was assigned by using the data obtained from an *in vitro* fermentation product of 3'-hydroxydaidzein.

4.5 Comparison of isoflavonoid profiles of urine samples collected after ingestion of soy or red clover isoflavones (II-IV)

Figures 14-16 present the total ion current chromatograms of three fractions collected from urine samples of an equol producer after soy and red clover supplementation. In general, the isoflavonoid profiles of urine samples after soy or red clover ingestion were found to be quite similar with few exceptions. The most abundant isoflavonoids excreted were daidzein **1** and genistein **2**, the principal isoflavones of soy and the main metabolites of formononetin **7** and biochanin A **8**, the isoflavones of red clover. The levels of formononetin and biochanin A were very low in urine samples collected after red clover supplementation and, naturally, were not detectable after soy supplementation.

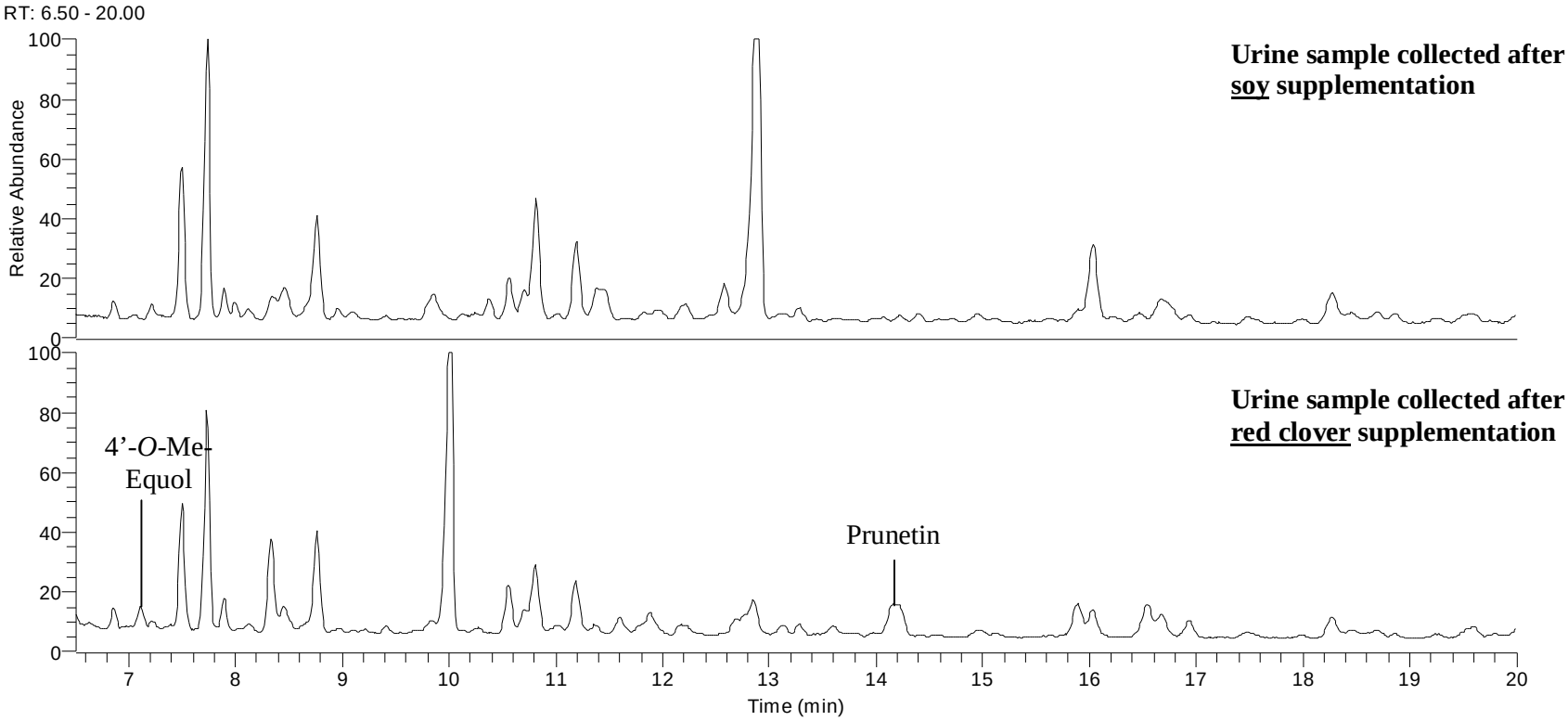


Figure 14. *Total ion chromatograms of urine sample extracts of an equol producer, after soy and red clover supplementation. First fraction after QAE-acetate chromatography.*

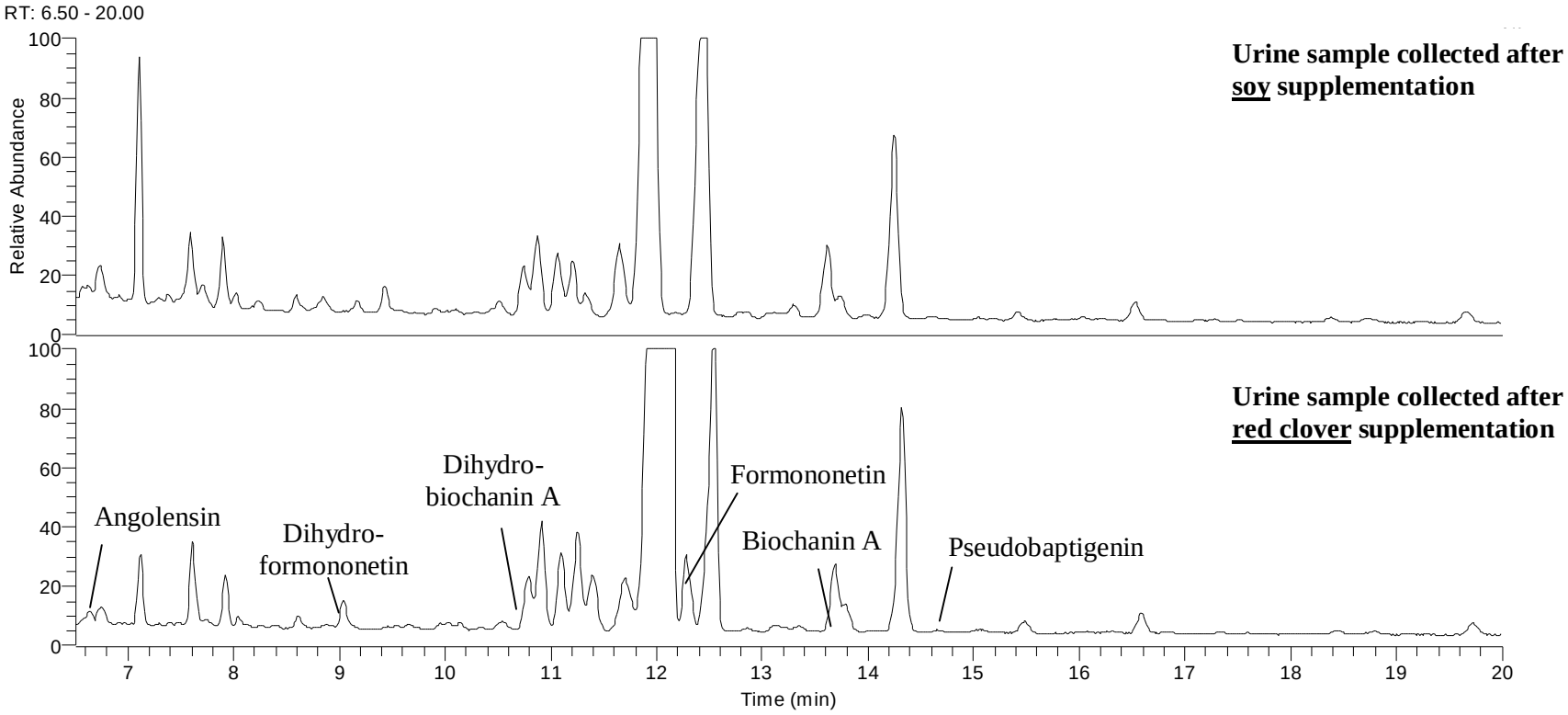


Figure 15. *Total ion chromatograms of urine sample extracts of an equol producer, after soy and red clover supplementation. Second fraction after QAE-acetate chromatography.*

