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# Metabolism of non-steroidal phytoestrogens: Ipriflavone and 5-Methyl-7-hydroxy-isoflavone

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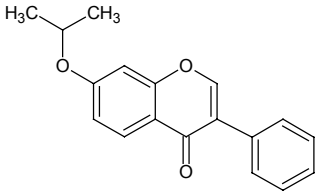
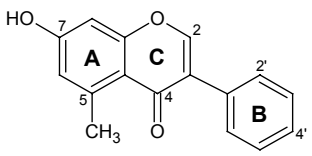
## Introduction

Isoflavones belong to a group of compounds that share a basic structure consisting of two benzyl rings joined by a three-carbon bridge, which may or may not be closed in a pyran ring. The structure is generally simplified as C6-C3-C6 [1,2]. Isoflavones are also classified as phytoestrogens due to their weak estrogenic activity in mammalian systems [3].




Ipriflavone




5-methyl-7-hydroxy-isoflavone

**Fig. 1.** The chemical structure of ipriflavone and 5-methyl-7-hydroxy-isoflavone

Recently a great deal of interest has been paid to anabolic activity of phytoestrogens. According to statements of supplement manufacturers isoflavones have potential anabolic activity, are safe and effective. Reportedly isoflavones are abused as concomitant drugs at the last stage of anabolic stacking, and their monitoring as indirect markers might be helpful in doping control routine. In this paper we investigated metabolic transformation of two isoflavones in human urine: ipriflavone and 5-methyl-7-hydroxy-isoflavone (Fig. 1).

The aim of this investigation was to examine the human urinary metabolites of ipriflavone and 5-methyl-7-hydroxy-isoflavone by LC-ESI(+)/MS Ion Trap and GC-MS following oral administration with a view to identify suitable targets for monitoring of those compounds.

## Experimental

*Instrumentation:* GC-MS analyses were carried out using GC-MS Agilent 6890N/5973inert system. Separation was performed on a HP-1 Ultra column (12 m x 0.2 mm x 0.33 μm). The GC-MS operating conditions were as follows: split mode 1:10, injector port

temp 280°C; oven temperature program: 190-234°C at 2°C/min then 234-300°C at 12°C/min with a final hold of 4.5 min. Helium was used as carrier gas at a flow-rate of 1 ml $min^{-1}$. The analyses were performed in the full-scan mode.

HPLC-MS experiments were carried out using a1100 Series LC/MSD Trap system from Agilent Technologies (Palo Alto, CA, USA) equipped with an autosampler. Chromatographic separations were performed using Ultra C-18 (100 mm x 2.1 mm x 5 µm) column (Restek Co. USA) connected to a guard column cartridge (2.1 × 12.5 mm) filled with the same packing material. Nitrogen was supplied from nitrogen generator with output pressure of 80 psi, and the ion source (nebulizer) inlet pressure was set at 30 psi. The drying gas was heated to 350°C at a flow rate of 8 litre per min. Capillary voltage was – 3500 V, skim trap drive and capillary exit were 46.4 and 104.0 V, respectively. The ion accumulation time was 300 ms, scan range 100 to 350 *m/z*.

The mobile phase was a mixture of **A**, 20 *mM* formic acid (pH = 3.0), and **B**, methanol, in a gradient elution mode. The starting mobile phase was 90 % **A** and 15 % **B**, and the linear gradient was run over 15 min to a proportion of 40 % **A** and 60 % **B** then the linear gradient was run over 25 min to a proportion of 15 % **A** and 85 % **B**. The flow rate was 0.25 ml $min^{-1}$.

*Administration:* Two healthy volunteers gave their informed consent to participate in the study. Blank urine was collected before the administration of a single oral dose of nutritional supplements: **Proxylon** (ipriflavone – 250 mg (one capsule), *Syntrax Innovations Inc.*, USA) and **AXIS HT** (5-methyl-7-hydroxy-isoflavone – 200 mg (one tablet), *Bio-Engineering Supplements and Nutrition*, USA) to 2 healthy volunteers (male, 21 years, 55 kg; male, 20 years, 60 kg). The samples urine were collected during 3 days and immediately frozen at -30°C. Collection time was: 3 hrs, 6hrs, 11 hrs, 24 hrs, 29 hrs, 35 hrs, 42 hrs, 49 hrs, 55 hrs, 63 hrs, 70 hrs.

