

Long-term kinetics of daidzein and its main metabolites in human equol-producers after soymilk intake: identification of equol-conjugates by UPLC-Orbitrap-MS and influence of the number of transforming bacteria on plasma kinetics

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Abstract

The primary objective of this study was to establish *in vivo* a correlation between equol production and number of bacteria able to perform the transformation. Secondary objective include the assessment of daidzein and its metabolites in plasma (0-99 h) and urine (0-96 h) after oral administration of 100 mg daidzein in soymilk. Healthy female volunteers were enrolled for their *in vitro* ability to convert slowly (n=6, 10^5 - 10^9 cell/ g wet faeces) or quickly (n=6, 10^{10} - 10^{12} cell/ g wet faeces) daidzein in equol. Plasma and urine samples were analysed by LC-MS/MS and UPLC-High Resolution-MS. Only for equol seemed to be a direct correlation between the number of transforming microorganisms and some parameters such as t_{max} and $t_{1/2}$ (p=0.027). Peak serum concentration time, C_{max} , AUC_{0-72h} and $t_{1/2}$ for total equol (n=12) were 36 ± 10 h, 89 ± 78 nM, 2.4 ± 1.7 ($\mu\text{mol} \times \text{h/L}$) and 15.6 ± 3.3 h, respectively. Equol metabolites were found only as glucuronide conjugates.

Keywords

Daidzein; Equol; pharmacokinetics; metabolism; human; mass spectrometry

Introduction

Soy isoflavones such as daidzein (DAI) and genistein have received attention for their potential prevention or treatment of estrogen- and androgen-mediated diseases (Travis et al., 2009). This plant-based class of phytoestrogens seems also to demonstrate a prophylactic role in the onset of some cancers such as prostate (Lee et al., 2009), breast (Rimbach et al., 2008) and colon (Coxam, 2009), cardiovascular disease (Kokubo et al., 2007) and osteoporosis (Euhara, 2013). Moreover, there are some data suggesting that soy isoflavones have beneficial effects on lipid homeostasis and glycemic control (Anderson et al.; 1993; Yang et al., 2004), and play a role in lowering blood concentrations of total cholesterol and LDL-cholesterol in human, limiting the development of atherosclerosis (Hodis et al., 2011) and inhibiting the transcriptional nuclear factor NF- κ B, the COX2 and aromatase (Rice et al., 2006).

On the other hand, studies of soy isoflavones intake in experimental animals suggest possible adverse effects such as enhancement of reproductive organ cancer, antithyroid effects (Xiao et al., 2014) and it seems correlated with lower sperm counts (Xia et al., 2013). Moreover, recent studies do not support the hypothesis that phytoestrogens prevent prostate or colorectal cancer (Bosland et al., 2013).

Metabolic investigations have observed a biphasic isoflavones appearance pattern in plasma and urine of humans after consumption of soy preparations, with two maximum concentrations at about two and six hours after intake (Franke et al., 2014). The time at which the first peak occurred (1-2 h) suggested that uptake occurred in the proximal small intestine (Walsh et al., 2007), particularly in the jejunum (Zubik and Meydani, 2003). The second peak at 4-6 h indicated that absorption took place in the large intestine and this suggested the involvement of the microflora. Thus, after ingestion, in the large intestine, anaerobic bacteria hydrolyse the glycosylated isoflavones to their respective aglycones and daidzein could be metabolized to dihydrodaidzein (DHD), O-desmethylangolensin (ODMA) and equol (EQU)

(Walsh et al., 2007) (Figure 1). To underline that in plasma and urine equol and ODMA are found as their S(-) and R(-) enantiomers, respectively, while there were both the enantiomers of the DHD. Once formed, these metabolites are absorbed and conjugated in the liver with glucuronic acid and/or sulphate (Shelnutt et al., 2000), which are then excreted predominantly in the urine (Shelnutt et al., 2000) (Figure 1). The conjugated forms of DAI, DHD, EQU and ODMA represent more than 90 and 95% of the plasmatic and urinary forms, respectively (Gardana et al., 2014).

Most of the isoflavones found in plasma and urine are present as glucuronides and in lower amount conjugated with sulfate (Shelnutt et al., 2000; Legette et al., 2014; Thomas et al., 2001), which could exert various health effects as well. Daidzein sulfate conjugates, but not daidzein, inhibited sterol sulfatase, which is involved in the development of breast cancer *in vitro* (Wong and Keung, 1997).

To date, there is limited information on the conjugation profile of daidzein (Doerge et al., 2000; Hosoda et al., 2011; Shelnutt et al., 2002; Zhang et al., 2003), and those relating to the equol are even less (Legette et al., 2014; Schwen et al., 2012; Redmon et al., 2016; Koh and Mitchell 2011). This is due to non-availability of reference standards and, until recently, to sensitive and specific analytical techniques. Therefore, it is generally determined the total amount of these compounds in biological fluids and tissues before and after hydrolysis with glucuronidase/sulphatase and the conjugated forms determined by difference.

In this research, we aim to explore the capabilities of the high-resolution mass spectrometry (HR-MS), in full-scan acquisition mode, in order to evaluate the equol conjugates forms present in plasma and urine samples after soymilk intake. Moreover, we found *in vitro* (Gardana et al., 2014) a possible direct correlation between the conversion rate of the DAI to ODMA and the number of bacteria that were able to perform the transformation. This correlation was found *in vivo* only for the time to reach the maximum

concentration but not for the other pharmacokinetic indexes evaluated. Thus, the purpose of this study was also to determine whether there was a correlation between EQU production and number of bacteria able to perform the transformation.

Materials and methods

Chemicals

Purchased chemicals included the standards daidzin, DAI, DHD, (R,S)-EQU and (R,S)-ODMA from Plantech (Reading, UK). Sakuranetin (SAK), formononetin (4'-methyldaidzein) and quercetin-glucuronide were obtained from Extrasynthese (Genay, France). Type H5 β -glucuronidase/sulfatase from *Helix pomatia* was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol, acetonitrile, sodium acetate and acetic acid were from Merck (Darmstadt, Germany). Water was obtained from a MilliQ apparatus (Millipore, Milford, MA). Soymilk from organic culture (NaturGreen Food, Murcia, E) was acquired in a local supermarket. Daidzein pure compound, approximately 95 mg, was dissolved in 100 mL of soymilk.

Subject and study design

In a previous work, the transformation of daidzein into DHD, EQU and ODMA was investigated in anaerobic batch cultures inoculated with mixed fecal bacteria from 90 healthy volunteers (Gardana et al., 2009). The results showed that in most subjects the number of microorganisms able to convert DAI in EQU were lesser than 10^9 cells/g wet faeces, and in few subjects the amount of equol-producing microbial populations were higher than 10^{10} cells/g wet faeces. The latter belonged to the sub-group at high rate conversion, whereas the other subjects had demonstrated slower equol-production in time-course experiments.

From the “equol-producer” subjects we have recruited six “fast equol producers” and six “slow equol producers” (aged=45-64 years, BMI=23.4 \pm 3.3 Kg/m²). The number of faecal microorganisms able to convert DAI to EQU was evaluated by the Most Probable Number method as described by Gardana et al. (2009).

They were not taking any supplement, drug or medication. Informal, written consent was obtained from each participant and the local ethical committee approved the protocol.

