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Lignan and Isoflavonoid Conjugates in Human Urine

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Lignans and isoflavonoids are two groups of diphenolic phytoestrogens of plant origin which have gained increasing interest because of their possible cancer protective properties. High excretion of these compounds occur in populations at low risk of breast, prostate and colon cancer consuming either high amounts of whole-grain (lignans and some isoflavonoids) or soy products (isoflavonoids and some lignans). We determined the pattern of conjugation of the phytoestrogens in four urine samples from vegetarian or semivegetarian women and in two samples from men. Seven compounds were investigated: enterodiol, enterolactone, matairesinol, daidzein, equol, genistein and *O*-desmethylangolensin. The fractions quantified are the free fraction, mono- and disulfate, as well as the mono-, di- and sulfoglucuronide fractions. For the fractionation and purification we used ion-exchange chromatography and the determination of the concentrations of each compound in all fractions was done by isotope dilution gas chromatography–mass spectrometry (GLC–MS) using deuterated internal standards of all diphenols. More than 60% of all compounds determined, occurred in the monoglucuronide fraction. Daidzein, enterodiol and equol are excreted to a relatively high extent as sulfoglucuronides and genistein as diglucuronide. We conclude that the general pattern of lignan and isoflavonoid conjugates in urine is similar to that of endogenous estrogens.

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INTRODUCTION

Recently there has been a growing interest in the possible cancer protective properties of mammalian lignans and isoflavonoid phytoestrogens [1–30]. These two groups of diphenols are found in high concentrations in all human biological materials studied so far, including blood, urine, bile and faeces. They occur in high amounts, usually in the form of glycosides, in hundreds of different edible plants but particularly in soybeans, some other beans, whole grain products, and seeds [5, 31, 32].

Until now approximately 15 lignans and isoflavonoids have been identified in human urine [3, 5, 31]. Nowadays even low concentrations of these compounds can be determined by GLC–MS in the selected ion monitoring mode. In this way it is possible to determine a complex profile of these compounds, including three lignans and four isoflavonoids in human urine [33]. In a recent study from this laboratory, the complete profile of seven compounds was also determined in plasma [34], including a separation of the compounds into the "biologically active" free and sulfate fractions and the "inactive" glucuronide and sulfoglucuronide fractions.

However, so far, little attention has been paid to the conjugation of lignans and isoflavonoids, except for the work concerning plasma [34]. In one publication [35] the authors concluded that enterolactone and enterodiol in urine occur mainly as glucuronides.

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Abbreviations: GCL–MS, Gas Chromatography–Mass spectrometry; DEAE-Ac$^-$, DEAE-Sephadex in acetate form; QAE-Ac$^-$, QAE-Sephadex in acetate form; QAE-Carb , QAE-Sephadex in carbonate form.

Previously, quantitative methods for the multicomponent analysis of estrogens in urine were developed in this laboratory [36, 37]. One of the methods [37] includes the fractionation and quantitation of the main estrogen conjugates. With slight modifications, this method can be used for the phytoestrogenic diphenols, fractionating every single compound into six different fractions (free, mono- and disulfate, mono-, di- and sulfoglucuronide fractions).

In this study we present the method for the determination of the phytoestrogen conjugate profile, and the pattern of conjugation in six human urine samples for three lignans (enterodiol, enterolactone, matairesinol) and for four isoflavonoids (daidzein, equol, genistein, *O*-desmethylangolensin).

MATERIALS AND METHODS

Samples

Four urine samples were collected from women and two from men. The men consumed an ordinary Finnish diet. Two samples were obtained from a woman who during the first sample collection period consumed soy products, and two samples were obtained from young vegetarian women because of expected higher phytoestrogen concentrations and in order to be able to also determine minor metabolites.

Collection of urine

The daily urines were collected in bottles containing ascorbic acid (1–2 g), and 0.1% sodium azide was added immediately after collection to minimize possible bacterial degradation. The samples were stored at −20°C until analysed.

Chemicals and standards

All chemicals used in this study have been described in detail previously [33, 34, 36, 37]. All the glassware including Pasteur pipettes and the glass liner of the injection system of the GLC–MS instrument was silanized by either Aqua-Sil (Pierce Chemical Co., Rockford, IL, U.S.A.) or dimethyldichlorsilane 2% in toluene to avoid creepage of active substances. Details of the synthesis of the standards and deuterium labelled compounds have also been described previously (or appropriate references given) [33, 38].