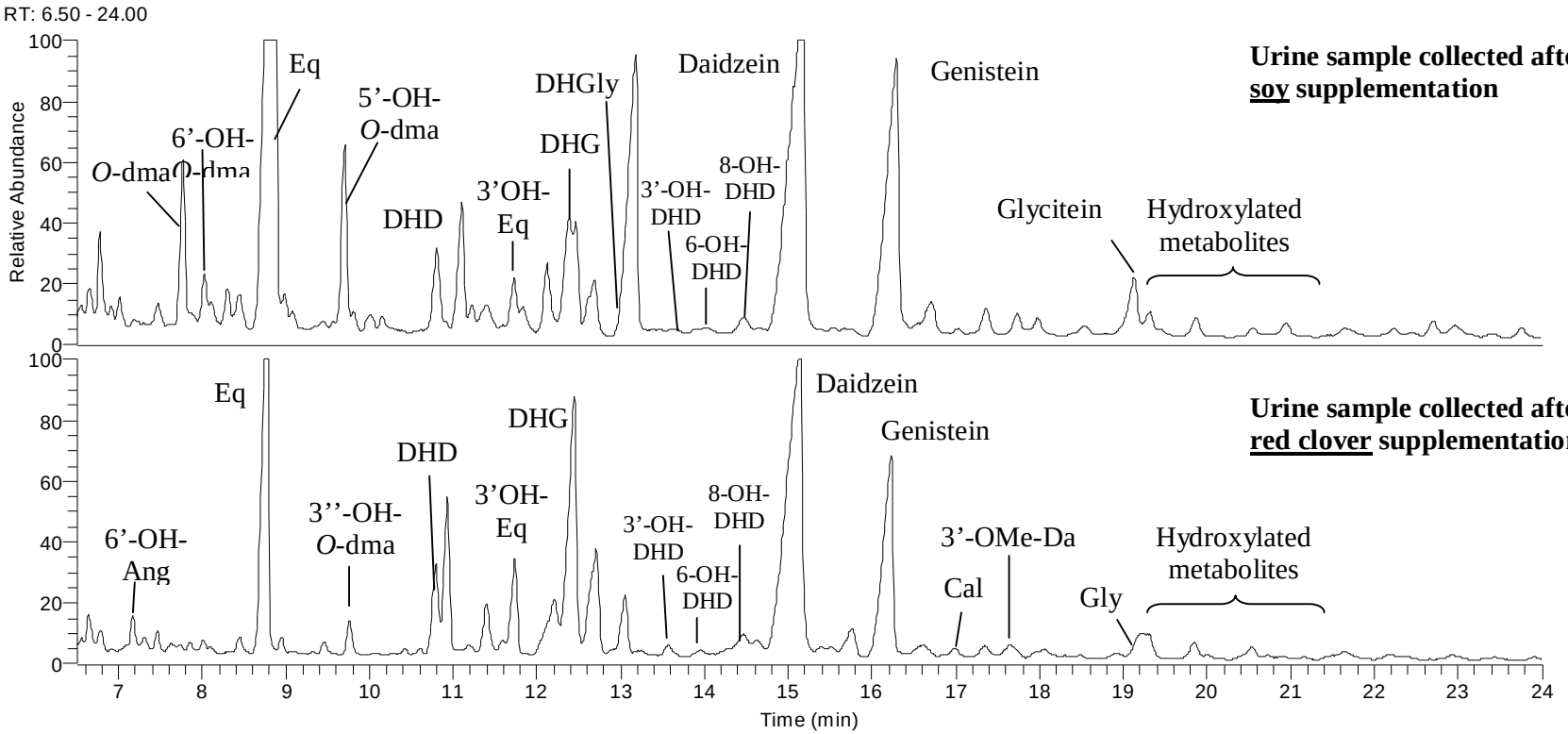


Figure 16. *Total ion ch romatograms of urine sample extracts of an equol producer, after soy and red clover supplementation. Second fraction after Sephadex LH-20 chromatography. Abbreviations: Eq, equol; DHD, dihydrodaidzein; DHG, dihydrogenistein; DHGly, dihydroglycitein; Ang, Angolensin; Cal, calycosin; Gly, glycitein.*

The most notable difference in urinary isoflavonoid profiles is the presence of moderate levels of glycitein **3** and its reduced metabolites after soy supplementation, whereas after red clover supplementation these compounds are detectable but in very low quantities. More pronounced excretion of glycitein and its metabolites (i.e. 6,7,4'-trihydroxyisoflavone, dihydroglycitein **21**, 6,7,4'-trihydroxyisoflavanone, 5'-methoxy-*O*-dma, 5'-hydroxy-*O*-dma , 6-methoxyequol, 6-hydroxyequol) has been noted in urine samples after ingestion of a dietary supplement containing high levels of glycitein (unpublished data).

Another difference between urinary profiles after soy and red clover supplementation is the presence of higher levels of metabolites with a hydroxyl substituent at the 3'-position after red clover supplementation. Furthermore, the urinary profile of isoflavonoids was more diverse after red clover supplementation, the number of different isoflavonoids excreted being larger than after soy supplementation. The difference can be explained by the presence of moderate amounts of calycosin **11** and other isoflavones, including prunetin **12** and pseudobaptigenin **14** (not quantitatively determined), in the red clover dietary supplement.

An interesting finding was that trace amounts of glycitein, which was not detected in ingested red clover tablets, appeared in urine samples after red clover supplementation. Most probably, glycitein is formed by the methylation of 6-hydroxydaidzein, the oxidized metabolite of daidzein, by COMT, as Kulling et al.[52] have demonstrated *in vitro*. The identification of 7,4'-dihydroxy-3'-methoxyisoflavan (3'-methoxyequol) and 3'-methoxyisoflavone in human urine after soy supplementation supports the view that vicinal hydroxy groups are methylated by COMT.

Furthermore, in addition to glycitein, calycosin, biochanin A, and prunetin, other isomeric compounds with the same molecular ion at *m/z* 428 were detected in the urine samples after red clover supplementation (Figure 12). There are two alternative origins for these metabolites. Either they are formed by the methylation of hydroxylated metabolites of daidzein, in the same way as glycitein was suggested to be formed by the methylation of 6-hydroxydaidzein, or the compounds are hydroxylated metabolites of formononetin. Recently, the oxidative *in vitro* metabolism of both formononetin and biochanin A by human liver microsomes has been investigated.[53,208] The results suggest that the liver microsomes mainly catalyze the demethylation of the 4´-methoxy group, the formation of hydroxylated formononetin and biochanin A metabolites being minor metabolic reactions. Some formation of hydroxylated metabolites does occur, and three hydroxylated metabolites of both formononetin and biochanin A, with additional hydroxy groups at the 3´-, 6-, and 8-positions, have been identified in liver microsomal extracts. The surprisingly high levels of calycosin, i.e. 3´-hydroxyformononetin and its reduced metabolites, could be explained by the formation of hydroxylated metabolites of formononetin. It should be noted, however, that because no tracer methods (radio, or more usually in humans, stable isotopes) were used, no conclusions about an association between the parent compounds and the metabolites can be drawn.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

A clear understanding of the metabolic fate of dietary compounds shown to possess biological activities is important since metabolism affects ingested compounds in many ways. Firstly, metabolic reactions can convert compounds into more bioactive forms. For instance, the intestinal microflora convert daidzein to the more active metabolite equol. Metabolic reactions may also decrease the biological activity of a compound, an example being deactivation by glucuronidation. Secondly, metabolism greatly affects the levels of circulating bioactive compounds; absorption of a compound can be enhanced or reduced by metabolic reactions, and extensive metabolism or conjugation decreases the levels of circulating biologically active free aglycones.

The aim of this study was to investigate the metabolism of isoflavones in humans – to identify all the urinary metabolites of isoflavonoids with an intact isoflavonoid structure. The metabolism of isoflavones was evaluated *in vivo* by two supplementation studies with soy and red clover isoflavones, and *in vitro* by fecal fermentation studies of pure isoflavone reference compounds. The qualitative methods, developed for analysis of isoflavonoid metabolites in human urine and fecal fermentation samples, were tested with reference compounds and showed good recoveries, specificity, and precision for analysis of ingested isoflavones and their metabolites. Identification and structural characterization of the metabolites were carried out using authentic reference compounds or by interpretation of the mass spectrum using deuterated TMS derivatives to aid and confirm interpretation. In addition, data obtained from *in vitro* experiments could be used to confirm identification of some of the urinary metabolites for which no authentic reference compounds were available.