Volunteers refrained from consuming food and beverages containing isoflavones 7 days prior to the study and 1 week after soymilk intake. On the day of the experiment, subjects were confined to the laboratory and between 6:00 and 6:30 a.m. the fasted volunteers received 100 mL of soymilk, containing 100 mg of DAI and 4 mg of daidzin, with jam (25 g) and bread (50 g). After dinner, the volunteers were allowed to leave the laboratory, returning the next four days for the blood and urine sampling.

Blood samples were collected in tubes containing lithium-heparin at the following time points: 0, 3, 5, 7, 9, 11, 27, 36, 51, 60, 75, 84 and 99 h after the soymilk consumption. Urine was collected into plastic bottles in five fractions (0-24, 24-48, 48-72 and 72-96 h) stored at 4 °C during the sampling period. The amount of urine in each fraction was measured, portioned in glass vials (1 mL) and frozen at -70 °C immediately after the participants had started to collect the next fraction.

Method validation

Calibration curves were constructed for each standard at five concentration levels and three independent determinations were performed at each concentration. LLOQ was determined by serial dilution of standard solutions and defined by the lowest injected inter-day concentration whose RSD% resulted to be <20% (Shah et al., 2000). LOD was defined by the lowest concentration that the assay can differentiate from background levels (S/N ratio > 3).

The accuracy (matrix effect) was evaluated according to a previously published paper (Gardana et al., 2007) using three sets of six standard lines (0.2 - 100 ng/mL). All the tests and LC-MS/MS analyses were carried out in triplicate and an internal standard (Sakuranetin) was used to correct the loss of analytes during sample preparation. Intra- and inter-day precision of the assay was verified by analyzing samples set 3 times on five consecutive days.

Repeatability was confirmed by evaluating standard deviations of the retention times and peak areas.

Determination of Daidzein and its glycosides in soymilk

One mL of soymilk was extracted by 5 mL of methanol and the resulting solution sonicated for 10 min, centrifuged at 1000 x g for 10 min and the residue was extracted twice by methanol. The supernatants were put together, the volume set to 20 mL with methanol and the resulting solution filtered through a 0.2 µm disk before injecting 5 µL in the LC-DAD-MS system. The chromatographic system consisted of an Alliance 2695 (Waters, Milford, MA) equipped with a model 2998 (Waters) photodiode array detector and a triple quadrupole mass spectrometer mod. Quattromicro (Micromass, Beverly, MA).

A 3.5 µm C₁₈ Symmetry column (150x2.1 mm, Waters) was used for the separation, which was performed by means of a linear gradient elution (eluent A, 0.05% acetic acid; eluent B, acetonitrile) at a flow rate of 250 µL/min. The column and the samples were maintained at 30°C and 20°C, respectively. The gradient was as follows: from 10 to 25% B in 10 min, from 25 to 35% B in 10 min and then from 35 to 55% B in 10 min. Chromatographic data were acquired in the 200-450 nm range and were integrated at 254 nm. Calibration curves were obtained from daidzin and daidzein stock solutions prepared by dissolving 20 mg of standard powder in 20 mL of methanol. They were measured in the range of 2-100 µg/mL.

The mass spectrometer operated in full-scan mode in the range (m/z)⁻ 200-1000 u. All data were acquired by Masslink 4.0 software (Micromass).

Plasma and urine preparation

Sample preparation for the quantitative analysis

Heparinized plasma (200 µL) was incubated with 100 µL glucuronidase/sulphatase (1 U/µL) and 50 µL I.S. (Sakuranetin, 50 ng/mL) in 0.1 mol/L CH₃COONa buffer (pH 5.2) at 37°C for 18 h. The reaction mixture was extracted with 500 µL ethylacetate, vortexed and centrifuged at 1000 x g for 1 min. The supernatant (350 µL) was dried under N₂ and the residue dissolved

in 100 μL methanol. A solution of quercetin-glucuronide (50 ng/mL) was incubated with 100 U glucuronidase/sulphatase and I.S. to assess the enzymatic activity.

To evaluate non-conjugated analytes, a plasma sample (200 μL) was incubated with 50 μL I.S. and 100 μL of 0.1 mol/L CH_3COONa buffer (pH 5.2) for 18 h at 37°C. The reaction mixture was then treated as described above.

Urine sample (200 μL) was incubated with 200 μL glucuronidase/sulphatase (1 U/ μL) and 50 μL I.S. (Sakuranetin, 50 ng/mL) in 0.1 mol/L CH_3COONa buffer (pH 5.2) at 37°C for 18 h. The reaction mixture was extracted with 800 μL ethylacetate, vortexed and centrifuged at 1000 x g for 1 min. The supernatant (600 μL) was dried under N_2 and the residue dissolved in 1 mL methanol. To evaluate non-conjugated analytes, a urine sample (200 μL) was incubated with 50 μL I.S. and 200 μL of 0.1 mol/L CH_3COONa buffer (pH 5.2) for 18 h at 37°C. The reaction mixture was then treated as described above.

Sample preparation for the qualitative analysis

For the determination of the equol conjugates in plasma, phospholipid and protein was removed by Phree tube (Phenomenex, Torrance, CA). Briefly, 200 μL of plasma was placed into a Phree tube and then 600 μL acetonitrile was pipetted directly into the plasma sample. The reaction mixture was vortexed and after 1 min the tube was loaded onto a manifold (Waters), to which was applied a negative pressure. The eluate was collected and analyzed by UPLC-HR-MS.

For the analysis of equol conjugates in urine, 200 μL of sample was diluted to 1 mL by a solution of 0.1% CH_3COOH in water and the resulting solution centrifuged at 1000 x g for 2 min before injecting 5 μL in the UPLC-HR-MS system.

LC-ESI-MS/MS analysis of DAI and its metabolites plasma and urine

The chromatographic system consisted of an HPLC mod. Alliance 2695 (Waters) coupled to a triple quadrupole mass spectrometer mod. Quattromicro (Micromass). A 3.5 μm C₁₈ Symmetry Shield column (150x2.1 mm, Waters) was used for the separation, which was performed by means of a linear gradient elution (eluent A, 0.02% CH₃COOH; eluent B, acetonitrile) at a flow rate of 250 $\mu\text{L}/\text{min}$. The gradient was as follows: 25% B for 5 min, from 25 to 50% B in 0.2 min and then 50% B for 10 min. The column was maintained at 30°C and 5 μL injected in LC-MS/MS system. The capillary voltage was set to 3.0 kV, the cone voltage and the collision energy was specific for each compound. The source temperature was 130°C, the desolvating temperature was 350°C and argon was used at 2.0×10^{-3} mbar to improve fragmentation in the collision cell.

Analytes were determined in multiple reaction monitoring mode (MRM) and data were acquired by Masslynx 4.0 with Quan-Optimize option for fragmentation study. The mass spectrometer operated in ESI negative mode and the fragmentation transitions were the following: (m/z)⁻ 253 to 91 for DAI, 255 to 149 for DHD, 241 to 119, 121, 135 for EQU, 257 to 109, 135 for ODMA and 285 to 165, 119 for SAK (I.S.). A dwell time of 0.2 s for transition was used.

The determinations were carried out in triplicate. Calibration curves were obtained from DAI, DHD, EQU, ODMA and I.S. stock solutions prepared by dissolving 20 mg of standard powder in 100 mL methanol. They were measured in the range of 5-100 ng/mL and for DAI it was prepared an additional calibration curve in the range 100-1000 ng/mL.