Anion exchange materials

DEAE- and QAE-Sephadex A-25 powder was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Before conversion to the appropriate form, 50 g of powder is washed successively with 1 l 20%, 50%, and absolute ethanol, respectively. Conversion to the acetate form is carried out by washing the chloride form with 10 bed volumes of 0.1 M NaOH in 70% methanol, 70% methanol (after washing the pH should be around 7.0), 0.5 M acetic acid in 70% methanol, and 70% methanol, respectively. The DEAE-Sephadex in acetate form (DEAE-Ac^-) is suspended in 70% MeOH and the QAE-Sephadex in acetate form (QAE-Ac^-) in MeOH (100%), both stored at 4°C. The carbonate form of QAE-Sephadex (QAE-$Carb^-$) should be prepared just before use because it decomposes rapidly. It is converted from the acetate form by washing with 10 bed volumes of 0.2 M $NaHCO_3$ in 40% methanol, 40% methanol, and absolute methanol, respectively.

Gas chromatography–mass spectrometry (GLC–MS)

The GLC–MS analysis was carried out using HP 5995 quadrupole instrument equipped with 0.2 mm × 12.5 m bonded phase BP-1 (SGE) capillary vitreous silica column, directly connected to the ionizing chamber. Helium was used as the carrier gas. The temperatures of the transfer line, ion source and analyser were 310, 250 and 250°C, respectively. The ionization energy was 70 eV. For each compound quantified, the corresponding deuterated compound was used as the internal standard. Other conditions, including the ions used for selected ion monitoring, have been described previously [33].

Procedure for analysis of phytoestrogen conjugates in urine

The whole procedure is shown in a flow-diagram (Fig. 1).

Extraction. A Sep-Pak C_{18} cartridge is used for the extraction. In order to prevent the loss of double conjugates, the pH of the sample is adjusted to 3.0 by adding 0.1 of sample volume of 1.5 M acetate buffer (pH 3) prior to the application to a primed Sep-Pak C_{18} cartridge. The cartridge is washed with 5 ml of 0.15 M acetate buffer (pH 3) and eluted with 3 ml of methanol.

Chromatography on DEAE-Ac^-. The multicomponent analysis of phytoestrogen conjugates in urine is carried out using a column of DEAE-Ac^- (2.5 cm × 0.5 cm in Pasteur pipettes) in 70% MeOH. After addition of 1.2 ml of distilled water to the methanol eluate, the sample is applied to the column. It is eluted with 7 ml of absolute methanol (free fraction), 10 ml of 0.2 M acetic acid (weak organic acid fraction which may be analysed in the same way as the free fraction to exclude loss of diphenols in this fraction), 15 ml of 0.4 M formic acid in 70% methanol (monoglucuronides), 15 ml of 0.4 M formic acid and 0.25 M KOH in 70% methanol (monosulfates), and 15 ml of 0.1 M formic acid and 0.3 M LiCl in 70% methanol (double conjugates). The free and monoglucuronide fractions are evaporated to dryness under nitrogen and to these fractions the deuterated internal standards are added. The monosulfate and double conjugate fractions are evaporated until only water is left (approx. 3 ml), the volumes are supplemented to 10 ml with distilled water, the pH adjusted to 3.0 by adding 0.1 of sample volume of 1.5 M acetate buffer (pH 3). The fractions are applied to primed Sep-Pak

