Detection and quantification of phyto-oestrogens in different matrices : development of a protocol based on UPLC®-MSMS method

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University Erlangen-Nuremberg

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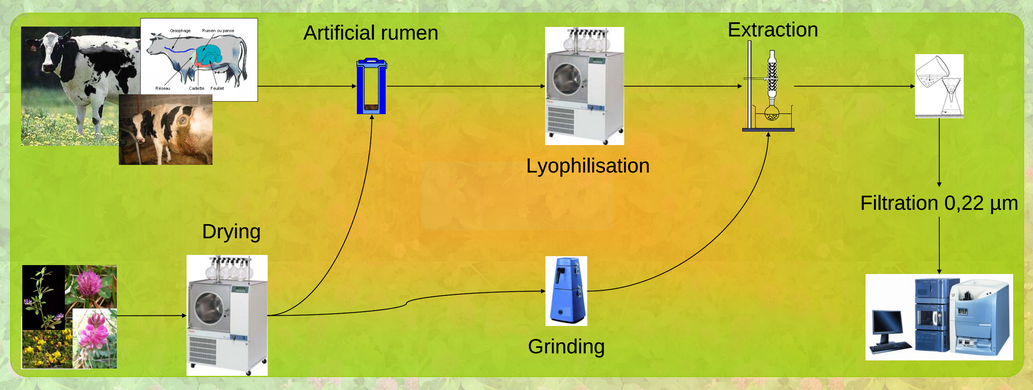
The main objective of this work describes the development of a simple analytical protocol making it possible to follow several phyto-oestrogens during their metabolization by a cow. The methodology associates an ultrasonic extraction with an ultra liquid chromatography, coupled to a tandem quadrupole mass spectrometer.

This analytical protocol could be applicable to the plant (food) and to the end product (milk, for example), but also to the blood plasma, the urine and the faeces.

Standards and samples preparation – UPLC®-MSMS Conditions



The samples of plants are dried by freeze-drying before being crushed with IKA mill. A 500 mg sample is weighed in a balloon, to which 40 mL of methanol 80% are added. A vigreux column makes it possible to avoid the solvent losses. The phyto-oestrogens are extracted from the plant by ultrasounds with 75°C during 1 hour. The solvent is recovered and filtered on 0.22 µm before injection in UPLC-MSMS.

The samples obtained for the juice of rumen are prepared in the following way : a bottle containing 25 mL of the juice of rumen taken on a cow, fitted with a rumen canula, and buffer in proportion 1:4 is put to incubate after addition of a given quantity of plant. At the end of incubation, the samples are conditioned by freeze-drying before extraction. The totality of freeze-drying is transferred in the balloon, to which the 40 ml of methanol 80% are added. The extraction itself and the UPLC-MSMS analysis are conducted as for the plants.

UPLC® conditions (Acquity® System - Waters)

Column : UPLC HSS T3 1.8 µm – 2.1 * 100 mm
Injection : 10 µL
Column temperature : 40°C
Run : 15,5 minutes
Flow : 0.600 mL·minutes^{-1}
Samples temperature : 4°C

Mobile phase

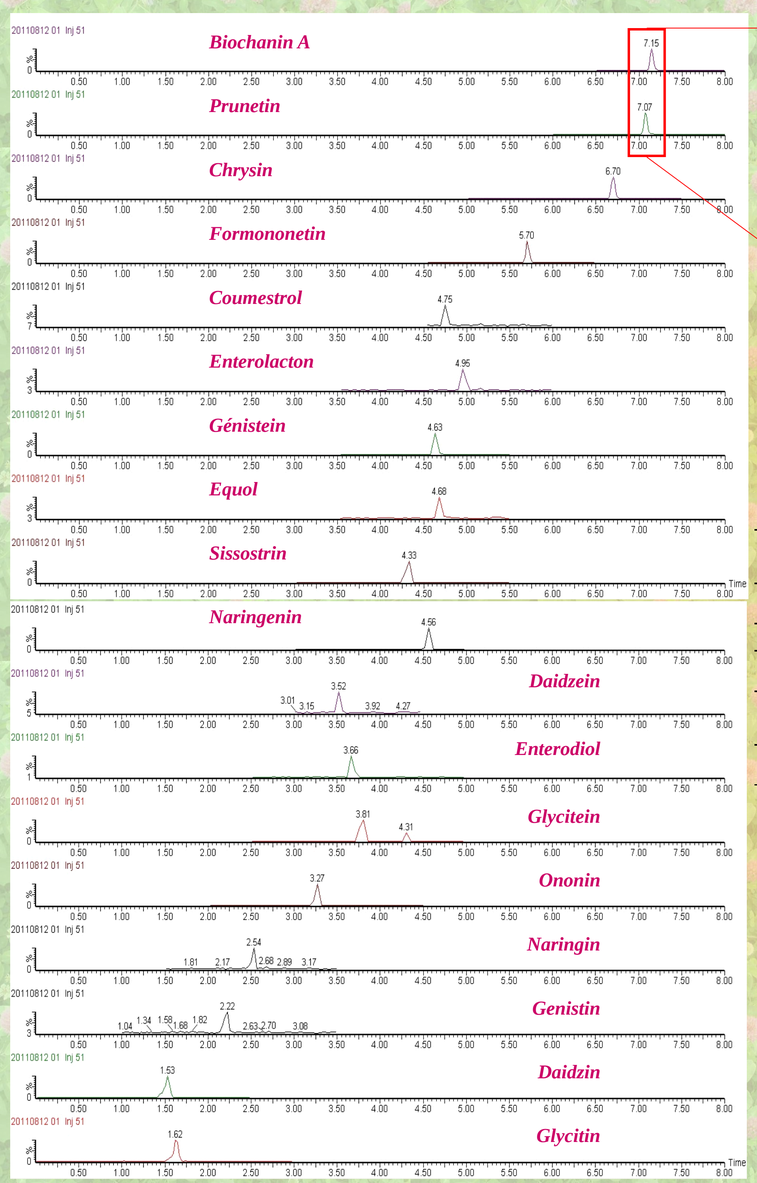
A : H_2O – Acetonitril – Formic Acid 95 - 4.5 - 0.5
B : Acetonitril – H_2O – Formic Acid 95 - 4.5 - 0.5