*Sample preparation procedure:* To 5 ml of urine 30 µl of internal standard (methyl-testosterone) was added. Then 1 ml of phosphate buffer (pH = 7.4) and 30 µl of β-glucuronidase *E. Coli* were added prior to enzymatic hydrolysis. The hydrolysis process was carried out at 55°C for 1 hour. The solution was cooled to room temperature and the pH was adjusted to 9 by adding solid buffer ($Na_2CO_3$/$NaHCO_3$, 1:1 mixture). Then, *ca*. 2 g of anhydrous sodium sulfate were added, and the mixture was extracted with 5mL of diethyl ether. After shaking (2 min, Vortex) and centrifugation (5 min, 3000 rpm), the organic layer was separated and taken to dryness at 60°C. The dry dried residue was derivatized with 50 µl

of MSTFA-$NH_4I$-DTE (1000:2:4, v/w/w) for 20 min at 60°C. One µl was injected into the GC-MS system. For HPLC-MS analysis the residue was dissolved in 50 µl of methanol, and 5 µl of this solution was injected into the LC-MS ion trap system.

**Results and discussion**

The first step in the work involved the characterisation of the GC-MS and HPLC-MS mass spectra properties of parent drug. The standard solutions of ipriflavone and 5-methyl-7-hydroxy-isoflavone were analysed by HPLC-MS with positive API-ESI, and by GC/EI-MS after trimethylsilylation.