Altogether 36 isoflavonoids (13 isoflavones, 9 isoflavanones, 7 isoflavans and 7 α-methyl-deoxybenzoins) were identified in human urine after soy or red clover supplementation, 18 of which were new metabolites reported for the first time. The possible role of these new metabolites in mechanisms and actions of isoflavones in human health remains to be investigated. In general, the levels of the newly identified metabolites were low and do not explain the overall poor recoveries of isoflavones encountered in some pharmacokinetic studies.

The methodology used in the analysis of urine samples limited the study to metabolites that have an intact isoflavonoid structure. Therefore, the analysis of metabolic products, formed by C-ring fission or conjugated sulfate and glucuronide metabolites, was not possible. The metabolism of isoflavones to smaller molecular weight metabolites and hitherto unknown compounds or conjugates thus requires further investigation. Another important task is to develop methods to analyze and determine the levels of actual forms of isoflavones and their metabolites, such as glucuronide and sulfate conjugates in biological fluids and tissues, to fully be able to assess any health benefits offered by isoflavones.

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APPENDIX 1

Table 1. *Analytical methods for isoflavonoids in plasma or serum samples applying mass spectrometry. Abbreviations: LOD, limit of detection; LOQ, limit of quantitation.*

Analyte(s)	Sample pretreatment	Instrumentation (mass analyzer)	LOD/LOQ	CV%	Recovery	Ref.
Daidzein, genistein, equol, *O*-dma (and lignans) ISTDs deuterated standards for all analytes	4 mL of plasma ISTD addition Solvolysis Ion exchange chromatography SPE with Sep-Pak C_{18} cartridges Enzymatic hydrolysis with purified *H. pomatia* ISTD addition Extraction with diethylether Ion exchange chromatography Derivatization (TMS)	GC-MS (quadrupole)	Sensitivity 0.2-1.0 nmol/L	Intra-assay: 1.4-30.8%	85.6-99.0%	Adlercreutz et al. (1993)[125] Adlercreutz et al. (1994)[134]
Equol, daidzein ISTDs d_4-equol and d_4-daidzein	1 mL of plasma ISTD addition Enzymatic hydrolysis with purified *H. pomatia* Extraction with diethylether Ion exchange chromatography Derivatization (TMS)	GC-MS (quadrupole)	0.05 ng/mL	Intra-assay: 2.5–10%	Not provided	Morton et al. (1994)[69]
Daidzein, [^{13}C]daidzein, genistein, [^{13}C]genistein ISTD dihydroflavone	0.25-0.50 mL of serum ISTD addition Heating with TEAS SPE with C_{18} Bond Elut cartridges Enzymatic hydrolysis with purified *H. pomatia* SPE with C_{18} Bond Elut cartridges Chromatography on Sephadex LH-20 Derivatization (tBDMS)	GC-MS (quadrupole)	No provided	Intra-assay: 0.5–1.0% Interassay: 1–17%	Not provided	Setchell et al. (2003)[115]

Table 1. *(Continued)*

Analyte(s)	Sample pretreatment	Instrumentation (mass analyzer)	LOD/LOQ	CV%	Recovery	Ref.
Daidzein, dihydrodaidzein, *O*-dma, genistein ISTDs: Phenolphthalein glucuronide, 4-methylumbelliferone-sulfate, biochanin A	1 mL of plasma ISTD addition Enzymatic hydrolysis with β-glucuronidase/sulfatase SPE with Sep-Pak C_{18} cartridges Extraction with hexane	LC-APCI/MS Negative mode (triple quadrupole)	Not provided	Intra-assay: 5.98-34.6% Interassay: 6.12-46.9%	87-99%	Coward et al. (1996)[140]
Daidzein, genistein, glycitein, *O*-dma (flavonoids and lignans) ISTD: Formononetin	0.45 mL of serum or plasma Enzymatic hydrolysis with β-glucuronidase/sulfatase ISTD addition Extraction with diethylether	LC-PDA-ESI/MS Negative mode (quadrupole iontrap)	2-39 nmol/L	Intra-assay: 0-14% Interassay: 18-23%	79-117%	Franke et al. (2002)[139]
Equol, daidzein, *O*-dma, genistein, glycitein ISTDs: $^{13}C_3$-labeled standards for all analytes except for *O*-dma	0.2 mL of serum ISTD addition Enzymatic hydrolysis with *H. pomatia* SPE with Strata C_{18} cartridges	LC-ESI/MS Negative mode (triple quadrupole)	<10 pg/mL <100 pg/mL for equol	Intra-assay: 2.9–5.7% Inter assay: 3.0–4.4%	92.5-100.7%	Grace et al. (2004)[135]
Daidzein, genistein, equol, *O*-dma, dihydrodaidzein, dihydrogenistein ISTDs: d_3-daidzein, d_4-genistein	0.1 mL of serum Enzymatic hydrolysis with *H. pomatia* Protein precipitation Centrifugation	LC-DAD-ESI/MS Negative mode (iontrap)	LOD 0.005 µmol/L LOQ 0.05 µmol/L	0.19-13.9% (low) 0.6-4.8% (high)	76-84%	Morandi et al. (2005)[137] Locati et al. (2005)[136]

Table 2. *Analytical methods for isoflavonoids in tissue samples applying mass spectrometry. Abbreviations: LOD, limit of detection; LOQ, limit of quantitation.*

Sample matrix	Analyte	Sample pretreatment	Instrumentation	LOD/LOQ	CV%	Recovery	Ref.
Mammary gland, uterus, ovary, testes, prostate, thyroid, liver, and brain	Genistein	Weighing Homogenization (20-mg portion of homogenate used for analysis) ISTD addition Sonication Enzymatic hydrolysis with *H. pomatia* Extraction with hexane[1] Centrifugation SPE (Oasis HLB)	LC-ESI/MS LC-ESI/MS/MS Positive mode	0.04–0.09 pmol/mg 0.01–0.03 pmol/mg	1-9%	40-78%	Chang et al. (2000)[107]
Liver, uterus, mammary glands, and brain	Daidzein, genistein	Pooling of the tissues 250-mg sample weighed (20-mg portion of homogenate used for analysis) Enzymatic hydrolysis with *H. pomatia* or Sequential hydrolysis or Acid hydrolysis Addition of 100 μL of glacial acetic acid[2] Extraction with hexane Aqueous phase dried under N_2 Extraction with ethyl acetate Evaporation to dryness ISTD added	LC-APCI/MS Negative mode	0.12 pmol (on column)	Enzymatic hydrolysis Intra-assay: 5.8% Interassay: 10.8% Acid hydrolysis Intra-assay: 6.7% Inter assay: 12.3%	Acid hydrolysis Daidzein: 98±2% Genistein: 94±4%	Gu et al. (2005)[106]

[1] *For fatty tissues such as mammary glands, testes, prostate and uterus*
[2] *Omitted for acid-hydrolyzed tissues.*

Table 3. *Analytical methods for isoflavonoids in urine samples applying mass spectrometry. Abbreviations: LOD, limit of detection; LOQ, limit of quantitation.*

Analyte(s)	Sample pretreatment	Instrumentation (mass analyzer)	LOD/LOQ	CV%	Recovery	Ref.
O-dma, equol, daidzein, genistein ISTDs d_5-O-dma, d_4-equol, d_4-daidzein, d_4-genistein	1/300 of 24-h urine [^{14}C]-estroneglucuronide added (ISTD) SPE on Sep-Pak C_{18} cartridges Ion exchange chromatography ISTD addition Enzymatic hydrolysis with *H. pomatia* SPE with Sep-Pak Ion exchange chromatography Derivatization (TMS)	GC-MS (quadrupole)	Sensitivity 3-4 nmol /24 h urine	Intra-assay: 7.3-12.4% (low) 0.8-9.7% (high) Interassay: 6.9-129%	100.9-105.5%	Adlercreutz et al. (1991)[27]
Daidzein, genistein, biochanin A, formononetin ISTDs: 6-hydroxyflavone robigenin	**Free isoflavones** 20 mL urine Centrifugation SPE with C_{18} cartridges ChemElut 1010 column Florsil cartridge ISTD addition Dervatization (TMS) **Total isoflavones** 20 mL urine Centrifugation SPE with C18 cartridges Enzymatic hydrolysis with *H. pomatia* ChemElut 1010 column Florsil cartridge ISTD addition Derivatization (TMS)	GC-MS (iontrap)	1.05-2.3 ng/mL	Not provided	61-89%	Tekel et al. (1999)[143]