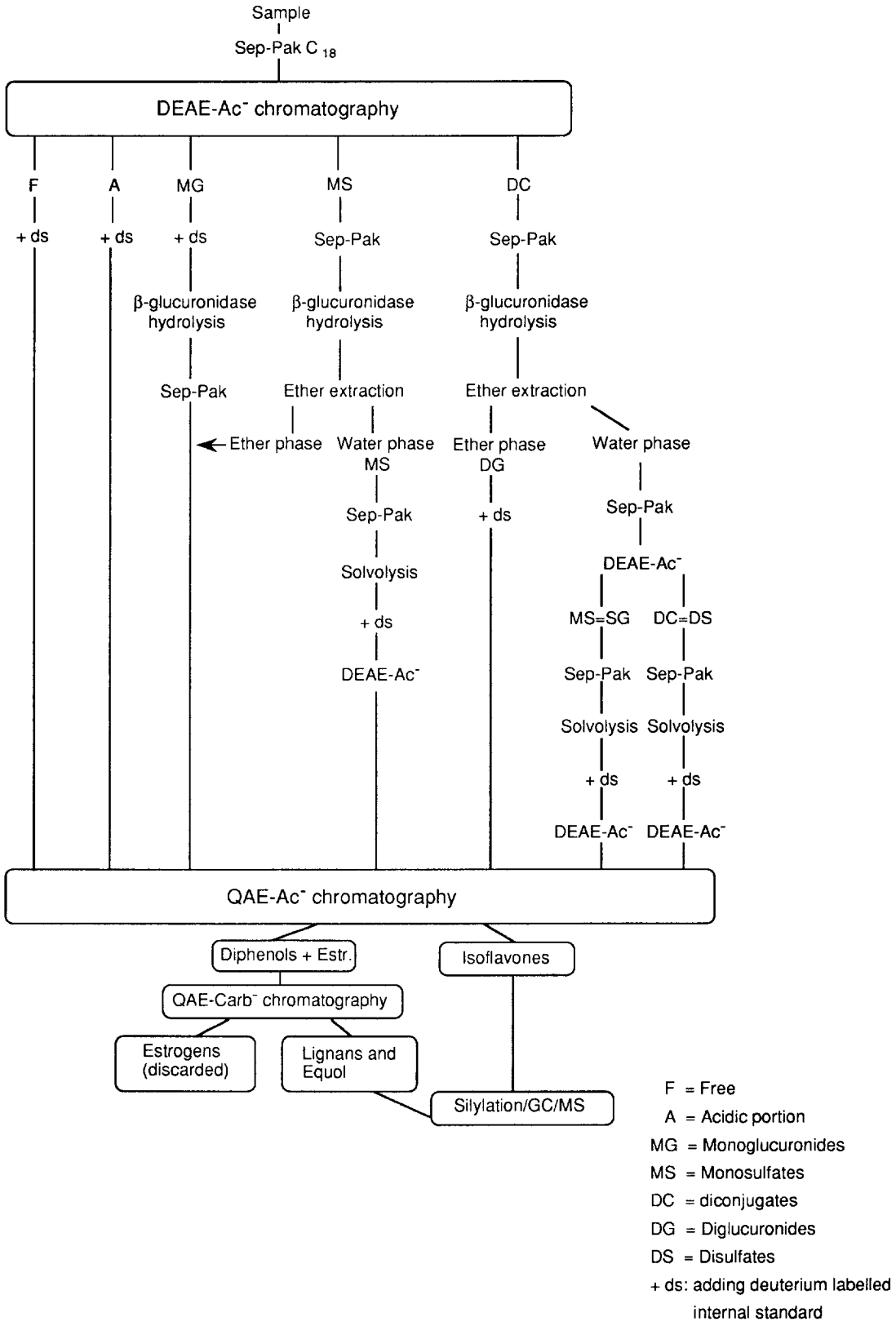

Fig. 1. Flow-diagram of method for the determination of lignan and isoflavonoid conjugates in urine.

C_{18} cartridges, washed with 5 ml of 0.15 M acetate buffer (pH 3), and eluted with 3 ml of methanol. Thereafter, the samples are evaporated to dryness under nitrogen.

Hydrolysis. Monoglucuronide, monosulfate and double conjugate fractions (dry samples) are dissolved in 1 ml of 0.15 M acetate buffer (pH 5) containing 100 μl (20 U) of β-glucuronidase solution from *E. coli*

(Boehringer Mannheim) and incubated at 37°C for 1 h. After the addition of 9 ml of distilled water the monoglucuronide fraction is applied to a primed Sep-Pak C_{18} cartridge, washed with 5 ml of 0.15 M acetate buffer (pH 3) and eluted with 3 ml of methanol. This fraction contains the hydrolysed monoglucuronides. For monosulfate and double conjugate fractions, ether extraction is carried out. The ether extract of the monosulfate fraction is combined with the monoglucuronide fraction. The water phases containing the monosulfates and the rest of the double conjugates are supplemented to 10 ml by the addition of 9 ml of distilled water, pH adjusted to 3.0 by adding 0.1 sample volume of acetate buffer 1.5 M (pH 3), and applied to a primed Sep-Pak C_{18} cartridge, which is washed with 5 ml of 0.15 M acetate buffer (pH 3) and eluted with 3 ml of methanol.

Chromatography on DEAE-Ac$^-$. To the methanol eluate of the double conjugate fraction 1.2 ml distilled water is added, and this fraction is applied to a DEAE-Ac$^-$ column. The column is eluted as described above, but the first three fractions are discarded. Fraction 4 contains the sulfoglucuronides (low monosulfates) and fraction 5 the disulfates. Both fractions are handled as described above.