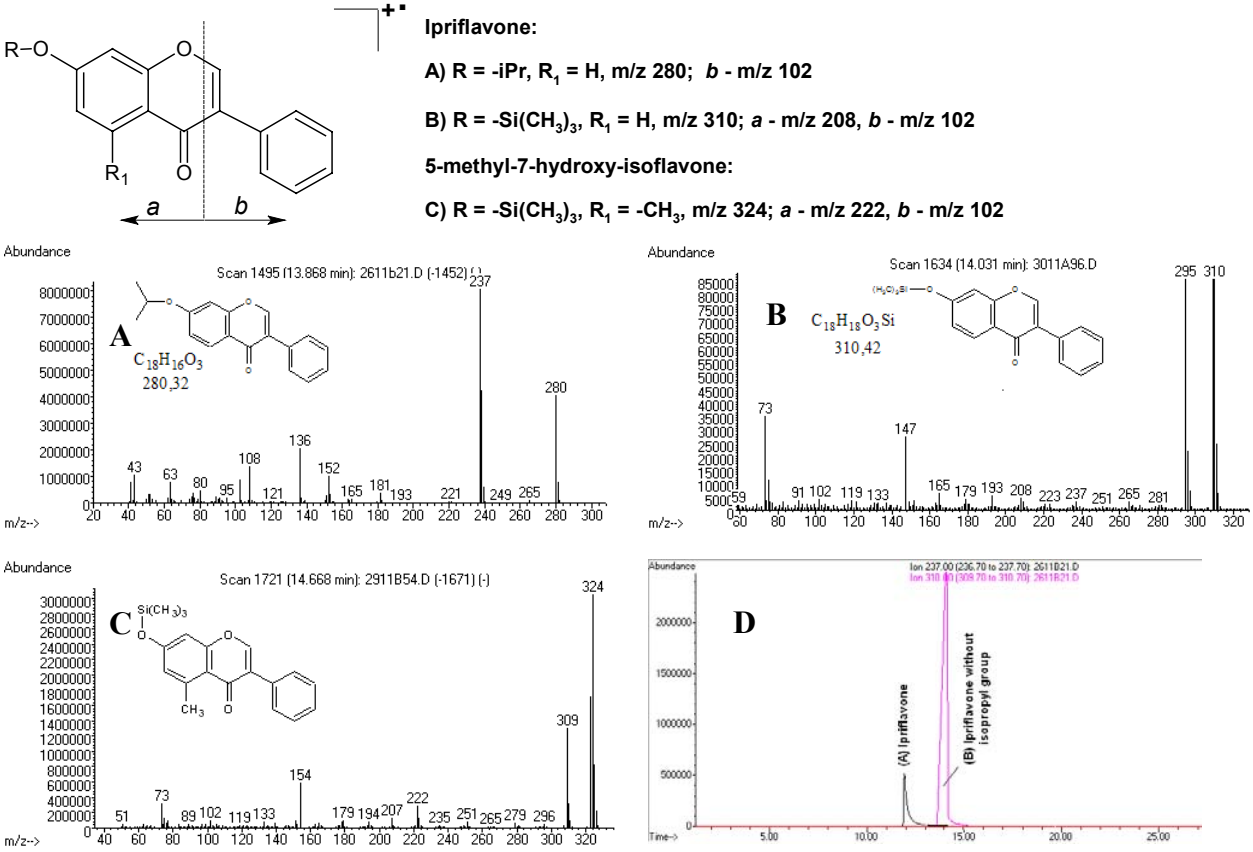



**Fig. 2.** Mass spectra of ipriflavone (**A**), ipriflavone without isopropyl group (**B**) and 5-methyl-7-hydroxy-isoflavone (**C**). Ion chromatograms of *m/z* 237 (Ipriflavone), *m/z* 310 (ipriflavone without isopropyl group). GC-MS method.

GC-MS analysis of the TMS derivative of ipriflavone shows two chromatography peaks with molecular ions at *m/z* 280 (trace) and *m/z* 310 (main peak) (Fig. 2D). Ipriflavone does not contain any center for TMS derivatisation, and the base ion at *m/z* 237 results from the loss of an isopropyl group (Fig. 2A). Figure 2 shows the EI mass spectra of ipriflavone (A), ipriflavone without isopropyl group (B), where the loss occurs during the derivatisation, and 5-methyl-7-hydroxy-isoflavone (C) after derivatisation with MSTFA/$NH_4I$/DTE.

Important fragments are formed via cleavage of C-ring with formation of two diagnostic fragments: *a* and *b*.

The HPLC-API/ESI mass spectrum displays single peak of ipriflavone with protonated molecular ion $[M+H]^+$ at *m/z* 281. Fragmentation of protonated ipriflavone in the ion trap leads to one product ion *m/z* 239 resulting from to the loss of the isopropyl group $[M+H\text{-}iPr]^+$ (Table 1).

The GC-MS analysis of the TMS derivative of 5-methyl-7-hydroxy-isoflavone shows a molecular ion at *m/z* 324 and the characteristic fragment ions: *m/z* 309, 222 and 102 (Fig. 2C). The positive HPLC-API/ESI mass spectrum displays a protonated molecular ion $[M+H]^+$ at *m/z* 253. Fragmentation of protonated 5-methyl-7-hydroxy-isoflavone in the ion trap leads to product ions *m/z* 235 $[M+H\text{-}H_2O]^+$, 197, 179, 225 and 210 (Table 2).

***Ipriflavone Metabolite Identification.***

The GC-MS and positive HPLC-API/ESI-MS methods were applied to the analysis of isoflavones and their metabolites in human urine. Possible positions for hydroxylation of ipriflavone are C-5, C-6 and C-8 in the aromatic A-Ring, C-2′, C-3′ (equivalent to C-6′ and C-5′, respectively) and C-4′ in the B-Ring, and C-2 in the C-Ring. We have not established direct correlation between peaks of metabolites which were found by HPLC-MS and GC-MS analyses. Therefore, we assigned numerical order as **M2**, **M3** to the metabolites found by HPLC-MS, and the similar but with «′» mark was done for the metabolites found by GC-MS: **M2′**, **M3′** etc.

Analysis by LC-ESI(+)/MS of extracts from enzymatic urine fraction gave the chromatogram of urine sample that shows a number of products appearing at retention times shorter than those of the parent drug i.e. more polar compounds. These results have shown that besides the unchanged parent drug (**1**) the following five metabolites were detected: four isomers of monohydroxy-ipriflavone **M2**, **M3**, **M4**, **M5** and one dihydroxy-ipriflavone **M6** (Table 1).

The unchanged ipriflavone (**1**) eluted at 24.0 min in the ipriflavone positive urine. The protonated molecular ion of ipriflavone is *m/z* 281 $[M+H]^+$. MS-MS mass spectra of ipriflavone show one product ion *m/z* 239 resulting of the loss of the isopropyl group $[M+H\text{-}iPr]^+$.