Table 3. *(Continued)*

Analyte(s)	Sample pretreatment	Instrumentation (mass analyzer)	LOD/LOQ	CV%	Recovery	Ref.
Daidzein, genistein, Equol, *O*-dma, glycitein ISTDs: $^{13}C_3$-daidzein, $^{13}C_3$-genistein, $^{13}C_3$-equol, anthraflavic acid (for O-dma and glycitein)	0.2 mL of urine ISTD addition Enzymatic hydrolysis with *H. pomatia* SPE with Strata C_{18} cartridges Addition of anthraflavic acid (ISTD) Derivatization (TMS)	GC-MS (quadrupole)	1.3-5.0 ng/mL	Intra-assay: 1.8-6.5% Interassay: 4.0-26.5%	94-104 % (low) 89-99% (high)	Grace et al. (2003)[142]
Daidzein, genistein, O-dma, equol, dihydrodaidzein, dihydrogenistein, glycitein, (coumestrol, estrogens, and lignans) ISTDs: Deuterated analogues	1/150 of 24-h volume, minimum 5 mL for a postmenopausal sample Ethoximation SPE with Sep-Pak C_{18} cartridges Ion exchange chromatography ISTD addition Enzymatic hydrolysis with *H. pomatia* SPE with Sep-Pak Ion exchange chromatography Chromatography with Lipidex 5000 Derivatization (TMS)	GC-MS (quadrupole)	Sensitivity 0.01–0.27 nmol/ 24 h urine	Intra-assay: 5.8-18.6% (low) 1.1-9.0% (high) Interassay: 7.1-12.8% (low) 4.7-9.6% (high)	88.9-101%	Adlercreutz et al. (2004)[138]
Daidzein, genistein, glycitein, dihydrodaidzein, dihydrogenistein, *O*-dma (flavonoids and lignans) ISTD: Formononetin	0.25 mL of urine Enzymatic hydrolysis with β-glucuronidase/sulfatase ISTD addition Extraction with diethylether	LC-PDA-ESI/MS Negative mode (quadrupole iontrap)	2-39 nmol/L	Intra-assay: 1-17% Interassay: 1-49%	93-118%	Franke et al. (2002)[139]

Table 3. *(Continued)*

Analyte(s)	Sample pretreatment	Instrumentation (mass analyzer)	LOD/LOQ	CV%	Recovery	Ref.
Daidzein, genistein, glycitein, equol, O-dma,dihydrodaidzein, dihydrogenistein ISTD: Biochanin A	1 mL of urine **Free aglycones:** Extraction with diethyl ether **Glucuronides + free aglycones:** Hydrolysis with β-Glucuronidase Extraction with diethyl ether **Sulfates + free aglycones:** Hydrolysis with sulfatase Extraction with diethyl ether **Total aglycones:** 1 mL urine + Hydrolysis with sulfatase/β-glucuronidase Extraction with diethyl ether	LC-APCI/MS Negative mode (quadrupole)	5 ng/mL 200 ng/mL for equol	Intra-assay: <10% Interassay: Free aglycones 39.3-66.1% Total aglycones 9.9-17.3% Glucuronide + free aglycones 10.8-29.4%	90.8±2.5%	Cimino et al. (1999)[141]
Daidzein, genistein, glycitein, formononetin, biochanin A, dihydrodaidzein, dihydrogenistein O-dma, equol, and their glucuronide and sulfate conjugates ISTDs: $^{13}C_3$-daidzein, $^{13}C_3$-genistein	1mL of urine ISTD addition No sample pre-treatment, enzymatic hydrolysis with β-glucuronidase (*E. coli*), or enzymatic hydrolysis with *H. pomatia* Filtration	LC-ESI/MS/MS Negative mode, except for equol aglycone	50 ng/mL	Intra-assay: 3-6% (semi-quantitative method 4-10%) Interassay: <15%	Not provided	Clarke et al. (2002)[118]

APPENDIX 2

Table 1. *Electron ionization mass spectra of nondeuterated (a) and deuterated (b) TMS derivatives of isoflavones studied. Peaks with intensities over 1% of the base peak are listed.*

Compound		t_R (min)	$M^{+\bullet}$	Ion[1] (abundance)
Formononetin	a	12.16	340	117 (10), 132 (32), 133 (5), 141 (11), 147 (11), 149 (4), 152 (5), 162 (32), 192 (4), 193 (5), 207 (10), 208 (12), 325 (36), 326 (8), 339 (39), 340 (100), 341 (25), 342 (6)
	b	11.68	349	117 (9), 132 (31), 133 (4), 144 (9), 150 (5), 165 (15), 166 (5), 198 (3), 207 (12), 217 (8), 331 (23), 332 (4), 348 (33), 349 (100), 350 (24), 351 (6)
Pseudopabtigenin	a	14.59	354	116 (3), 119 (3), 121 (3), 129 (2), 133 (2), 135 (1), 137 (3), 139 (4), 140 (2), 145 (16), 146 (38), 147 (6), 149 (3), 150 (3), 151 (3), 162 (3), 165 (4), 166 (1), 169 (24), 170 (11), 171 (1), 179 (3), 193 (5), 195 (1), 207 (4), 208 (5), 209 (8), 210 (1), 223 (2), 251 (3), 253 (3), 281 (3), 282 (1), 309 (2), 311 (1), 339 (25), 353 (24), 354 (100), 255 (25), 356 (8), 357 (1)
Daidzein	a	14.25	398	115 (3), 119 (2), 121 (2), 133 (1), 135 (1), 137 (1), 145 (1), 147 (3), 149 (2), 152 (2), 159 (1), 161 (1), 162 (2), 163 (1), 165 (1), 169 (3), 170 (2), 175 (11), 176 (3), 177 (2), 178 (1), 179 (1), 184 (38), 190 (5), 191 (3), 192 (2), 193 (1), 207 (1), 208 (1), 209 (1), 237 (2), 253 (2), 281 (2), 283 (4), 295 (1), 311 (3), 325 (3), 327 (1), 353 (1), 355 (9), 356 (2), 367 (1), 383 (70), 384 (20), 385 (7), 386 (1), 397 (10), 398 (100), 399 (30), 400 (11), 401 (2)
	b	13.86	416	117 (1), 118(1), 127 (1), 138 (1), 143 (1), 150 (3), 151 (1), 152 (1), 155 (1), 162 (1), 163 (1), 172 (3), 176 (1), 181 (14), 182 (3), 187 (3), 188 (1), 190 (34), 199 (8), 200 (1), 290 (3), 299 (2), 306 (1), 318 (3), 334 (3), 335 (1), 342 (1), 370 (8), 371 (2), 389 (5), 390 (1), 395 (1), 398 (57), 399 (16), 400 (6), 401 (1), 407 (8), 408 (2), 413 (2), 414 (1), 415 (10), 416 (100), 417 (28), 418 (11), 419 (2)
Biochanin A	a	13.81	428	117 (1), 132 (2), 133 (1), 163 (1), 177 (7), 199 (19), 207 (3), 312 (2), 340 (2), 341 (4), 370 (7), 371 (2), 398 (3), 413 (100), 414 (31), 415 (12), 416 (2), 428 (<1)
	b	13.54	446	117 (1), 132 (2), 184 (5), 205 (16), 214 (2), 223 (1), 318 (2), 346 (2), 356 (2), 384 (1), 385 (6), 386 (2), 413 (2), 428 (100), 429 (30), 430 (12), 431 (2), 446 (<1)