UPLC-HR-MS analysis of the EQU-conjugates in plasma and urine

The UPLC-HR-MS analysis was carried out on an UPLC model Acquity (Waters) coupled with a high-resolution Fourier Transform mass spectrometer (HR-FT-MS) model Exactive

(Thermo Scientific, San Jose, CA, USA) equipped with an HESI-II probe for electrospray ionization and a collision cell (HCD). The operative conditions were: spray voltage -3.0 kV, sheath gas flow-rate 55, auxiliary gas flow-rate 10, capillary temperature 275 °C, capillary -37.5 V, tube lens -125 V, Skimmer -26 V, and heater temperature 130 °C. Five μL was injected on a 1.7 μm BEH Shield C₁₈ column (150 \times 2.1 mm, Waters) kept at 50 °C. The eluents were 0.02% acetic acid in MilliQ-treated water (solvent A) and acetonitrile (solvent B). The UPLC separation was performed by the following linear elution gradient: 5 to 35% of solvent B in 10 min and then from 35 to 80 % in 10 min at a flow-rate of 0.45 mL/min.

The identification of the conjugated metabolites was performed in full-scan mode in the range (m/z) 80-1000 u, using an isolation window of ± 2 ppm. The AGC target, injection time, mass resolution and energy in the collision cell were 1×10^6 , 100 ms, 50 K and 30 V, respectively. The MS data were processed using Xcalibur software (Thermo Scientific). The peak identity was ascertained by evaluation of both the accurate mass that the fragments obtained in the collision cell.

Statistical analysis

Statistical analyses were performed with the STATISTICA software (Statsoft Inc, Tulsa, OK, USA). A one-way repeated measure analysis of variance (ANOVA) with the time as the independent factor was used. The kinetic indexes and the amount excreted by urine have been expressed as mean \pm s.d. The terminal half-life was calculated as $0.6935/K$. The area under the plasma concentration time curve (AUC_{0-t}) was calculated using the linear trapezoidal rule in the range 0-51 h (DAI) or 0-72 h (DHD, EQU and ODMA).

The \log_{10} -transformed urinary [EQU]/[DAI] ratio was used to define *in vivo* equol producer status (Setchell and Cole, 2006). Each analyte was analyzed separately for each kinetic parameter for assessing if there were any significant differences between slow- and

quick-equal producers using the Wilcoxon matched pairs test. P values < 0.05 were considered significant.

Results

Equol production in vitro

The accuracy of the extraction for EQU from spiked sterilised faecal samples was 96-103 and 93-105% for intra-day and inter-day assays, respectively. The controls did not produce compounds overlapping with EQU. No changes were detected in EQU amount after 96 h incubation with bacteria from faeces of EQU-producing volunteers.

The comparison of the different time-course experiments allowed identification of a sub-group of EQU-producers able to convert 50% of DAI to EQU in about 6 h (Supplemental 1). In these volunteers, the number of microorganisms able to convert DAI in EQU was in the range 10^{10} - 10^{12} cells/g wet faeces. These volunteers belonged to the sub-group at high conversion rate.

The other EQU-producers reached the highest concentration of EQU between 20 and 30 h (Supplemental 1) and 50% of DAI was converted to EQU after about 12-16 h of incubation. These volunteers had a number of microorganisms in the range 10^5 - 10^9 cells/g wet faeces, and therefore belonging to a sub-group at lower conversion rate.

Quantitative determination of daidzein and its metabolites in plasma and urine

During the direct infusion of DAI, DHD, ODMA, EQU and IS solutions in the mass spectrometer, all were easily deprotonated in the negative ionization mode, providing ions with appropriate intensity except for the EQU for which the signal intensity was lower by about 50% compared to that of daidzein.

Thus, ESI in negative mode was chosen for the qualitative and quantitative analysis of all the analytes and IS in human plasma and urine. The $[M-H]^-$ ion was then used as the precursor to obtain typical product ion spectra. The MRM fragmentation transitions were $(m/z)^-$ 253→91, 255→149, 257→109 and 285→165 u for DAI, DHD, ODMA and IS, respectively.

Regarding EQU, to increase the specificity of the analysis three product ions were chosen such as 241→119, 241→93 and 241→135 u (Supplemental 2). Figure 2 shows an example of DAI, DHD, EQU, ODMA and IS peaks detected by LC-MS/MS in an enzymatically hydrolysed plasma sample obtained 27 h after soy milk intake.

The recovery of the extraction for DAI, DHD, EQU, ODMA and IS from spiked plasma samples was 94±3, 87±4, 91±3, 88±3 and 95±4%, respectively. The precision of the method was tested by both intra-day (n=6) and inter-day (5 days, n=6) reproducibility, and the coefficient of variation was below 11%. The DAI, DHD, EQU and ODMA concentrations were calculated by external standardisation from calibration curves and their levels in plasma and urine were evaluated before and after the enzymatic hydrolysis.

The conjugated forms of these analytes represented more than 95% of the plasmatic and urinary forms. None of the subjects had detectable amounts of DAI or its metabolites at baseline. All subjects absorbed DAI and EQU, while DHD and ODMA were found in nine and seven subjects, respectively.

Regarding the evaluated pharmacokinetic parameters, great inter-individual differences were detected. The C_{max} of EQU was reached in 36±10 h (n=12) after the DAI ingestion and only in one subject it was found after 60 h. Table 1 reports the values of the kinetics indexes calculated from the DAI, DHD, EQU and ODMA plasma levels. The half-life ($t_{1/2}$) for DAI, DHD, EQU and ODMA was about 9, 7, 16 and 9 h, respectively. The amount of the analytes excreted in urine following the intake of 100 mL of soymilk containing 100 mg of DAI is reported in table 2.

Identification of Equol-conjugates in plasma and urine

The metabolites of equol produced *in vivo* by human were determined by UPLC coupled to high resolution mass spectrometry detector. In figure 3 was reported the extracted ions, contained in a sample of urine (24-48h), with $(m/z)^-$ 417.1185, 321.0438, and 241.0870, corresponding to EQU-glucuronide, EQU-sulfate and EQU, respectively. The ions corresponding to equol (Fig. 3, peak A1 and A2) were obtained after fragmentation in the collision cell, and were used to confirm the identity of the equol-conjugates.

In this regard, the EQU-glucuronide (Fig. 3, peak B1 and B2) gave the product ions with $(m/z)^-$ 241.0865, corresponding to EQU, whilst EQU-sulfate (Fig. 3, peak C) did not. This could be due to the lower amount of EQU-sulfate, or it may not be EQU-sulfate. The latter hypothesis seems supported by the absence, after fragmentation in the collision cell, of the ions with $(m/z)^-$ 96.9601 or 80.9652 corresponding to HSO_4^- and HSO_3^- , respectively. Thus, it seems that in urine and plasma glucuronides were the major EQU-metabolite. Conversely, EQU and its other conjugates such as disulfates, diglucuronides, sulfate-glucuronides or methylated were not detected.

In the urine, a major glucuronide peak was found for equol (Fig. 3, peak B2) and a second glucuronide peak was present in all subjects in much lower quantities (Fig. 3, peak B1). The results obtained do not allow defining the position of the glucuronic acid in the equol molecule. At this regard, Schwen et al. (2012) suggested that the major metabolite of the EQU in rat plasma and urine was the 4'-glucuronide conjugate, with lesser amounts of the 7-sulfate conjugate, and the 4'-glucuronide-7-sulfate diconjugate. On the contrary, Clarke et al. (2002) identified in human urine, by authentic standard, EQU-7-glucuronide, EQU-4'-glucuronide and EQU-4'-glucuronide and the main peak was related to the 7-glucuronide. Redmon et al. (2016) reported that in dog, cat and human the 7-OH glucuronide was the major phase II

metabolite of the EQU. Thus, based on these reported data we suppose that the main EQU-glucuronide (Fig. 3, peak B2) found in plasma and urine was the 7-glucuronide.