The molecular ion *m/z* 296 of metabolites **M2**, **M3**, **M4** and **M5** was shifted by 16 Da compare to that of the unchanged ipriflavone *m/z* 281, indicating mono-hydroxylation of ipriflavone. MS-MS mass spectra of these metabolites are not informative, further fragment

ions are *m/z* 255 and 237, which originate from the loss of isopropyl group and $H_2O$, respectively.

**Table 1.** Product ions for ipriflavone and its metabolites, protonated molecule $[M+H]^+$, retention time (RT), changes in observed mass for the metabolites (ΔM) and fragmentation results from MS-MS spectrum when were obtained via fragmentation of molecular ions. HPLC-ESI(+)/MS results.

| | $[M+H]^+$ | RT [min] | MS-MS |
|---|---|---|---|
| **(1)** Ipriflavone | 281 | 24.0 | **281**->239 $[M+H\text{-}iPr]^+$ |
| *Mono-hydroxyl metabolites* | | | |
| **M2** | 297 | 19.8 | **297**->255, 237 |
| **M3** | 297 | 21.6 | **297**->255, 237 |
| **M4** | 297 | 22.4 | **297**->255, 237 |
| **M5** | 297 | 22.7 | **297**->255, 237 |
| *Dihydroxy metabolite* | | | |
| **M6** | 313 | 16.3 | **313**->271,253,235 |

The metabolite **M6** eluted at 16.3 min. The molecular ion *m/z* 313 of metabolites **M6** was increased by 32 Da compared to that of the unchanged ipriflavone, confirming dihydroxylation of ipriflavone (Table 1).

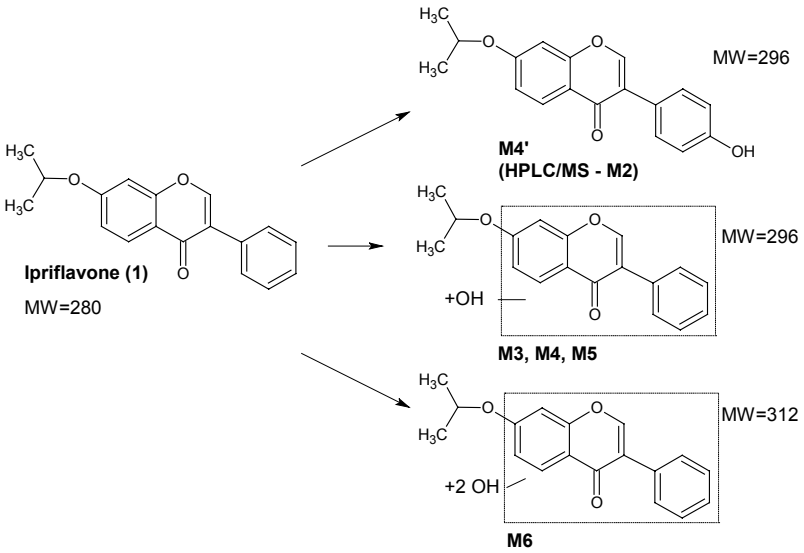



**Fig. 3.** Structural assignments for Ipriflavone metabolites.

GC-MS data of the ipriflavone positive urine showed ambiguous results (Fig 4). The parent ipriflavone without isopropyl group eluted at 14.06 min. The isopropyl group was lost during the derivatisation step. Unchanged ipriflavone was not detected. GC-MS analysis of ipriflavone urine showed four compounds: two isomeres of monohydroxy-ipriflavone **M2′** and **M3′** with retained isopropyl group, one monohydroxy-ipriflavone without isopropyl group **M4′** and one dihydroxy-ipriflavone without isopropyl group **M6′** (Fig 4). The GC-MS analysis of the TMS derivative of the major metabolite **M4′**showe a molecular ion at *m/z* 398 (di-TMS), a fragment ion at *m/z* 208, implaying an unchanged A-Ring, and a fragment ion at *m/z* 190 (102 ->190, adding –OTMS group) indicating a mono-hydroxylated ipriflovone

structure in the B-Ring. The characteristic ions are *m/z* 383, 208, 184, 190. The retention time and EI mass spectrum of **M4′** were same with those of Daidzein (4',7-dihydroxy-isoflavone) standard. Therefore, the metabolite **M4′** of ipriflavone without isopropyl group corresponds with the structure with a hydroxyl group at C-4′ position in the B-Ring (p-hydroxylated metabolite of ipriflavone, Fig. 3).