[1] *Ions that do not have any isotopic peaks are underlined.*

Table 1. *(Continued)*

Compound		t_R (min)	$M^{+\bullet}$	Ion[1] (abundance)
Prunetin	a	14.19	428	175 (9), 177 (11), 199 (29), 340 (3), 341 (4), 356 (2), 370 (8), 413 (100), 414 (31), 415 (12), 416 (2), 428 (<1)
	b	13.95	446	181 (4), 184 (9), 205 (26), 346 (3), 356 (1), 365 (2), 385 (6), 428 (100), 429 (31), 430 (10), 431 (1), 446 (<1)
Glycitein	a	18.29	428	101 (1), 115 (4), 119 (1), 121 (1), 133 (1), 135 (2), 137 (1), 139 (2), 141 (1), 145 (2), 146 (2), 147 (1), 149 (2), 150 (2), 151 (1), 152 (6), 153 (1), 154 (1), 155 (1), 159 (1), 161 (2), 162 (3), 163 (2), 165 (2), 167 (1), 169 (2), 170 (2), 175 (9), 176 (6), 177 (5), 178 (2), 179 (2), 180 (5), 181 (1), 184 (23), 191 (41), 199 (15), 206 (7), 208 (9), 209 (2), 221 (1), 223 (3), 237 (1), 238 (2), 239 (1), 253 (5), 267 (2), 269 (1), 279 (1), 281 (2), 295 (1), 297 (2), 309 (3), 310 (2), 311 (2), 323 (1), 325 (6), 326 (1), 327 (2), 339 (2), 340 (1), 341 (1), 353 (2), 354 (2), 355 (12), 356 (3), 357 (2), 367 (1), 369 (1), 370 (2), 383 (13), 384 (4), 385 (7), 386 (1), 397 (7), 398 (62), 399 (21), 400 (8), 401 (2), 413 (31), 414 (10), 415 (4), 427 (11), 428 (100) 429 (32), 430 (12), 431 (2)
	b	17.72	446	101 (1), 102 (5), 105 (1), 117 (2), 126 (1), 138 (2), 139 (2), 143 (1), 149 (2), 150 (2), 151 (1), 152 (2), 156 (1), 157 (2), 158 (3), 159 (2), 161 (1), 162 (1), 165 (2), 166 (1), 167 (1), 168 (1), 169 (2), 170 (1), 174 (1), 175 (1), 178 (1), 179 (5), 180 (3), 181 (12), 182 (2), 183 (1), 186 (6), 187 (2), 188 (1), 189 (18), 197 (19), 205 (13), 214 (12), 215 (2), 222 (1), 223 (1), 247 (3), 259 (4), 260 (1), 275 (1), 287 (1), 303 (1), 315 (1), 317 (2), 331 (6), 332 (1), 339 (1), 345 (1), 348 (1), 364 (2), 366 (2), 367 (9), 368 (4), 385 (2), 395 (14), 396 (3), 397 (1), 400 (5), 412 (8), 413 (63), 414 (19), 415 (7), 416 (2), 417 (2), 427 (1), 428 (20), 429 (8), 430 (3), 443 (2), 444 (2), 445 (13), 446 (100) 447 (30), 448 (10), 449 (3)
Calycosin	a	17.33	428	119 (2), 160 (4), 169 (2), 175 (10), 184 (20), 190 (26), 191 (37), 199 (5), 207 (2), 209 (3), 253 (2), 325 (2), 355 (3), 383 (4), 398 (100), 399 (30), 400 (11), 413 (11), 414 (3), 428 (68), 429 (21), 430 (6)
7,4'-dihydroxy-3'-methoxyisoflavone	a	17.68	428	160 (4), 175 (9), 176 (7), 177 (5), 184 (20), 190 (23), 191 (42), 199 (7), 355 (3), 383 (4), 397 (8), 398 (100), 399 (31), 400 (12), 413 (14), 414 (5), 428 (70), 429 (23), 430 (9)
	b	17.36	446	160 (4), 178 (10), 179 (7), 180 (4), 188 (17), 196 (30), 198 (43), 205 (6), 364 (2), 395 (3), 412 (8), 413 (100), 414 (30), 415 (12), 416 (2), 428 (10), 429 (3), 446 (74), 447 (24), 448 (7)
Genistein	a	15.38	486	133 (1), 147 (2), 175 (1), 177 (1), 192 (1), 228 (22), 309 (1), 327 (2), 369 (1), 370 (1), 383 (2), 397 (1), 398 (1), 399 (11), 400 (3), 401 (1), 414 (4), 415 (1), 416 (4), 441 (1), 469 (2), 471 (100), 472 (37), 473 (17), 474 (4), 475 (1), 486 (<1)
	b	14.99	513	142 (1),162 (1), 181 (1), 183 (1), 198 (1), 239 (24), 335 (1), 385 (1), 396 (1), 413 (2), 414 (3), 415 (8), 416 (3), 432 (4), 433 (1), 459 (1), 492 (3), 493 (1), 494 (2), 495 (100), 496 (36), 497 (17), 498 (4), 499 (1) , 513 (<1)

[1]*Ions that do not have any isotopic peaks are underlined.*

Table 1. *(Continued)*

Compound		t_R (min)	$M^{+\cdot}$	Ion (abundance)
6,7,4'-trihydroxy-isoflavone	a	19.12	486	105 (2), 115 (2), 133 (4), 135 (1), 147 (3), 175 (4), 179 (1), 191 (1), 193 (1), 207 (2), 208 (3), 209 (2), 223 (2), 253 (3), 281 (9), 282 (2), 283 (1), 325 (2), 355 (6), 356 (2), 383 (8), 384 (2), 385 (1), 397 (2), 398 (3) , 399 (2), 413 (2), 443 (2), 471 (68), 472 (27), 473 (12), 474 (2), 485 (5), 486 (100), 487 (41), 488 (18), 489 (5), 490 (1)
	b	18.53	513	108 (1), 142 (4), 162 (2), 181 (4), 188 (1), 207 (1), 214 (3), 222 (1), 259 (2), 296 (8), 297 (2), 298, 331 (1), 367 (5), 368 (1), 395 (7), 396 (2), 413 (3), 414 (1), 415 (1), 431 (2), 467 (2), 495 (53), 496 (20), 497 (9), 498 (2), 510 (2), 511 (2), 512 (5), 513 (100), 514 (38), 515 (18), 516 (5), 517 (1)
7,8,4'-trihydroxy-isoflavone	a	19.62	486	115 (2), 131 (1), 133 (3), 147 (3), 152 (1), 165 (1), 175 (4), 191 (1), 208 (1), 209 (1), 223 (2), 253 (3), 279 (1), 281 (2), 296 (1), 309 (1), 325 (2), 353 (1), 355 (6), 356 (2), 367 (1), 383 (9), 384 (3), 385 (1), 397 (7), 398 (6), 399 (3), 413 (1), 443 (2), 455 (1),458 (1), 471 (63), 472 (25), 473 (11), 474 (3), 485 (11), 486 (100), 487 (40), 488 (18), 189 (5), 490 (1)
	b	19.23	513	142 (2), 158 (1), 162 (3), 181 (4), 207 (2), 214 (1), 223 (1), 259 (2), 314 (1), 331 (1), 367 (5), 368 (2), 395 (7), 396 (3), 412 (2), 413 (4), 414 (2), 415 (7), 416 (2), 431 (1), 467 (2), 492 (1), 495 (48), 496 (19), 497 (8), 498 (2), 510 (2), 511 (3), 512 (10), 513 (100), 514 (39), 515 (18), 516 (5), 517 (1)
7,3',4'-trihydroxy-isoflavone	a	18.45	486	115 (2), 133 (3), 147 (6), 149 (1), 160 (2), 161 (1), 165 (1), 175 (6), 176 (2), 177 (1), 190 (8), 193 (2), 207 (2), 209 (1), 253 (3), 309 (1), 327 (2), 353 (1), 367 (1), 383 (7), 384 (2), 385 (1), 395 (1), 396 (1), 397 (5), 398 (5), 399 (3), 400 (1), 411 (1), 413 (3), 414 (1), 455 (1), 469 (2), 471 (85), 472 (34), 473 (15), 474 (4), 485 (7), 486 (100), 487 (39), 488 (18), 489 (5), 490 (1)
	b	18.09	513	118 (1), 142 (3), 160 (3), 162 (3), 178 (5), 179 (2), 196 (7), 197 (2), 207 (2), 223 (1), 259 (3), 339 (2), 395 (7), 396 (3), 397 (1), 412 (2), 413 (4), 414 (2), 415 (3), 416 (1), 431 (3), 432 (1), 476 (1), 492 (2), 494 (2), 495 (74), 496 (30), 497 (13), 498 (3), 510 (2), 511 (2), 513 (100), 514 (41), 515 (18), 516 (5), 517 (1)
5,7,3',4'-tetrahydroxy-isoflavone	a	20.04	574	133 (1), 147 (3), 175 (1), 207 (1), 268 (1), 369 (1), 399 (5), 400 (1), 471 (12), 472 (5), 473 (2), 487 (5), 488 (2), 502 (2), 557 (1), 559 (100), 560 (48), 561 (25), 562 (8), 563 (2), 573 (1), 574 (5), 575 (2), 576 (1)
	b	19.45	610	142 (1), 162 (1), 178 (1), 207 (1), 412 (5), 413 (2), 492 (11), 493 (12), 494 (2), 495 (1), 512 (4), 513 (2), 514 (1), 528 (2), 589 (3), 590 (1), 591 (3), 592 (100), 593 (47), 594 (24), 595 (8), 596 (2), 610 (5), 611 (2), 612 (1)