Discussion

To the best of our knowledge, this is the first paper reporting the *in vivo* correlation between production rate of equol and the number of bacteria able to metabolise daidzein.

Among the twelve EQU-producing volunteers, six were able to convert 50% of DAI to EQU within about 6 hours of fermentation, while the other six EQU-producers reached the same level within about 12-14 hours. The different rates of formation seem to depend on the number of bacterial able to metabolise DAI to EQU. Indeed, in the faster EQU-producers, the number of microorganisms able to convert DAI in EQU was 10^{10} (n=2), 10^{11} (n=3) and 10^{12} (n=1) cells/g wet faeces. These volunteers belonged to the sub-group with the higher rate of conversion. For other EQU-producers, the bacterial populations were 10^5 (n=1), 10^7 (n=4) and 10^9 (n=1) cells/g wet faeces. These volunteers, who demonstrated slower EQU production in time-course experiments, belonged to the sub-group with the lower rate of conversion. Thus, it seems that the different rate of EQU production could be related to inter-individual differences in the number of EQU-producing microorganisms.

After 24 h of incubation, approximately 50% of the initial DAI was converted to EQU and once formed it was stable over 96 h. On the other hand, a percentage of DAI (40-50%) disappeared without the production of known metabolites. Thus, as already reported for the degradation of the DAI to ODMA (Gardana et al., 2014), the stoichiometric assessments suggest that the DAI degradation pathway responsible for forming EQU is not yet fully identified.

Equol was detected in the plasma and urine of all the subjects after enzymatic hydrolysis by glucuronidase/sulphatase. The kinetic data show that all healthy EQU-producers absorb

DAI rapidly and the time taken to attain peak plasma concentrations after ingestion is about 8 h. At the contrary, slow- and high-EQU producers reach maximum plasma concentrations of EQU at about 45 and 34 h, respectively. At these times, the mean maximum plasma concentration (C_{max}) of DAI, DHD and EQU was about 2.9 μM , 0.1 μM and 0.09 μM , respectively. Thus, DAI C_{max} was about 30-times higher than that of EQU. For the latter, the plasma profiles showed that there is a time lag in its appearance and that, after a single DAI ingestion, it takes at least 9-11 h before EQU appears in plasma. This observation is consistent with a colonic origin for its formation. In particular, our data suggest a direct correlation between $t_{1/2}$, the conversion rate of the DAI to EQU and the number of bacteria that are able to perform the transformation. In fact, the $t_{1/2}$ and t_{max} was 13 and 18 h and 45 and 34 h for the subjects with lower and higher conversion rates, respectively.

One volunteer belonging to the subgroup with the lower rate conversion had t_{max} of 60 h, which was significantly different to that of other subjects of the same sub-group. At this regard, it should be highlighted that the latter had lowest number of microorganisms (10^5 cells/g wet faeces) able to convert DAI to EQU. It was therefore possible that this subject was not an EQU-producer. To define the EQU-producer status, Setchell et al. (2006) suggest using the urinary \log_{10} ([EQU]/[DAI]) ratio. In this way, values higher than -1.75 define equol producer status. Thus, we have calculated the urinary \log_{10} ([EQU]/[DAI]) ratio and for the subject with the lowest number of transforming bacteria the value was -2.5, suggesting that was not an EQU-producer. On the contrary, the other subjects had a value higher than -1.3 suggesting that they were EQU-producer.

The evaluation of the transforming bacteria could be an easy and non-invasive measurement to determine whether a subject is EQU-producer or not. Thus, it should be assessed the minimum number of transforming bacteria that determines the state of EQU-

producer. Regarding C_{\max} and AUC, it seems that there is no correlation between these parameters and the number of bacteria able to produce EQU.

Most DAI is excreted into the urine within the first 24 h, with a high individual variability, ranging from 11 to 45% of the ingested amount. This finding is in agreement with Chang et al. (2013), which found a daidzein urinary excretion within 24 h in the range of 43-60%. Thus, the recovery of DAI in urine was low, also considering that the excretion of DHD and EQU produced by catabolism of DAI did not account for more than 10% of the dose ingested.

Bayer et al. (2001) reported that in rat $85\pm 24\%$ of the orally administered daidzein was excreted unchanged with feces and only approximately 9% was recovered in urine, in part as parent compound and as conjugates. At the contrary, Watanabe et al. (1998) reported that in human about 4.6 and 36% of the DAI were excreted in the feces and urine, respectively. Moreover, the authors reported that the total amounts of DAI recovered in urine and feces were about 55% of the ingested dose. These last data are in good agreement with our results and seem suggest that approximately 50% of the daidzein disappeared without being associated to the appearance of known metabolites.

Glucuronide peaks on the chromatogram were identified by accurate mass and product ions determination. In the urine of all the subjects, a major glucuronide peak was identified for equol, which was tentatively identified as the 7-O-glucuronide according to Clarke et al. (2002). In addition, a second glucuronide peak present in much lower quantities and eluting shortly before the EQU-7-O-glc peak was found, and it was tentatively identified as EQU-4'-O-glucuronide.

It should be noted that a peak with molecular formula corresponding to EQU-sulfate (Fig. 3, peak C) was detected in urine in some sample but, after fragmentation, the product ions related to EQU were not found. Thus, the peak with $(m/z)^+$ 321.0438 (Fig. 3, peak C) was not

considered EQU-sulfate. Regarding plasma, EQU-7-glucuronide was the only metabolite found, and EQU pharmacokinetics studies in human and animals such as pig, rat, dog, horse and monkey show similar metabolism (Redmon et al., 2016).

On the contrary, the deprotonated ions corresponding to possible metabolites of the EQU, such as -sulfate-methylated, -diglucuronide, -disulfate and -methylated were not detected. This is not in agreement with several authors that reported the presence of EQU-sulfate in urine and plasma sample (Shelnutt et al., 2002; Legette et al., 2014).

The first appearance of EQU-glucuronides in plasma occurred around 9-11 h after soymilk intake, and their concentration peaked around 40 h. The late appearance was due to time required for bacterial conversion of DAI to EQU in the large intestine. These findings are in agreement with those reported in literature (Legette et al., 2014). Moreover, data obtained in the range 27-51 h increase the knowledge concerning plasma concentrations and kinetic of the EQU-conjugates.

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends to the figures

Figure 1. Degradation of the daidzein (DAI) to racemic dihydrodaidzein (RS-DHD), R(-)-O-desmethylangolensin [R(-)-ODMA) and S(-)-equol [S(-)-EQU] by intestinal bacteria and subsequent conjugation of the EQU with sulfate or glucuronic acid by hepatocytes.

Figure 2. Typical LC-MS/MS chromatogram (MRM mode) of a plasma sample obtained 27 h after soymilk intake. 1: DAI (m/z)⁻ 253→91, 2: DHD 255→149, 3: EQU, (a) 241→119, (b) 241→121, (c) 241→135, 4: ODMA 257→109, 5: SAK (IS) 285→165 u.