Solvolysis. After evaporation of the methanol eluates from the Sep-Pak C_{18} under nitrogen, the monosulfate, sulfoglucuronide and disulfate fractions are solvolysed by the addition of 300 μl of dimethylformamide, 5 μl of 6 M HCl and 3 ml of dichloromethane and incubated at 37°C overnight. After incubation, 300 μl of distilled water is added to the samples and evaporated under nitrogen until only water remains ($\approx$300 μl). Then 700 μl of methanol is added, and after receiving the deuterated internal standard the samples are subjected to anion exchange chromatography of DEAE-Ac$^-$ and the free fraction is collected [37].

Chromatography on QAE-Ac$^-$. The combined fraction, containing the monoglucuronides, and the diglucuronide fraction are evaporated to dryness under nitrogen, and the deuterated internal standards are added to the diglucuronide fraction. The free forms of all metabolites, in 0.5 ml of methanol, are applied to QAE-Ac$^-$ (5 cm × 0.5 cm in Pasteur pipettes) in methanol, to separate the lignans enterodiol, enterolactone and matairesinol, all estrogens and the isoflavonoid equol from the isoflavonoids, daidzein, genistein and *O*-desmethylangolensin. The former compounds are eluted with 7 ml of absolute methanol, and the latter compounds with 7 ml of 0.2 M acetic acid in methanol. The samples are then dried under nitrogen. The isoflavonoids are stored in 0.5 ml of methanol at −20°C until silylated.

Chromatography on QAE-Carb$^-$. To separate the lignans and equol from estrogens we used freshly prepared QAE-Carb$^-$, (4 cm × 0.5 cm in Pasteur pipettes) in methanol, as previously described [33]. The samples are stored in 0.5 ml of methanol at −20°C until silylated.

Silylation. The trimethylsilyl derivatives of the compounds are formed by adding 100 μl of pyridine–hexamethyldisilazane–trimethylchlorosilane solution (9:3:1, v/v) to the dry samples and incubation at room temperature for 30 min. Then the samples are evaporated in a sand bath, dissolved in 400 μl of n-hexane and 10 μl of Silyl 8^{TM} column conditioner (Pierce Chemical Co., Rockford, IL, U.S.A.) and analysed by GCL–MS.

RESULTS AND DISCUSSION

The methodology is based on two carefully evaluated methods for estrogens [36, 37]. Because no conjugated lignans or isoflavonoid standards are available no recovery experiments can be carried out. The losses in the first steps including hydrolysis cannot be corrected for. However, methodological studies showed that the hydrolysis is quantitative and the losses during the first steps are about 5–15% as judged from studies with radioactive estrogen conjugates [37]. Lignans and isoflavonoids are much more stable than many of the estrogens and after hydrolysis the deuterated internal standards correct for all losses. The sensitivity is good. The lowest point of the standard curves (50–246 pg, depending on compound) shows a signal-to-noise ratio of more than 20 to 1 for all compounds. The amount of urine may be increased to increase sensitivity, if necessary.

The total excretion (nmol/24 h) of the seven compounds in the different fractions are shown for all subjects in Tables 1 and 2, and the percentage distribution of the various fractions in Table 3. Two samples (Urines no. 1 and 2, Table 1) were obtained from a woman who consumed soy products at the time of the first sample collection (Urine no. 1). Consequently, the total amounts of diphenols were very different in the two samples (13–58 μmol/24 h) from this subject.

The principal conjugate of all compounds is the monoglucuronide, approx. 85–90% of total diphenols is excreted as monoglucuronides. The three lignans (enterodiol, enterolactone, matairesinol) show quite a similar pattern. They are excreted mainly as monoglucuronides (73–94%), and a small part occurs as monosulfates (2–10%). Hardly any (0.3–1%) free lignans are found, nor diconjugates, except for urine no. 3 in which 14% of enterodiol occurs in the sulfoglucuronide fraction.

For the isoflavonoids the pattern is somewhat different for the four compounds. *O*-desmethylangolensin occurs as monoglucuronide (97%) and the rest is divided over the other fractions. Equol is found as the monoglucuronide (32–93%), 0–43% as the sulfoglucuronide, 0–15% as the monosulfate and 0–10% as the disulfate. Daidzein occurs as monoglucuronide (79–82%) and 6–17% as sulfoglucuronide. The rest is