Table 2. *Electron ionization mass spectra of nondeuterated (a) and deuterated (b) TMS derivatives of isoflavanones studied. Peaks with intensities over 1% of the base peak are listed.*

Compound		t_R (min)	$M^{+\bullet}$	Ion[1] (abundance)
Dihydroformononetin	a	8.99	342	119 (9), 121 (3), 134 (100), 135 (8), 137 (1), 149 (1), 193 (2), 208 (3), 209 (1), 327 (1), 342 (5), 343 (1)
	b	8.72	351	119 (9), 121 (1), 134 (100), 135 (8), 143 (1), 199 (2), 217 (3), 218 (1), 324 (1), 333 (1), 351 (4), 352 (1)
Dihydrodaidzein	a	10.18	400	103 (1), 115 (1), 117 (2), 119 (2), 121 (2), 135 (2), 137 (1), 147 (1), 149 (4), 151 (5), 152 (1), 161 (3), 163 (1), 177 (27), 178 (4), 179 (2), 185 (7), 192 (100), 193 (16), 194 (4), 208 (1), 209 (1), 235 (1), 281 (26), 282 (6), 327 (1), 385 (4), 386 (1), 400 (3)
	b	9.86	418	119 (2), 127 (1), 138 (2), 143 (1), 152 (1), 155 (3), 157 (3), 164 (2), 183 (25), 184 (3), 185 (1), 191 (6), 199 (1), 201 (100), 202 (14), 203 (4), 217 (1), 218 (1), 244 (1), 299 (31), 300 (7), 301 (3), 336 (1), 400 (2), 418 (3)
Dihydroglycitein	a	13.02	430	103 (4), 109 (3), 115 (1), 116 (2), 117 (4), 119 (2), 133 (4), 135 (5), 147 (7), 149 (7), 151 (9), 152 (4), 153 (2), 161 (6), 177 (38), 178 (9), 179 (10), 180 (6), 191 (3), 192 (95), 193 (18), 194 (6), 195 (3), 200 (4), 207 (15), 208 (28), 209 (7), 210 (2), 223 (6), 238 (100), 239 (15), 240 (69, 267 (2), 280 (5), 281 (7), 282 (3), 311 (20), 312 (3), 415 (4), 416 (2), 430 (16), 431 (5), 432 (2)
	b	12.76	448	103 (2), 105 (2), 106 (1), 112 (4), 119 (3), 131 (3), 133 (3), 138 (7), 147 (3), 152 (3), 155 84), 156 (3), 157 (5), 158 (3), 162 (7), 163 (2), 183 (36), 184 (6), 186 (7), 188 (9), 199 (4), 201 (92), 202 (13), 203 (4), 204 (2), 207 (19), 208 (2), 209 (4), 214 (25), 215 (4), 222 (4), 235 (2), 247 (100), 248 (16), 249 (6), 281 (2), 299 (3), 329 (19), 330 (19), 331 (2), 388 (2), 448 (16), 449 (5), 450 (1)
Dihydrobiochanin A	a	10.78	430	119 (5), 121 (12), 134 (13), 135 (2), 147 (4), 207 (1), 222 (1), 239 (4), 268 (1), 279 (2), 281 (7), 282 (1), 296 (100), 297 (22), 298 (9), 299 (2), 415 (14), 416 (5), 417 (2), 430 (<1)
	b	10.47	448	119 (5), 121 (10), 134 (14), 135 (2), 142 (2), 161 (1), 162 (4), 206 (1), 224 (1), 235 (1), 256 (4), 286 (6), 296 (3), 297 (2), 314 (100), 315 (21), 316 (9), 317 (1), 430 (14), 431 (5), 432 (2), 448 (<1)
Dihydrogenistein	a	11.62	488	103 (1), 115 (1), 133 (4), 135 (2), 147 (5), 148 (2), 147 (5), 148 (2), 149 (1), 151 (5), 161 (3), 163 (1), 166 (1), 177 (14), 178 (1), 179 (8), 180 (1), 192 (20), 193 (5), 194 (2), 207 (2), 225 (1), 229 (5), 235 (1), 239 (4), 251 (1), 267 (1), 268 (5), 269 (1), 279 (2), 281 (6), 282 (1), 296 (100), 297 (22), 298 (9), 299 (2), 325 (1), 340 (3), 369 (9), 370 (3), 371 (2), 415 (1), 473 (12), 474 (5), 475 (2), 488 (<1)
	b	11.40	515	183 (21), 201 (47), 233 (3), 240 (7), 286 (6), 296 (5), 297 (1), 314 (100), 315 (21), 316 (8), 317 (2), 352 (3), 396 (12), 434 (2), 497 (13), 498 (5), 515 (<1)

[1]*Ions that do not have any isotopic peaks are underlined.*

Table 2. *(Continued)*

Compound		t_R (min)	$M^{+\bullet}$	Ion (abundance)
6,7,4'-trihydroxy-isoflavanone[1]	a	13.57	488	103 (2), 115 (2), 117 (2), 119 (1), 121 (1), 131 (2), 133 (5), 134 (2), 135 (1), 147 (6), 149 (3), 151 (5), 152 (2), 161 (3), 166 (1), 175 (1), 176 (4), 177 (12), 178 (5), 179 (10), 180 (3), 181 (1), 191 (1), 192 (18), 193 (5), 194 (1), 205 (1), 207 (3), 208 (4), 209 (2), 223 (1), 267 (6), 268 (2), 280 (1), 281 (9), 282 (2), 296 (100), 297 (21), 298 (10), 369 (3), 473 (3), 474 (1), 488 (11), 489 (4), 490 (2)
	b	13.33	515	137 (5), 143 (3), 183 (12), 184 (1), 188 (2), 201 (18), 214 (2), 226 (2), 295 (2), 296 (6), 297 (1), 314 (100), 315 (18), 316 (5), 317 (1), 328 (3), 329 (1), 365 (1), 382 (1), 397 (1), 471 (2), 497 (2), 515 (9), 516 (4), 517 (2), 518 (1)
7,8,4'-trihydroxy-isoflavanone[1]	a	13.18	488	103 (3), 105 (2), 115 (1), 117 (2), 119 (1), 133 (6), 135 (1), 136 (1), 147 (3), 149 (3), 150 (1), 151 (3), 161 (2), 176 (3), 177 (9), 178 (1), 179 (6), 180 (1), 192 (8), 193 (3), 207 (3), 208 (3), 209 (3), 267 (2), 280 (2), 281 (100), 282 (14), 283 (7), 284 (1), 296 (7), 297 (2), 298 (1), 442 (1), 445 (5), 446 (2), 447 (1), 473 (1), 488 (5), 489 (2)
	b	12.89	515	105 (2), 112 (3), 117 (2), 121 (2), 126 (2), 129 (1), 138 (2), 139 (1), 141 (3), 142 (5), 143 (2), 152 (3), 157 (3), 158 (1), 161 (4), 162 (5), 163 (2), 166 (2), 182 (3), 183 (10), 184 (2), 185 (2), 186 (1), 187 (1), 188 (7), 189 (2), 197 (1), 199 (1), 201 (7), 202 (2), 204 (1), 209 (1), 214 (4), 215 (2), 216 (1), 218 (1), 222 (2), 223 (1), 225 (1), 231 (1), 232 (1), 235 (1), 285 (5), 286 (1), 293 (2), 294 (2), 295 (16), 296 (100), 297 (16), 298 (10), 299 (2), 314 (11), 315 (2), 316 (1), 338 (2), 351 (1), 400 (2), 469 (2), 514 (1), 515 (4), 516 (2), 517 (1)
7,3',4'-trihydroxy-isoflavanone[2]	a	12.88	488	103 (1), 105 (3), 109 (1), 115 (2), 121 (2), 131 (2), 133 (3), 137 (2), 147 (4), 149 (2), 162 (4), 163 (1), 177 (6), 179 (5), 192 (16), 193 (4), 205 (2), 207 (3), 208 (1), 209 (1), 265 (4), 267 (4), 280 (100), 281 (37), 282 (12), 283 (3), 442 (1), 473 (3), 474 (1), 488 (9), 489 (2)
	b	12.65	515	105 (4), 112 (1), 119 (1), 120 (1), 127 (1), 138 (1), 142 (3), 143 (2), 162 (9), 163 (1), 166 (1), 180 (5), 181 (1), 185 (3), 188 (1), 189 (2), 198 (14), 199 (4), 200 (1), 216 (2), 217 (1), 218 (1), 280 (4), 282 (4), 285 (1), 295 (1), 298 (100), 299 (38), 300 (12), 301 (3), 497 (2), 515 (10), 516 (4), 517 (2)
5,7,3',4'-tetrahydroxy-isoflavanone[1]	a	15.45	576	103 (3), 133 (6), 143 (1), 147 (11), 179 (10), 192 (7), 205 (1), 207 (13), 267 (5), 280 (100), 281 (30), 282 (11), 283 (3), 294 (6), 295 (1), 296 (14), 297 (19), 369 (25), 370 (8), 371 (4), 473 (1), 561 (7), 562 (2), 576 (5), 577 (2)
	b	15.18	612	105 (3), 112 (2), 123 (3), 142 (3), 147 (3), 149 (8), 161 (4), 162 (9), 163 (3), 188 (7), 207 (12), 208 (2), 209 (2), 281 (2), 298 (100), 299 (25), 301 (2), 314 (3), 315 (1), 365 (3), 378 (4), 379 (7), 396 (23), 397 (7), 398 (5), 399 (1), 416 (4), 592 (4), 594 (6), 595 (3), 596 (1), 612 (5), 613 (2)