Figure 3. Chromatograms relative to a urine sample collected in the range 24-48 h after soymilk intake containing about 100 mg of daidzein. A1, A2: EQU (m/z)⁻ 241.0870 obtained after fragmentation at 30 V in the collision cell. B1, B2: EQU-glucuronides (m/z)⁻ 417.1191, C: EQU-sulfate (m/z)⁻ 321.0438 u. The peaks B1, B2 and C were obtained in scan mode without fragmentation (HCD off).

Table 1. Plasma kinetic indexes in 12 healthy volunteers for Daidzein and its metabolites after ingestion of about 100 mg daidzein in soy milk.

Analyte	t_{\max} (h)		C_{\max} (μM or nM)		AUC ($\mu\text{mol} \times \text{h} / \text{L}$)		$t_{1/2}$ (h)	
	S	H	S	H	S	H	S	H
t-DAI	8.0±1.7	7.0±2.6	2.8±1.2	3.0±1.1	45.1±15.6	41.0±14.3	12.4±4.8	9.2±4.7
t-DHD	27.0±0.0	17.4±10.4	66.5±65.8	117.0±82.1	1.8±1.9	2.1±1.3	5.2±3.4	6.5±3.4
t-EQU	45.0±6.6 ^a	34.5±12.7 ^a	81.1±72.7	87.2±71.9	2.2±1.5	2.3±1.6	12.8±1.6 ^b	18.4.4±1.7 ^b
t-ODMA	33.0±12.0	28.1±11.7	84.4±78.9	66.9±57.2	2.2±2.1	1.7±1.5	9.9±1.9	8.7±2.8

t-DAI, total Daidzein; t-DHD, total Dihydrodaidzein; t-EQU, total Equol; t-ODMA, total O-Desmethylangolensin.

S, Slow EQU producer (n=6); H, Quick EQU producer (n=6).

Values with the same letters within the same line are significantly different ($P < 0.05$). t_{\max} : $p=0.027$, $t_{1/2}$: $p=0.027$

t_{\max} , time taken to reach peak concentration; C_{\max} , peak concentration (μM for DAI, nM for DHD, EQU and ODMA); AUC area under the curve (0-51 h for DAI, 0-72 h for DHD, EQU and ODMA); $t_{1/2}$, terminal half-life.

Table 2. Urinary excretion (mg, mean±s.d.)[#] of daidzein and its metabolites after ingestion of about 100 mg daidzein in soymilk.

Time (h)	total DAI	total DHD	total ODMA	total EQU
Basal	0.0±0.0 ^a	0.0±0.0 ^a	0.0±0.0 ^a	0.0±0.0 ^a
0-24	20.6±12.1 ^b	2.0±2.5 ^a	1.1±1.0 ^a	1.0±0.9 ^b
24-48	4.0±2.7 ^c	1.2±0.6 ^a	1.3±1.1 ^a	2.4±2.1 ^c
48-72	1.7±4.6 ^c	0.0±0.1 ^a	0.2±0.3 ^a	0.6±0.5 ^{ab}
72-96	0.0±0.0 ^a	0.0±0.0 ^a	0.0±0.0 ^a	0.0±0.0 ^a
Total	26.2±10.3	3.2±2.5	2.6±2.1	4.0±3.3

[#] No significant difference was detected between slow and quick EQU producers ($P < 0.05$).

Means between groups not having the same letters were statistically different.

Effect of time was significant by ANOVA for DAI and EQU ($p < 0.001$).

DAI: found in all subjects in the 0-24 and 24-48 h sample and in three volunteers also detected in the 48-72 h sample.

DHD: not found in one subject belonging to the “slow EQU-producers” sub-class.

ODMA: not found in two subjects. After 72 h was present in the urine of five subjects.

EQU: found in all subjects in the 24 and 48 h sample and detected in eleven volunteers after 72 h. In one subject, the $\log ([EQU]/[DAI])$ was -2.5, suggesting that was not an equal-producer.