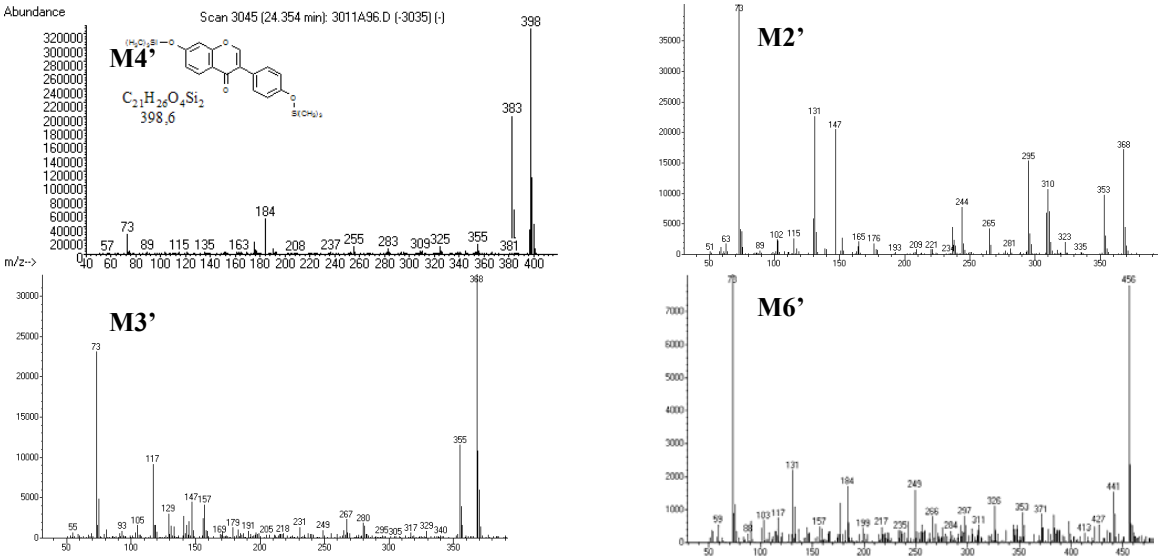



**Fig. 4** Electron impact mass spectra of the TMS derivatives of ipriflavone metabolites.

***5-Methyl-7-hydroxy-isoflavone Metabolite Identification.***

Possible positions for the hydroxylation of 5-methyl-7-hydroxy-isoflavone are C-6 and C-8 in the aromatic A-Ring, C-2′, C-3′ (equivalent to C-6′ and C-5′, respectively) and C-4′ in the B-Ring, and C-2 in the C-Ring. LC-ESI/MS analysis showed a number of products appearing at retention times shorter than those of parent drug (i.e. more polar compounds).

**Table 2.** Product ions for 5-methyl-7-hydroxy-isoflavone and its metabolites, protonated molecule $[M+H]^+$, retention time (RT), changes in observed mass for the metabolites (ΔM) and fragmentation results from MS-MS spectrum when were obtained via fragmentation of molecular ions. HPLC-ESI(+)/MS results.

| | $[M+H]^+$ | RT [min] | MS-MS |
|---|---|---|---|
| **(1)** 5-methyl-7-hydroxy-isoflavone | 253 | 19.7 | **253**->235, 210, **197** |
| *Metabolite with reduced C=O bond* | | | |
| **M2** | 255 | 15.3 | **255**->237, 227, 199, 137 |
| *Mono-hydroxyl metabolites* | | | |
| **M3** | 269 | 17.1 | **269**->251, 223, **213** |
| **M4** | 269 | 18.1 | **269**->251, 223, **213** |
| **M5** | 269 | 19.1 | **269**->251, 223, **213** |
| *Dihydroxy metabolite* | | | |
| **M6** | 285 | 14.1 | **285**->267, 249, 211, 177 |
| **M7** | 285 | 15.0 | **285**->267, 249, 211, 177 |
| **M8** | 285 | 15.5 | **285**->267, 249, 211, 177 |
| *Dihydroxy metabolite with reduced C=O bond* | | | |
| **M9** | 287 | 12.2 | **287**->269, 251 |

These results confirmed the presence of the metabolite with reduced C=O bond, monohydroxylated and dihydroxylated metabolites and dihydroxylated metabolite with reduced C=O bond in human urine (Fig. 5).