[1] *Authentic reference compound not available.*

Table 3. *Electron ionization mass spectra of nondeuterated (a) and deuterated (b) TMS derivatives of isoflavans studied. Peaks with intensities over 1% of the base peak are listed.*

Compound		t_R (min)	$M^{+\bullet}$	Ion[1] (abundance)
4'-*O*-methylequol	a	7.13	328	103 (1), 105 (2), 115 (1), 119 (19), 121 (19), 122 (3), 134 (100), 135 (15), 136 (1), 147 (2), 149 (1), 151 (2), 157 (2), 163 (1), 164 (2), 165 (2), 179 (3), 191 (5), 193 (1), 206 (9), 207 (11), 208 (1), 298 (3), 313 (3), 328 (30), 329 (5), 330 (1)
	b	7.05	337	103 (1), 105 (2), 119 (14), 121 (17), 122 (3), 134 (100), 135 (14), 136 (1), 197 (4), 216 (10), 215 (1), 304 (5), 319 (1), 337 (29), 338 (4), 339 (1)
Equol	a	8.09	386	103 (1), 105 (2), 115 (2), 117 (2), 119 (2), 135 (2), 147 (1), 149 (3), 151 (6), 161 (3), 163 (2), 165 (3), 177 (25), <u>178</u> (16), 179 (11), 180 (3), 186 (1), 192 (100), 193 (15), 194 (4), 195 (2), 205 (1), 206 (8), 207 (8), 208 (1), 267 (10), 268 (2), 371 (5), 372 (2), 386 (16), 387 (5), 388 (2)
	b	7.74	404	103 (1), 105 (2), 119 (2), 138 (2), 152 (1), 155 (2), 157 (3), 164 (2), 166 (1), 169 (1), 171 (2), 183 (23), <u>184</u> (23), 185 (4), 188 (6), 189 (3), 193 (1), 197 (2), 201(100), 202 (15), 203 (4), 204 (1), 215 (8), 216 (8), 217 (1), 295 (10), 285 (10), 286 (2), 386 (2), 404 (16), 405 (5), 406 (2)
7,3'-dihydroxyisoflavan	a	7.34	386	103 (1), 105 (2), 115 (2), 117 (2), 119 (2), 135 (2), 147 (1), 149 (2), 151 (4), 161 (2), 163 (1), 165 (3), 177 (24), <u>178</u> (16), 179 (10), 192 (100), 193 (14), 194 (4), 195 (2), 205 (1), 206 (9), 207 (8), 208 (1), 267 (10), 268 (2), 371 (4), 372 (1), 386 (16), 387 (5), 388 (2)
	b	7.02	404	103 (1), 105 (2), 119 (2), 138 (2), 152 (1), 155 (2), 157 (3), 164 (2), 166 (1), 169 (1), 171 (2), 183 (23), <u>184</u> (23), 185 (4), 188 (6), 189 (3), 193 (1), 197 (2), 201 (100), 202 (15), 203 (4), 204 (1), 215 (8), 216 (8), 217 (1), 295 (11), 285 (10), 286 (2), 386 (2), 404 (15), 405 (4), 406 (2)
7,4'-dihydroxy-6-methoxyisoflavan (6-OMe-equol)	a	10.64	416	119 (3), 133 (9), 149 (7), 151 (14), 161 (5), 163 (3), 165 (5), 166 (4), 177 (47), 178 (17), 179 (26), 180 (7), 185 (16), <u>186</u> (13), 192 (100), 193 (20), 194 (14), 205 (5), 206 (11), 207 (14), 209 (13), 210 (1), 224 (8), 225 (3), 236 (6), 237 (22), 238 (4), 297 (5), 386 (10), 387 (5), 401 (4), 416 (74), 417 (23), 418 (9)
	b	10.32	434	133(6), 183 (44), 184 (13), 185 (2) 188 (30), <u>192</u> (28), 201 (100), 202 (21), 203 (13), 246 (22), 247 (3), 315 (4), 401 (7), 402 (2), 416(3), 418 (2), 434 (77), 435 (25), 436 (6)

[1]*Ions that do not have any isotopic peaks are underlined.*

Table 3. *(Continued)*

Compound		t_R (min)	$M^{+\bullet}$	Ion (abundance)
7,4'-dihydroxy-3'-methoxyisoflavan (3'-OMe-equol)	a	10.52	416	103 (2), 105 (3), 135 (2), 147 (4), 149 (3), 151 (3), 162 (4), 163 (2), 166 (2), 177 (10), 178 (4), 179 (12), 180 (7), 181 (2), 185 (11), 186 (2), 191 (3), 192 (73), 193 (17), 194 (5), 195 (5), 205 (2), 206 (2), 207 (21), 208 (4), 209 (7), 210 (14), 211 (3), 219 (2), 222 (100), 223 (16), 224 (5), 267 (3), 401 (4), 416 (21), 417 (7), 418 (3)
	b	10.24	434	105 (2), 112 (1), 142 (2), 149 (1), 162 (10), 180 (5), 185 (14), 186 (3), 198 (14), 213(16), 231 (100), 232 (14), 233 (3), 285 (4), 416 (2), 418 (4), 434 (23), 435 86), 436 (3)
Trans-4-OH-equol	a	9.48	474	117 (1), 133 (1), 135 (1), 147 (2), 149 (2), 151 (2), 161 (1), 163 (1), 177 (9), 178 (2), 179 (13), 180 (2), 192 (13), 193 (3), 195 (2), 267 (100), 268 (21), 269 (8), 270 (1), 282 (11), 283 (2), 355 (5), 385 (3), 474 (1)
Cis-4-OH-equol	a	10.07	474	117 (1), 133 (1), 135 (1), 147 (2), 149 (2), 151 (2), 161 (1), 163 (1), 177 (9), 178 (2), 179 (12), 180 (2), 192 (13), 193 (3), 195 (2), 267 (100), 268 (21), 269 (8), 270 (1), 282 (9), 283 (2), 355 (5), 385 (2), 474 (1)
8,7,4'-trihydroxy-isoflavan[1] (8-OH-equol)	a	11.57	474	103 (5), 117 (3), 129 (5), 133 (9), 147 (13), 149 (5), 151 82), 177 (15), 179 (57), 180 (9), 181 (1), 191 (3), 192 (16), 193 (7), 194 (3), 204 (1), 205 (2), 207 (15), 209 (1), 265 (19), 267 (100), 268 (22), 269 (7), 280 (1), 281 (15), 282 (3), 294 (13), 295 (16), 296 (3), 474 (32), 475 (13), 476 (4)
	b	11.19	501	105 (5), 107 (7), 119 (5), 125 (5), 133 (3), 183 (29), 188 (81), 189 (15), 190 (5), 198 (3), 199 (4), 201 (45), 202 (12), 203 (4), 282 (100), 283 (21), 284 (9), 285 (10), 297 (5), 298 (13), 300 (3), 312 (18), 313 (19), 314 (10), 315 (7), 316 (3), 501 (37), 502 (16), 503 (8)
6,7,4'-trihydroxy-isoflavan[1] (6-OH-equol)	a	11.97	474	103 (3), 105 (2), 115 (4), 117 (3), 119 (2), 121 (4), 129 (1), 131 (4), 133 (6), 135 (4), 145 (1), 147 (15), 148 (2), 149 (7), 151 (10), 152 (1), 161 (6), 163 (4), 165 (3), 177 (39), 178 (4), 179 (66), 180 (11), 181 (3), 189 (2), 190 (2), 192 (71), 193 (17), 194 (6), 195 (3), 205 (5), 206 (1), 207 (6), 209 (1), 219 (2), 221 (1), 229 (6), 230 (2), 237 (3), 239 (2), 251 (3), 252 (3), 267 (34), 268 (7), 269 (3), 279 (1), 281 (3), 282 (34), 283 (9), 284 (3), 294 (11), 295 (26), 296 (7), 297 (2), 355 (4), 356 (1), 401 (1), 474 (100), 475 (39), 476 (19), 477 (4), 478 (1)
	b	11.45	501	119 (2), 121 (2), 138 (2), 142 (4), 147 (1), 152 (3), 154 (4), 155 (4), 157 (4), 161 (1), 162 (8), 163 (2), 164 (3), 169 (3), 183 (33), 184 (5), 185 (10), 186 (2), 188 (42), 189 (6), 190 (2), 200 (3), 201 (63), 202 (12), 203 (3), 207 (2), 212 (2), 213 (2), 214 (4), 242 (6), 251 (2), 263 (2), 266 (2), 282 (22), 283 (5), 284 (2), 300 (31), 301 (7), 302 (3), 312 (9), 313 (22), 314 (6), 315 (2), 382 (4), 500 (2), 501 (100), 502 (40), 503 (18), 504 (4), 505 (1)