Figure 1

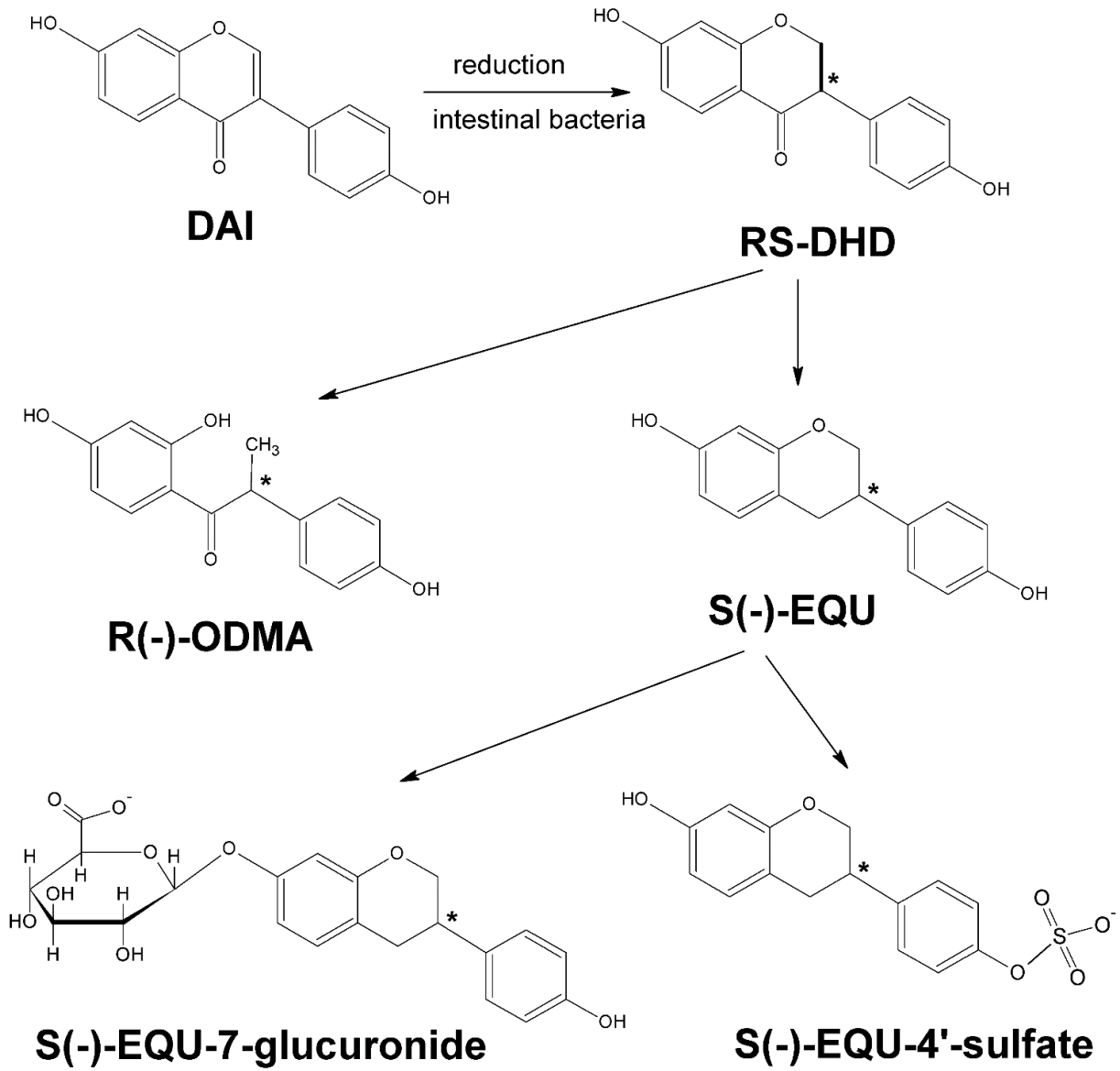


Figure 2

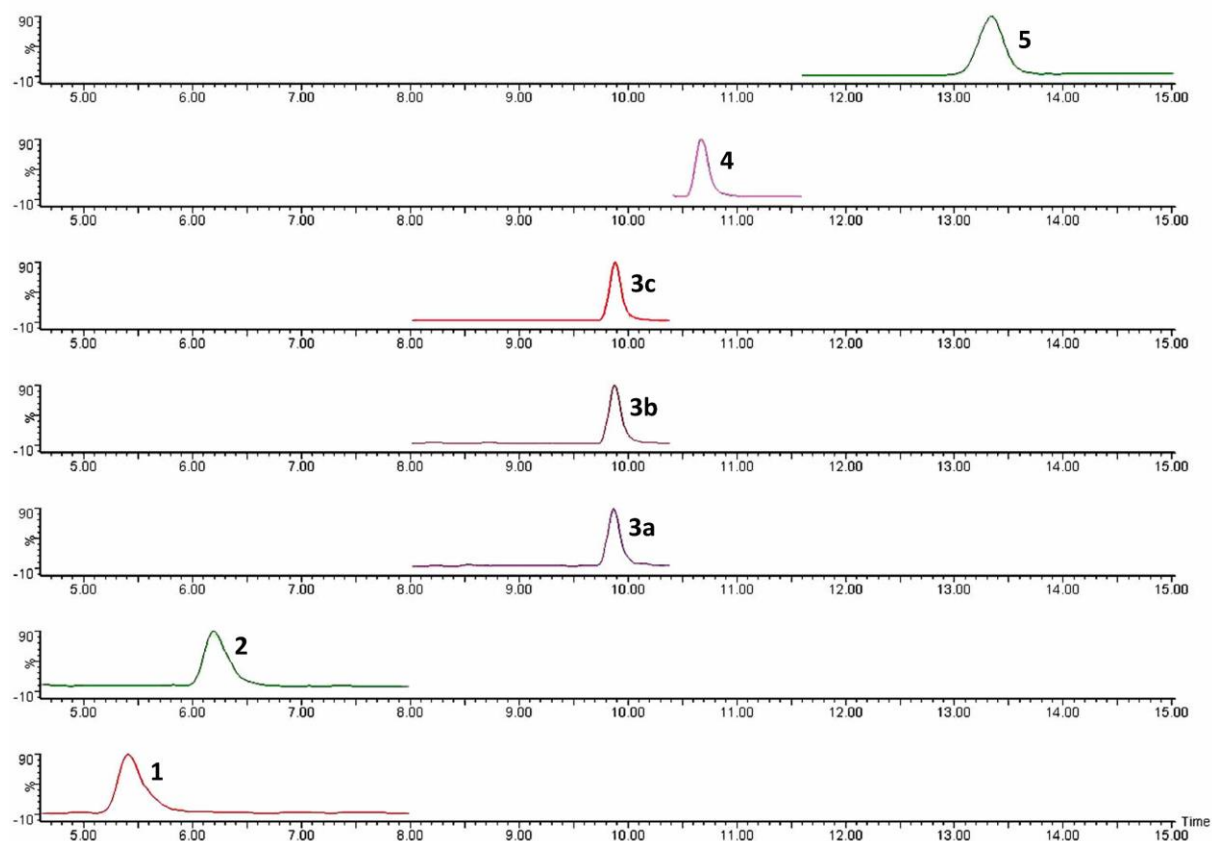