Table 1. Lignan and isoflavonoid conjugates in four female urine samples

	Matairesinol (nmol/24 h)	Enterodiol (nmol/24 h)	Enterolactone (nmol/24 h)	Daidzein (nmol/24 h)	Genistein (nmol/24 h)	*O*-Desmethylangolensin (nmol/24 h)	Equol (nmol/24 h)
Urine no. 1							
Free	0	115	152	812	239	26.6	0
Monoglucuronides	44.6	23550	7530	14500	2970	1704	79.5
Diglucuronides	0	52.0	119	238	867	11.1	0
Sulfoglucuronides	0	621	142	1121	116	4.2	5.9
Monosulfates	8.1	1250	279	340	45.4	7.9	0
Disulfates	0	38.5	120	615	395	3.8	0
Total	52.7	25627	8342	17626	4632	1758	85.4
Urine no. 2							
Free	0	7.5	24.4	168	73.5	7.7	1.4
Monoglucuronides	51.8	1005	4930	3990	1154	646	58.6
Diglucuronides	0	3.4	13.3	63.4	222	0.3	0
Sulfoglucuronides	0	32.9	42.5	480	160	5.0	0
Monosulfates	0.6	33.5	98.9	96.6	90.2	2.4	9.7
Disulfates	0	3.7	29.8	80.5	86	0.9	0
Total	52.4	1086	5139	4879	1786	662	69.7
Urine no. 3							
Free	0	0	106	238.5	75.9	19.7	0
Monoglucuronides	11.4	187	6400	18290	10210	2360	70.4
Diglucuronides	0	0	79.8	305	2190	1.8	0
Sulfoglucuronides	0	35.7	184	2840	557	21.4	93.3
Monosulfates	0	24.8	174	1190	148	19.8	32.4
Disulfates	0	1.9	20.4	206	338	5.7	21.1
Total	11.4	249	6964	23070	13519	2428	217
Urine no. 4							
Free	0.4	0	0	229	36.2	13.2	0
Monoglucuronides	33.9	243	4310	9210	3160	998	125
Diglucuronides	0	0	0	150	1560	1.7	0
Sulfoglucuronides	1.0	51.2	5.2	1430	629	7.2	86.0
Monosulfates	3.5	14.6	56.5	221	184	6.3	23.1
Disulfates	0	0	2.8	204	396	5.0	16.7
Total	38.8	309	4375	11444	5965	1031	251

distributed over the other fractions, with a relatively high amount in the free fraction (1–5%).

A different pattern is shown for genistein, of which only 53–76% is in the monoglucuronide fraction. Almost a quarter (12–26%) is found in the diglucuronide, 2–15% in the sulfoglucuronide and 1–4% in the disulfate fractions. This difference is probably due to the fact that genistein has three free hydroxyl groups and is therefore more likely to form diconjugates than the other compounds.

Table 2. Lignan and isoflavonoid conjugates in urine of two men

	Matairesinol (nmol/24 h)	Enterodiol (nmol/24 h)	Enterolactone (nmol/24 h)	Daidzein (nmol/24 h)	Genistein (nmol/24 h)	*O*-Desmethylangolensin (nmol/24 h)	Equol (nmol/24 h)
Urine no. 5							
Free	0	4.0	13.9	55.1	22.4	0	6.1
Monoglucuronides	256	328	4771	3614	1519	86.4	402
Diglucuronides	0	0	0	24.5	277	0.3	5.8
Sulfoglucuronides	0	30.7	124	490	203	0.6	52.2
Monosulfates	13.2	49.5	284	182	80.9	0.6	18.6
Disulfates	0	4.8	146	14.8	70.9	0.2	0
Total	269	417	5339	4380	2173	88.1	485
Urine no. 6							
Free	0	0	32.2	70.4	12.6	10.9	0
Monoglucuronides	228	627	1168	3295	873	1261	65.5
Diglucuronides	0	—	34.8	24.9	187	3.7	5.2
Sulfoglucuronides	0	243	59.9	712	203	12.5	21.4
Monosulfates	9.6	68.3	58.0	96.4	30.9	10.0	12.9
Disulfates	0	1.3	78.1	25.8	57.6	0	4.8
Total	238	940	1431	4225	1364	1298	110

Table 3. Mean percentage distribution of lignan and isoflavonoid conjugates in five female and two male urine samples