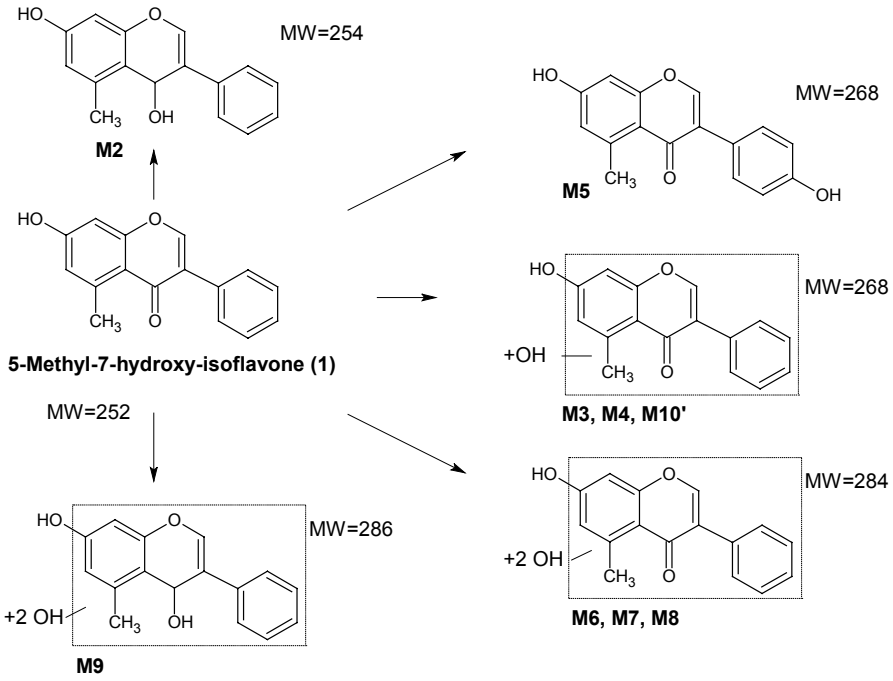



**Fig. 5.** Structural assignments for 5-methyl-7-hydroxy-isoflavone metabolites.

GC-MS analysis: The metabolites **M3′** and **M4′** eluted at 19.48 min and 22.89 min, respectively. The spectra of these two metabolites differ in the intensities of the fragment ions only (Fig. 6). The molecular ion of metabolites **M3′** and **M4′** was increased by 16 Da compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (di-TMS) is *m/z* 412. The characteristic ions are *m/z* 397, *m/z* 323, *m/z* 222. The ion *m/z* 222 of these metabolites was the same as the fragment ion of the parent drug (Fig 2). Therefore, this indicates that the C-, B-Ring of parent drug was hydroxylated.

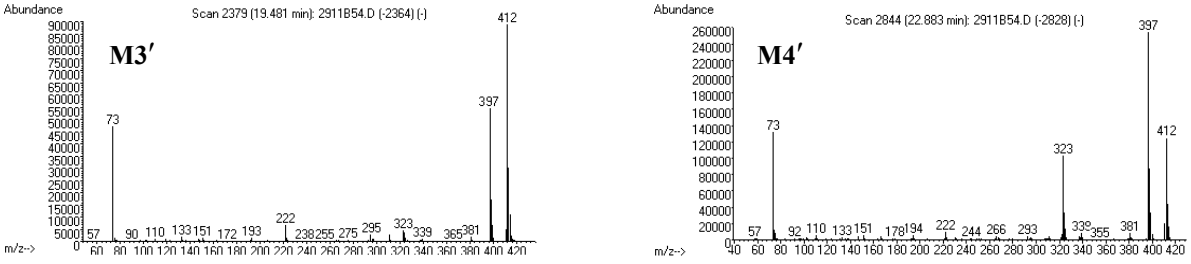



**Fig. 6** Electron impact mass spectra of the TMS derivatives of mono-hydroxylated 5-methyl-7-hydroxy-isoflavone metabolites.