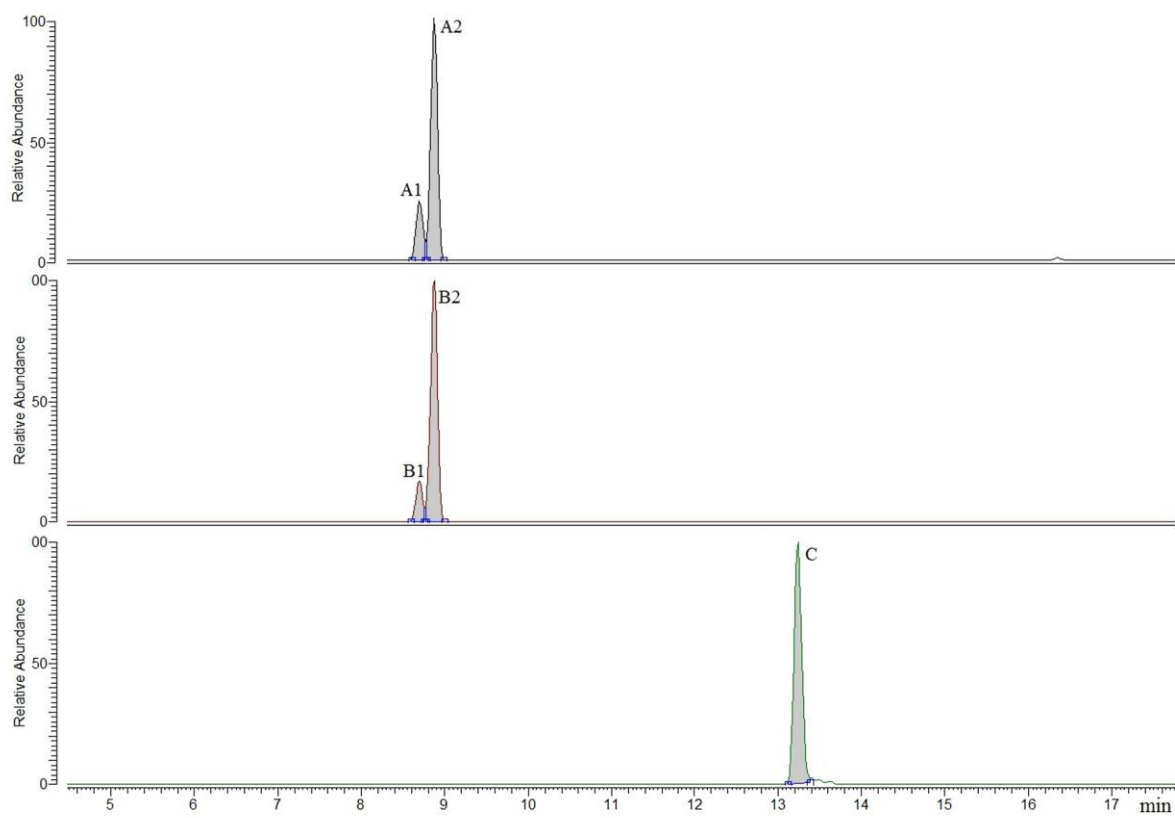
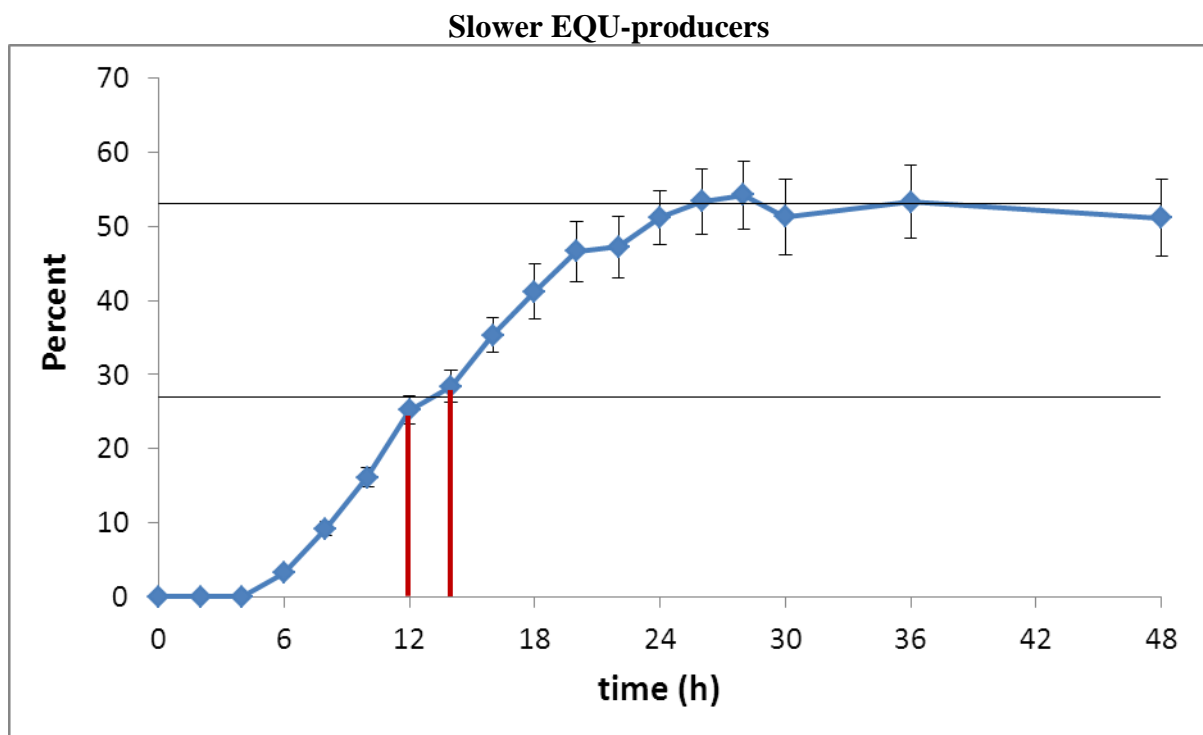
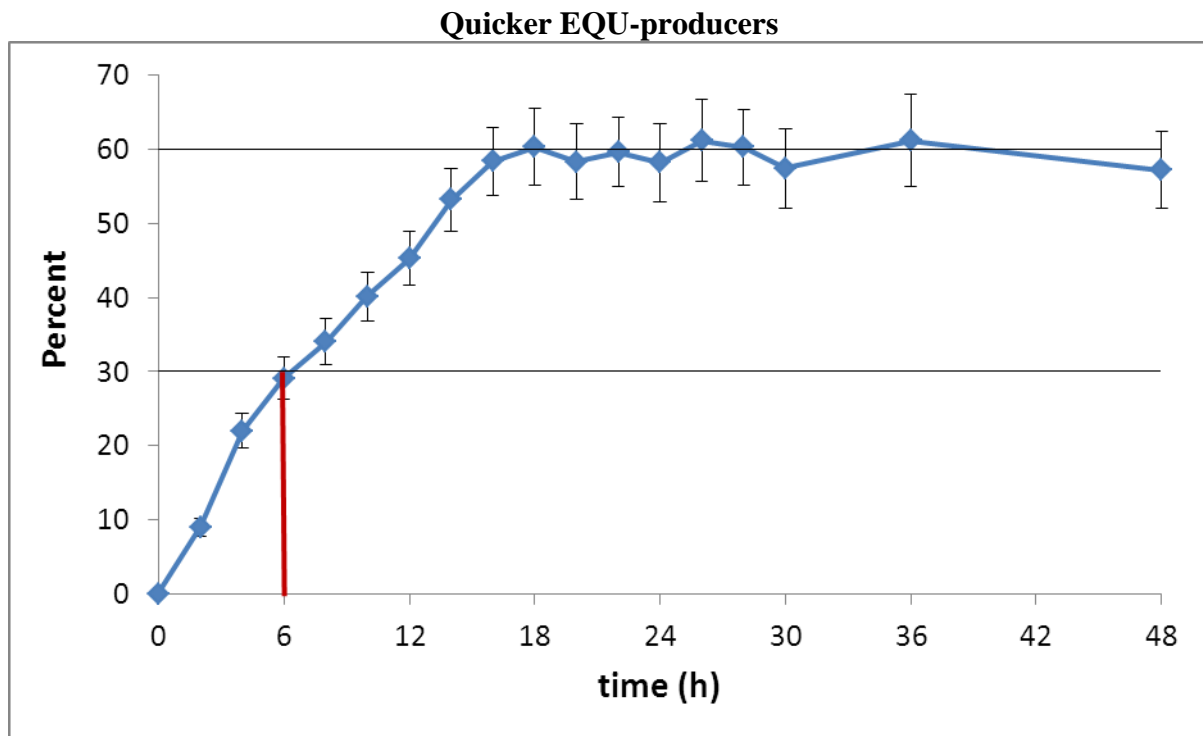