[1]*Authentic reference compound not available.*

Table 3. *(Continued)*

Compound		t_R (min)	$M^{+\bullet}$	Ion (abundance)
7,3',4'-trihydroxy-isoflavan (3'-OH-equol)	a	10.85	474	103 (3), 105 (3), 115 (2), 117 (3), 119 (2), 129 (1), 131 (4), 133 (4), 135 (2), 147 (9), 148 (2), 149 (2), 151 (2), 161 (2), 162 (3), 163 (2), 165 (1), 177 (8), 179 (30), 180 (5), 181 (2), 189 (1), 191 (3), 192 (24), 193 (8), 194 (2), 195 (3), 203 (1), 204 (3), 205 (4), 206 (2), 207 (11), 208 (2), 209 (1), 219 (3), 220 (3), 221 (1), 237 (2), 249 (1), 251 (1), 253 (1), 265 (5), 267 (26), 268 (23), 269 (6), 270 (2), 280 (100), 281 (27), 282 (9), 283 (12), 293 (1), 355 (3), 356 (1), 395 (5), 396 (2), 408 (1),444 (1), 445 (1), 474 (22), 475 (9), 476 (4)
	b	10.52	501	105 (3), 112 (12), 135 (3), 138 (6), 141 (3), 162 (19), 163 (3), 166 (2), 178 (8), 180 (6), 185 (21), 186 (4), 198 (21), 199 (3), 201 (3), 204 (4), 207 (4), 208 (1), 209 (5), 210 (2), 211 (2), 216 (4), 217 (1), 222 (3), 223 (2), 228 (1), 229 (3), 232 (2), 235 (23), 236 (4), 237 (2), 247 (2), 248 (2), 249 (1), 251 (2), 261 (4), 262 (1), 263 (1), 280 (3), 281 (1), 282 (1), 285 (19), 286 (23), 287 (6), 288 (2), 289 (7), 290 (2), 295 (2), 297 (1), 298 (100), 299 (24), 300 (9), 301 (1), 346 (3), 358 (2), 388 (48), 389 (14), 390 (7), 391 (2), 473 (8), 474 (3), 475 (2), 487 (2), 501 (25), 502 (9), 503 (4)

Table 4. *Electron ionization mass spectra of nondeuterated (a) and deuterated (b) TMS derivatives of α-methyldeoxybenzoins studied. Peaks with intensities over 1% of the base peak are listed.*

Compound		t_R (min)	$M^{+\bullet}$	Ion (abundance)
Angolensin	a	6.71	416	105 (3), 119 (1), 133 (2), 135 (7),147 (1), 149 (2), 163 (1), 179 (1), 193 (1), 195 (1), 209 (9), 210 (1), 223 (2), 237 (1), 251 (1), 265 (1), 266 (1), 281 (100), 282 (12), 283 (8), 284 (1), 371 (1), 386 (1), 401 (3), 402 (1), 416 (<1)
	b	6.48	434	105 (2), 120 (1), 135 (5), 142 (1), 155 (1), 162 (2), 171 (1), 199 (1), 218 (2), 219 (6), 220 (1), 235 (1), 252 (1), 279 (1), 296 (1), 299 (100), 300 (21), 301 (7), 302 (1), 386 (1), 401 (1), 416 (3), 417 (1), 434 (<1)
O-dma	a	7.32	474	133 (1), 147 (1), 149 (1), 163 (1), 177 (1), 193 (4), 209 (6), 223 (1), 237 (1), 281 (100), 282 (20), 283 (8), 284 (1), 459 (3), 460 (1), 474 (<1)
	b	7.02	501	183 (1), 202 (4), 219 (5), 220 (1), 235 (1), 296 (1), 299 (100), 300 (20), 301 (8), 302 (1), 483 (2), 501 (<1)
6'-OH-angolensin	a	7.01	504	103 (1), 105 (2), 133 (2), 135 (12), 147 (9), 148 (1), 193 (1), 207 (1), 251 (1), 265 (1), 281 (4), 297 (3), 298 (1), 337 (1), 339 (1), 353 (2), 354 (1), 369 (100), 370 (30), 371 (14), 372 (3), 373 (1), 416 (1), 459 (1), 474 (1), 489 (5), 490 (2), 504 (<1)
	b	6.72	531	105 (2), 135 (6), 142 (2), 161 (1), 162 (5), 163 (1), 202 (1), 216 (1), 268 (1), 296 (1), 316 (2), 317 (1), 360 (1), 376 (2), 393 (2), 396 (100), 397 (30), 398 (15), 399 (2), 483 (1), 498 (1), 513 (5), 514 (2), 531 (<1)
5'-OMe-*O*-dma[1]	a	8.20	504	130 (1), 147 (1), 149 (1), 180 (1), 193 (6), 194 (1), 209 (10), 210 (1), 211 (1), 223 (3), 224 (1), 237 (2), 238 (2), 239 (2), 257 (2), 279 (1), 281 (24), 282 (4), 311 (100), 312 (21), 313 (7), 385 (2), 400 (5), 401 (1), 489 (4), 490 (1), 504 (<1)
	b	7.98	531	202 (7), 216 (2), 296 (25), 299 (30), 329 (100), 330 (20), 331 (9), 332 (1), 382 (4), 513 (2), 531 (<1)
5'-OH-*O*-dma[1]	a	9.18	562	133 (2), 147 (4), 163 (1), 177 (2), 179 (2), 191 (1), 193 (8), 194 (1), 281 (8), 282 (2), 369 (100), 370 (29), 371 (14), 372 (3), 457 (1), 547 (2), 562 (1)
	b	8.97	598	142 (1), 162 (3), 188 (2), 202 (6), 296 (8), 297 (2), 393 (2), 396 (100), 397 (27), 398 (3), 399 (3), 580 (1), 598 (1)

[1]*Authentic reference compound not available.*

Table 4. *(Continued)*

Compound		t_R (min)	$M^{+\bullet}$	Ion (abundance)
6'-OH-*O*-dma	a	7.59	562	133 (3), 147 (8), 148 (1), 149 (3), 163 (2), 177 (2), 191 (1), 193 (7), 207 (3), 251 (1), 265 (1), 281 (2), 297 (3), 337 (1), 339 (2), 353 (3), 369 (100), 370 (29), 371 (15), 372 (3), 459 (1), 547 (3), 548 (2), 562 (<1)
	b	7.33	598	142 (2), 161 (2), 162 (7), 163 (1), 169 (1), 201 (2), 202 (6), 296 (1), 316 (2), 376 (2), 393 (2), 395 (4), 396 (100), 397 (26), 398 (12), 399 (3), 580 (2), 581 (1), 598 (<1)
3''-OH-*O*-dma[1]	a	9.32	562	117 (1), 133 (2), 147 (4), 149 (1), 193 (4), 207 (2), 209 (7), 221 (1), 223 (3), 251 (1), 281 (100), 282 (22), 283 (9), 284 (1), 355 (1), 369 (1), 547 (1), 562 (<1)
	b	9.05	598	142 (1), 162 (2), 199 (3), 219 (5), 232 (2), 296 (2), 299 (100), 300 (21), 301 (8), 302 (1), 580 (1), 598 (<1)

[1]*Authentic reference compound not available.*