The metabolites **M10′** and **M5′** eluted at 23.04 min and 24.46 min, respectively. The spectra of these two metabolites differ in the intensities of the fragment ions only (Fig. 7). The molecular ion of metabolites **M10′** and **M5′** was increased by 16 Da compared to that of the

unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (di-TMS) is *m/z* 412, the characteristic ions are *m/z* 397, 222, 191. The ion *m/z* 191 of these metabolites was increased by 89 Da (102->191, added –OTMS group) compared to that of the unchanged 5-methyl-7-hydroxy-isoflavon. The ion *m/z* 222 of these metabolites was same as the fragment ion of parent drug (Fig 2). Therefore, this indicates that the B-Ring of parent drug was hydroxylated. Possible positions of 5-methyl-7-hydroxy-isoflavone for the hydroxylation at the B-Ring are C-2′ (C-6′), C-3′ (C-5′) and C-4′. The metabolites **M10′** and **M5′** are mono-hydroxy metabolites of 5-methyl-7-hydroxy-isoflavone.

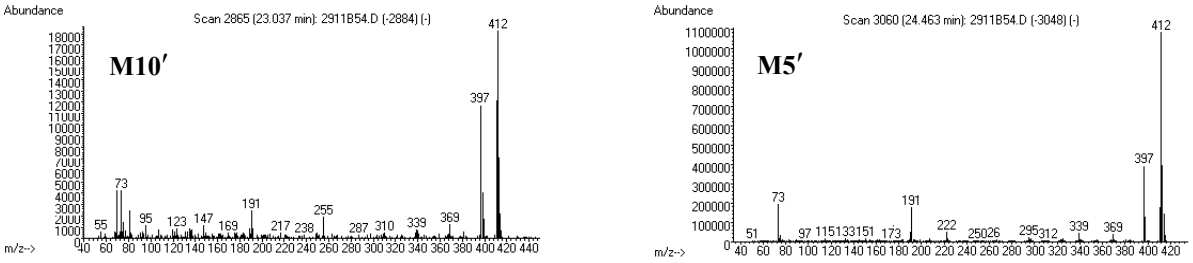



**Fig. 7** Electron impact mass spectra of the TMS derivatives of mono-hydroxylated 5-methyl-7-hydroxy-isoflavone metabolites.

The metabolite **M6′** eluted at 26.25 min. The molecular ion of metabolite **M6′** was increased by 32 Da compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (tri-TMS) is *m/z* 500 (Fig. 8). The characteristic ions are *m/z* 485, 397, 190. The ion *m/z* 190 of thise metabolite was increased by 88 Da (102->190, added –OTMS group) compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The ion at *m/z* 222 was not detected in mass spectrum (Fig 2). Therefore, this indicates that the A-Ring and B-Ring of parent drug were hydroxylated. Hence the metabolite **M6′** is di-hydroxy metabolite of 5-methyl-7-hydroxy-isoflavone, which is formed by the introduction of a hydroxyl group in the A- or B-Ring.

The metabolites **M7′** and **M8′** eluted at 26.44 min and 27.41 min, respectively. The spectra of these two metabolites differ in the intensities of the fragment ions only (Fig. 8). The molecular ion of metabolites **M7′** and **M8′** was increased by 32 Da compared to that of the unchanged 5-methyl-7-hydroxy-isoflavone. The molecular ion of these metabolites (di-TMS) is *m/z* 500. The characteristic ions are *m/z* 485, 411, 222, 147. The ion at *m/z* 222 of these metabolites was same as the fragment ion of parent drug (Fig 2). The ion at *m/z* 147 indicates that hydroxylation has taken place in the *meta* position relative to the second hydroxy group. Therefore, we suppose that the B-Ring of parent drug was dihydroxylated. Possible positions of 5-methyl-7-hydroxy-isoflavone for the dihydroxylation at the B-Ring are C-2′ (C-6′), C-3′

(C-5′) and C-4′. Hence the metabolites **M7′** and **M8′** are dihydroxy- metabolites of 5-methyl-7-hydroxy-isoflavone.