	Matairesinol (% ± SD)	Enterodiol (% ± SD)	Enterolactone % ± SD)	Daidzein (% ± SD)	Genistein (% ± SD)	*O*-Desmethylangolensin (% ± SD)	Equol (% ± SD)
Women							
Free	0.3 ± 0.5	0.3 ± 0.4	1.0 ± 0.8	2.8 ± 1.6	2.6 ± 2.3	1.2 ± 0.3	0.3 ± 0.7
Monoglucuronides	92.7 ± 7.9	84.5 ± 9.0	94.0 ± 3.7	81.0 ± 1.3	64.2 ± 9.2	97.2 ± 0.4	66.3 ± 30.0
Diglucuronides	0	0.1 ± 0.2	0.7 ± 0.7	1.3 ± 0.1	18.8 ± 5.3	0.3 ± 0.20	0
Sulfoglucuronides	0.7 ± 1.3	9.1 ± 7.4	1.4 ± 1.1	10.3 ± 2.8	6.4 ± 3.7	0.7 ± 0.3	21.1 ± 20.8
Monosulfates	6.4 ± 7.2	5.7 ± 2.9	2.3 ± 0.8	2.8 ± 1.6	2.5 ± 1.9	0.6 ± 0.2	8.3 ± 6.2
Disulfates	0	0.4 ± 0.3	0.6 ± 0.6	2.0 ± 1.1	5.7 ± 2.5	0.3 ± 0.2	4.1 ± 4.9
Men							
Free	0	0.5 ± 0.7	1.3 ± 1.4	1.5 ± 0.3	0.9 ± 0	0.4 ± 0.6	0.6 ± 0.9
Monoglucuronides	95.5 ± 0.6	72.7 ± 8.4	85.5 ± 5.4	80.3 ± 3.2	62.5 ± 2.1	97.6 ± 0	71.3 ± 16.5
Diglucuronides	0 ± 0	0 ± 0	1.2 ± 1.7	0.6 ± 0	12.5 ± 1.9	0.3 ± 0	3.0 ± 2.5
Sulfoglucuronides	2.1 ± 2.9	16.6 ± 13.1	3.3 ± 1.3	14.0 ± 4.0	11.5 ± 4.7	0.9 ± 0.2	15.1 ± 6.2
Monosulfates	2.5 ± 3.5	9.6 ± 3.3	4.7 ± 0.9	3.2 ± 1.3	2.8 ± 0.7	0.7 ± 0.1	7.8 ± 5.6
Disulfates	0	0.6 ± 0.7	4.1 ± 1.9	0.5 ± 0.2	3.5 ± 1.0	0.1 ± 0	2.2 ± 3.1

The acidic fraction is not mentioned in Table 1. It is used to elute organic acids (e.g. bile acids). The fraction was analysed to see if there is any loss of diphenols, but none was found (0–1%). The ether phase obtained after ether extraction of the monosulfate fraction containing possibly co-eluted monoglucuronides in the monosulfate fraction was analysed separately: For the most of the compounds this amount was very low (0–1%). However, 1.1–5.5% of daidzein and 3.5–16.5% of genistein were found in this fraction. The explanation for this phenomenon is that these two compounds have a higher polarity and bind more tightly to the column. Therefore, the ether extract was combined with the monoglucuronide fraction.

The results found in this study for the conjugation of enterodiol and enterolactone agree with an earlier study [35] in which the two compounds were fractionated into only four different fractions (free, glucuronide, monosulfate and disulfate), and finding 92% of enterodiol and 98% of enterolactone in the glucuronide fraction. We found 84.6% (84.5% as monoglucuronide and 0.1% as diglucuronide) of enterodiol and 95% (94% as monoglucornide and 1% as diglucuronide) of enterolactone in the glucuronide fractions.

The pattern of conjugation is similar to that found for endogenous estrogens [37]. For genistein with three hydroxyl groups the pattern is similar to that of estrogens with the same number of hydroxyl groups.

The extensive amount of work needed to carry out one analysis prohibits the use of the method for larger studies. However, for specific analytical problems, e.g. in cell cultures, the method or a part of it may well be used to shed light on the pattern of phytoestrogen conjugation [19, 39].

Information regarding the mode of conjugation is important in the understanding of the biological effects of phytoestrogens. Our studies in plasma show that the free and monosulfate fractions form a very high proportion of plasma phytoestrogens, particularly with regard to lignans (up to 20% of total) [34]. In Japanese male subjects, the proportion of free and monosulfate fractions for enterolactone is even higher (45%, mean of 14 subjects), despite the fact that the urinary excretion of enterolactone in these subjects is lower than in Finnish men (unpublished observation). In order to understand these differences it would be important to determine the complete conjugation pattern in several other body fluids such as saliva and bile, and also in faeces.