Figure 3

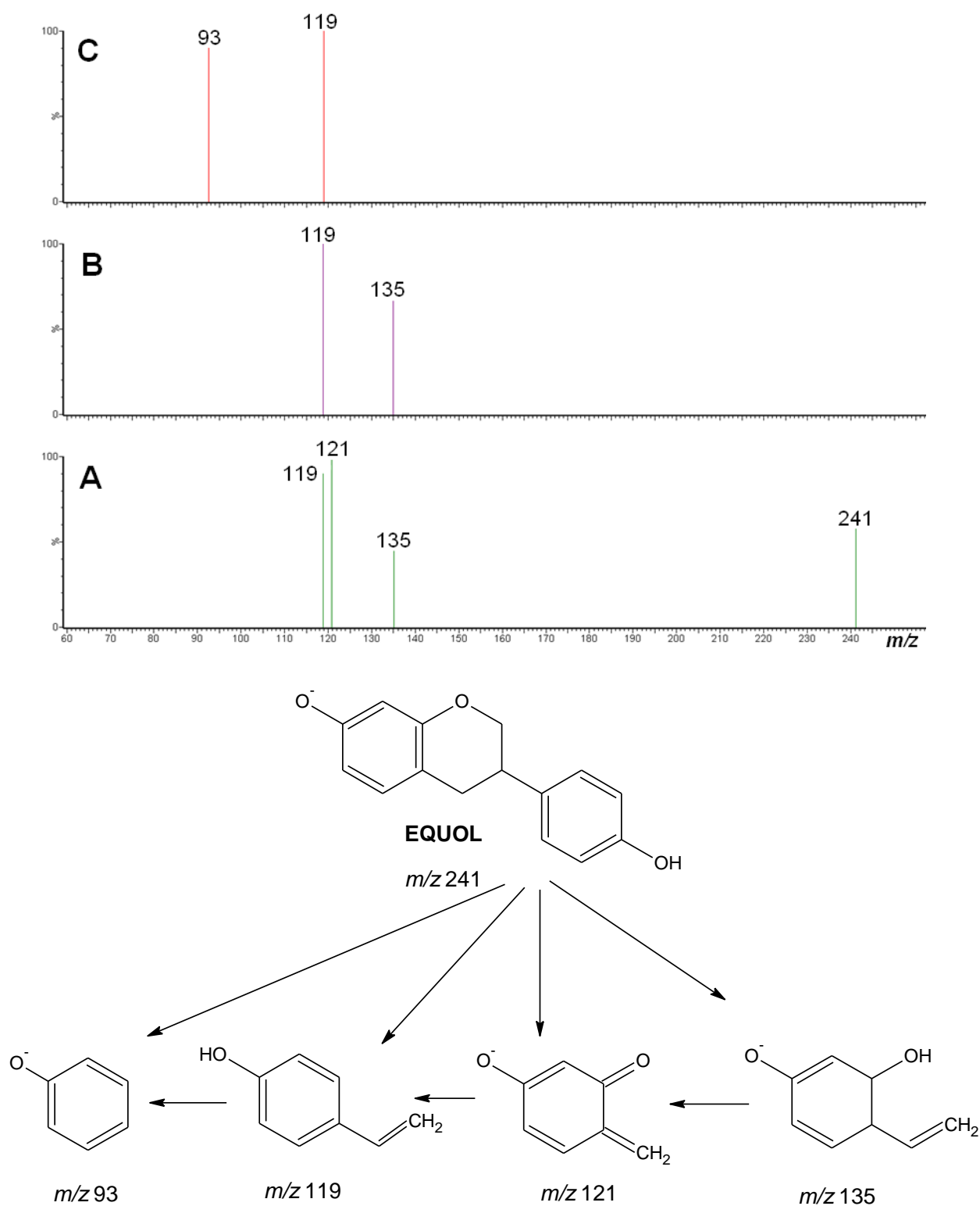


Supplementary 1



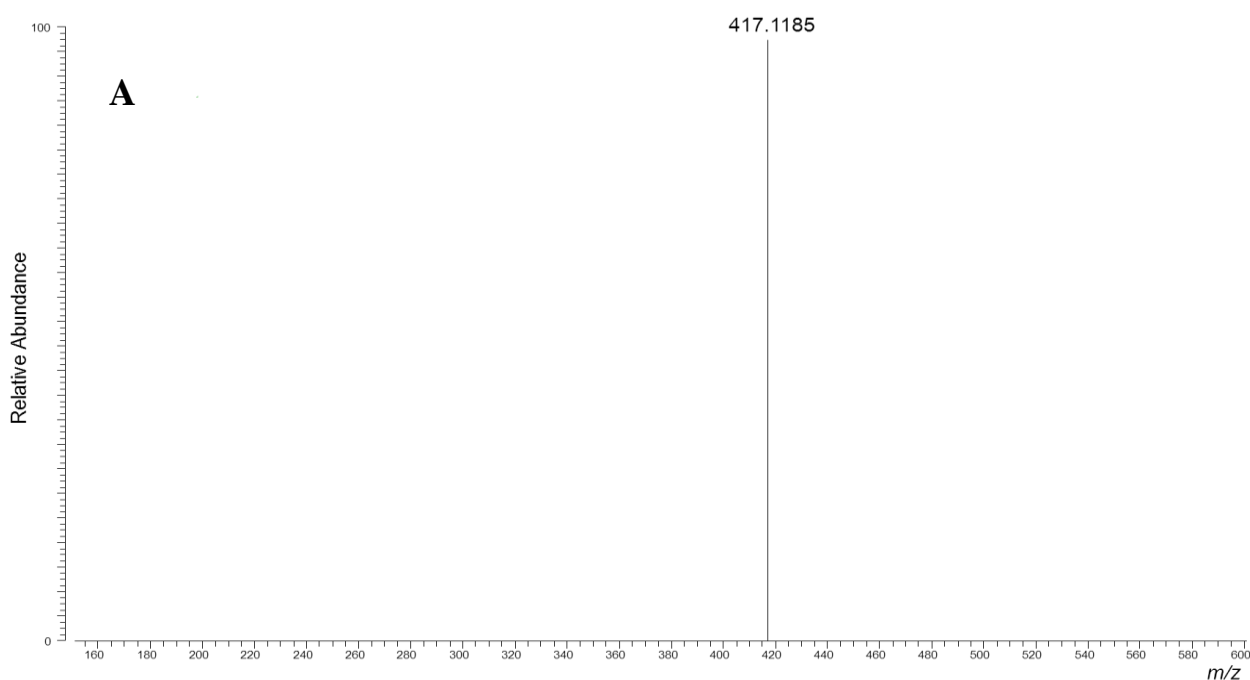
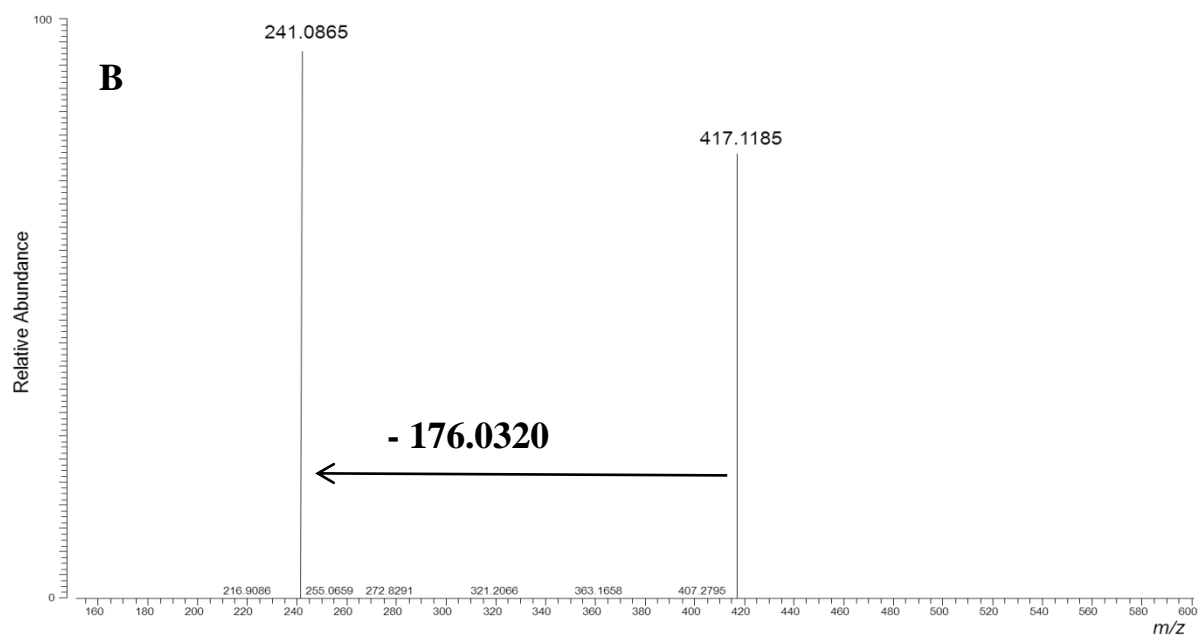
Y value (Percent) \equiv Equal percentage than expected value

Supplementary 2



Equol fragmentation pattern at 10, (A), 15 (B) and 20 eV

Supplementary 3



Extracted ions at m/z 417.1185 u (A), contained in a sample of urine, corresponding to EQU-glucuronide (error 1.4 ppm). In the fragmentation spectra (B) of the EQU-glucuronides (see Fig. 3, peak B1 and B2), obtained by a collision energy of 30 V, the main product ion had m/z 241.0865 u (error 2.1 ppm), which correspond to EQU. The fragmentation pattern (B) show the loss of the glucuronic moiety (-176.0320 u, error 0.6 ppm).

Graphic vision