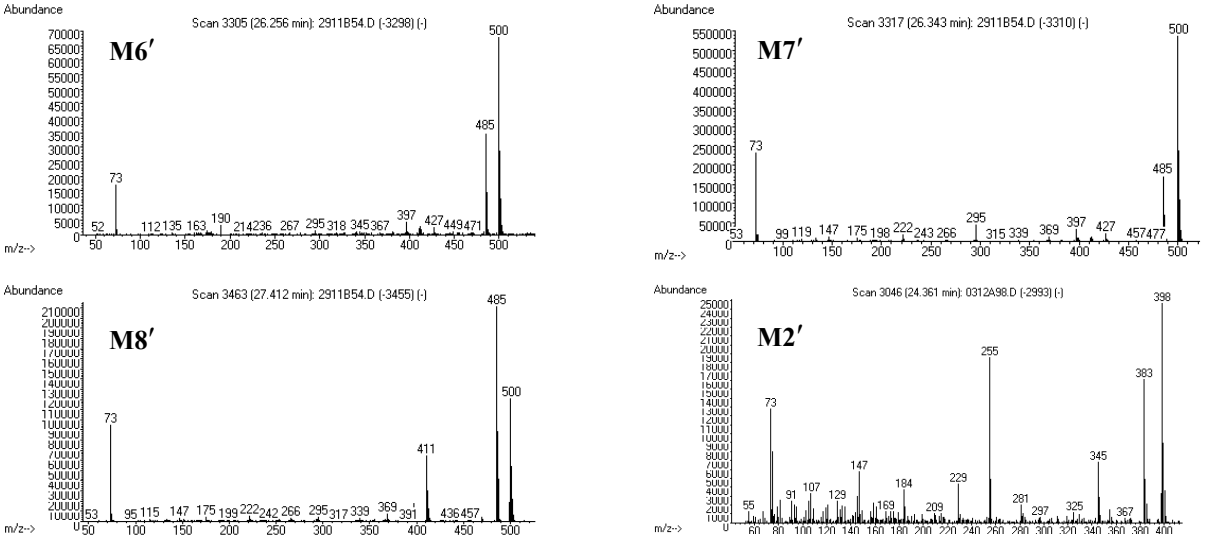



**Fig. 8** Electron impact mass spectra of the TMS derivatives of dihydroxylated 5-methyl-7-hydroxy-isoflavone (M6′, M7′ and M8′) metabolites and metabolite with reduced C=O bond (M2′).

The LC-ESI/MS and GC-MS results have shown that besides the unchanged parent drug (**1**) the following nine metabolites were detected: one metabolite with reduced C=O bond **M2**, four isomeres of monohydroxylated **M3**, **M4**, **M10′**, **M5**, three isomeres of dihydroxylated **M6**, **M7**, **M8** and one dihydroxylated metabolite with reduced C=O bond **M9** (Fig. 5).

Ipriflavone and 5-methyl-7-hydroxy-isoflavone urine samples were analysed by routine doping control procedure for anabolic steroids and was found that dihydroxylated metabolite **M8′** (Fig. 8) of 5-methyl-7-hydroxy-isoflavone has the same retention time as canrenone. Moreover, this metabolite has characteristic ions (*m/z* 412 and *m/z* 397) in the mass spectrum which are also ions for canrenone. Therefore, the 5-methyl-7-hydroxy-isoflavone may be a potential masking agent of canrenone in human urine.

**Conclusions**

High-performance liquid chromatography and gas chromatography coupled with mass-spectrometry were used to study the phytoestrogens ipriflavone and 5-methyl-7-hydroxy-isoflavone. In humans ipriflavone is metabolized on follows: parent drug ipriflavone, four monohydroxylated metabolites and one dihydroxylated metabolite. The main metabolites for 5-methyl-7-hydroxy-isoflavone are four monohydroxylated metabolites, three dihydroxylated metabolites, one metabolite with reduced C=O bond and one dihydroxylated

metabolite with reduced C=O bond. The HPLC-MS and GC-MS procedures allows for the identification up to 60 hrs after ingestion of a single 200-250 mg dose.

**Acknowledgements**

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