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REFERENCES

1. Adlercreutz H., Fotsis T., Heikkinen R., Dwyer J. T., Woods M., Goldin B. R. and Gorbach S. L.: Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian women and in women with breast cancer. *Lancet* 2 (1982) 1295–1299.
2. Adlercreutz H.: Does fiber-rich food containing animal lignan precursors protect against both colon and breast cancer? An extension of the "fiber hypothesis". *Gastroenterology* **86** (1984) 761–764.
3. Adlercreutz H., Fotsis T., Bannwart C., Wähälä K., Mäkelä T., Brunow G. and Hase T.: Determination of urinary lignans and phytoestrogen metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. *J. Steroid Biochem.* **25** (1986) 791–797.
4. Adlercreutz H.: Lignans and phytoestrogens. Possible preventive role in cancer. In *Progress in Diet and Nutrition* (Edited by Horwitz C. and Rozen P.). S. Karger, Basel (1988) pp. 165–176.
5. Adlercreutz H.: Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. *Scand. J. Clin. Lab. Invest.* **50**, Suppl 201 (1990) 3–23.
6. Adlercreutz H., Honjo H., Higashi A., Fotsis T., Hämäläinen E., Hasegawa T. and Okada H.: Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming traditional Japanese diet. *Am. J. Clin. Nutr.* **54** (1991) 1093–1100.
7. Adlercreutz H., Hämäläinen E., Gorbach S. and Goldin B.: Dietary phyto-oestrogens and the menopause in Japan. *Lancet* **339** (1992) 1233.
8. Adlercreutz H., Markkanen H. and Watanabe S.: Plasma concentrations of phyto-oestrogens in Japanese men. *Lancet* **342** (1993) 1209–1210.

9. Akiyama T., Ishida J., Nakagawa S., Ogawara H., Watanabe S.-I., Itoh N., Shibuya M. and Fukami Y.: Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **262** (1987) 5592–5595.
10. Arakaki N., Hirono S., Kawakami S., Tsubouchi H., Ishii T., Hara H. and Daikuhara Y.: Effects of protein kinase inhibitors on the mitogenic activity of human hepatocyte growth factor on rat hepatocytes in primary culture. *Biochem. Biophys. Res. Commun.* **185** (1992) 22–28.
11. Fotsis T., Pepper M., Adlercreutz H., Fleischmann G., Hase T., Montesano R. and Schweigerer L.: Genistein, a dietary-derived inhibitor of invitro angiogenesis. *Proc. Natn. Acad. Sci. U.S.A.* **90** (1993) 2690–2694.
12. Honma Y., Okabe-Kado J., Kasukabe T., Hozumi M. and Umezawa K.: Inhibition of *abl* oncogene tyrosine kinase induces erythroid differentiation of human myelogenous leukemia K562 cells. *Jpn. J. Cancer Res.* **81** (1990) 1132–1136.
13. Kondo K., Tsuneizumi K., Watanabe T. and Oishi M.: Induction of *in vitro* differentiation of mouse embryonal carcinoma (F9) cells by inhibitors of topoisomerases. *Cancer Res.* **51** (1991) 5398–5404.
14. Linassier C., Pierre M., Le Pecq J.-B. and Pierre J.: Mechanisms of action in NIH-3T3 cells of genistein, an inhibitor of EGF receptor tyrosine kinase activity. *Biochem. Pharmac.* **39** (1990) 187–193.
15. Makishima M., Honma Y., Hozumi M., Sampi K., Hattori M., Umezawa K. and Motoyoshi K.: Effects of inhibitors of protein tyrosine kinase activity and/or phosphatidylinositol turnover on differentiation of some human myelomonocytic leukemia cells. *Leukemia Res.* 15 (1991) 701.
16. Markovits J., Linassier C., Fossé P., Couprie J., Pierre J., Jacquemin-Sablon A., Saucier J.-M., Le Pecq J.-B. and Larsen A. K.: Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. *Cancer Res.* **49** (1989) 5111–5117.
17. Matsukawa Y., Marui N., Sakai T., Satomi Y., Yoshida M., Matsumoto K., Nishino H. and Aoike A.: Genistein arrests cell cycle progression at G(2)-M. *Cancer Res.* **53** (1993) 1328–1331.
18. Messina M. and Barnes S.: The role of soy products in reducing risk of cancer. *J. Natn. Cancer Inst.* **83** (1991) 541–546.
19. Mousavi Y. and Adlercreutz H.: Enterolactone and estradiol inhibit each other's proliferative effect on MCF-7 breast cancer cells in culture. *J. Steroid Biochem. Molec. Biol.* 41 (1992) 615–619.
20. Mousavi Y. and Adlercreutz H.: Genistein is an effective stimulator of SHBG production in Hep-G2 human liver cancer cells and suppresses proliferation of these cells in culture. *Steroids* 58 (1993) 301–304.
21. Ogawara H., Akiyama T., Watanabe S.-I., Ito N., Kobori M. and Seoda Y.: Inhibition of tyrosine protein kinase activity by synthetic isoflavones and flavones. *J. Antibiot.* **XLII** (1989) 340–343.
22. Okura A., Arakawa H., Oka H., Yoshinari T. and Monden Y.: Effect of genistein on topoisomerase activity and on the growth of [VAL 12]Ha-*ras*-transformed NIH 3T3 cells. *Biochem. Biophys. Res. Comm.* **157** (1988) 183–189.
23. Peterson G. and Barnes S.: Genistein inhibition of the growth of human breast cancer cells—independence from estrogen receptors and the multi-drug resistance gene. *Biochem. Biophys. Res. Comm.* **179** (1991) 661–667.
24. Peterson G. and Barnes S.: Genistein and Biochanin-A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate* 22 (1993) 335–345.
25. Schweigerer L., Christeleit K., Fleischmann G., Adlercreutz H., Wähälä K., Hase T., Schwab M., Ludwig R. and Fotsis T.: Identification in human urine of a natural growth inhibitor for cells derived from solid paediatric tumours. *Eur. J. Clin. Invest.* 22 (1992) 260–264.
26. Takeda Y., Nishio K., Morikage T., Kubota N., Kojima A., Kubo S., Fujiwara Y., Niitani H. and Saijo N.: Reversal of multidrug resistance by genistein in non-P-glycoprotein mediated multidrug-resistant cell line (K562/TPA). *Proc. Ann. Meet. Am. Assoc. Cancer Res.* **33** (1992) 476.
27. Thompson L. U. and Serraino M. R.: Protective effect of flaxseed as suggested by risk markers for mammary carcinogenesis. *FASEB J.* 5 (1991) A928 no. 3242.
28. Thompson L. U. and Serraino M. R.: Protective effect of flaxseed as suggested by risk markers for mammary carcinogenesis. *FASEB J.* 5 (1991) A928 no. 3242.
29. Traganos F., Ardelt B., Halko N., Bruno S. and Darzynkiewicz Z.: Effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. *Cancer Res.* 52 (1992) 6200–6208.
30. Versantvoort C. H. M., Schuurhuis G. J., Pinedo H. M., Eekman C. A., Kuiper C. M., Lankelma J. and Broxterman H. J.: Genistein modulates the decreased drug accumulation in Non-P-Glycoprotein mediated multidrug resistant tumour cells. *Br. J. Cancer* **68** (1993) 939–946.
31. Setchell K. D. R. and Adlercreutz H.: Mammalian lignans and phyto-oestrogens. Recent studies on their formation, metabolism and biological role in health and disease. In *Role of the Gut Flora in Toxicity and Cancer* (Edited by Rowland I.). Academic Press, London (1988) pp. 315–345.
32. Thompson L. U., Robb P., Serraino M. and Cheung F.: Mammalian lignan production from various foods. *Nutr. Cancer* **16** (1991) 43–52.
33. Adlercreutz H., Fotsis T., Bannwart C., Wähälä K., Brunow G. and Hase T.: Isotope dilution gas chromatographic–mass spectrometric method for the determination of lignans and isoflavonoids in human urine, including identification of genistein. *Clin. Chim. Acta* **199**, (1991) 263–278.
34. Adlercreutz H., Fotsis T., Lampe J., Wähälä K., Mäkelä T., Brunow G. and Hase T.: Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas-chromatography mass-spectrometry. *Scand. J. Clin. Lab. Invest.* 53 (1993) 5–18.
35. Axelson M. and Setchell K. D. R.: Conjugation of lignans in human urine. *FEBS Lett.* **122** (1980) 49–53.
36. Fotsis T. and Adlercreutz H.: The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS-I. Quantitation of estrogens after initial hydrolysis of conjugates. *J. Steroid Biochem.* 28 (1987) 203–213.
37. Fotsis T.: The multicomponent analysis of estrogens in urine by ion exchange chromatography and GC-MS-II. Fractionation and quantitation of the main groups of estrogen conjugates. *J. Steroid Biochem.* 28 (1987) 215–226.
38. Wähälä K., Brunow G., Hase T., Bannwart C. and Adlercreutz H.: Synthesis of deuterium labelled ethoxyamine for derivatization of estrogens as stable-isotope internal standards. *Finn. Chem. Lett.* 14 (1987) 198–201.
39. Adlercreutz H., Mousavi Y., Clark J., Höckerstedt K., Hämäläinen E., Wähälä K., Mäkelä T. and Hase T.: Dietary phytoestrogens and cancer: *in vitro* and *in vivo* studies. *J. Steroid Biochem. Molec. Biol.* 41 (1992) 331–337.