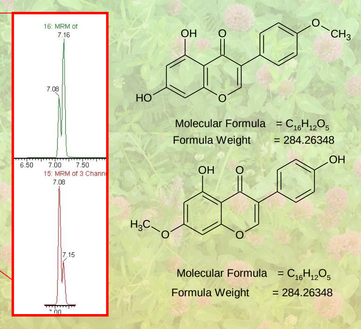
	Time (min.)	% A	% B
1	*Initial*	90.0	10.0
2	15.00	10.0	90.0
3	15.30	90.0	10.0

Mass spectrometer conditions (Quatro Premier XE – Micromass)

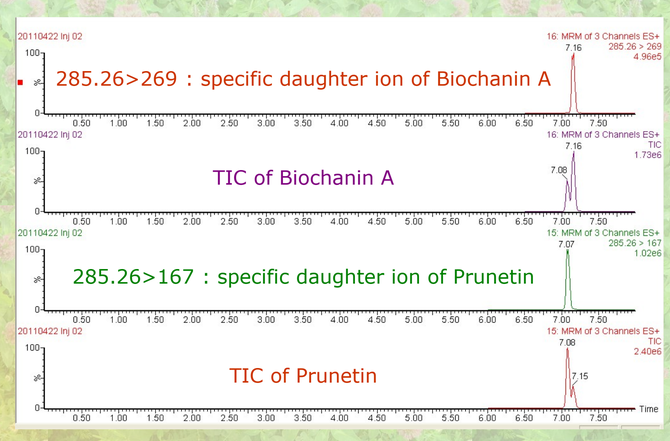
Collision gas : argon
Desolvation gas : nitrogen
Source temperature : 120 °C
Desolvation temperature : 350 °C
Desolvation gas flow : 650 L·H^{-1}
Cone gas flow : 50 L·H^{-1}
Collision gas flow : 0.13 mL·min^{-1}

Results





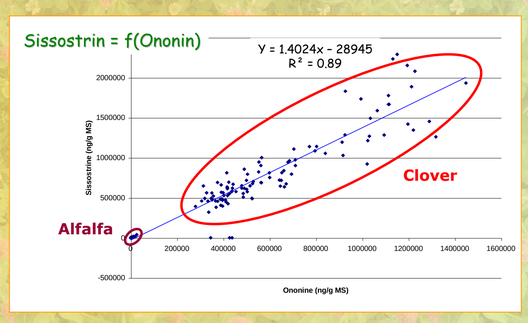
Bad resolution between biochanin A and prunetin, solved using specific transition of each component

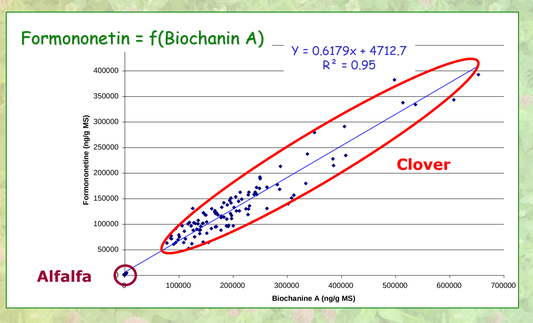


Analysis of a 18 phyto-oestrogens mix, including Prunetin and Biochanin A
Use of developped UPLC-MSMS method with specific transitions for both components

Plant analysis

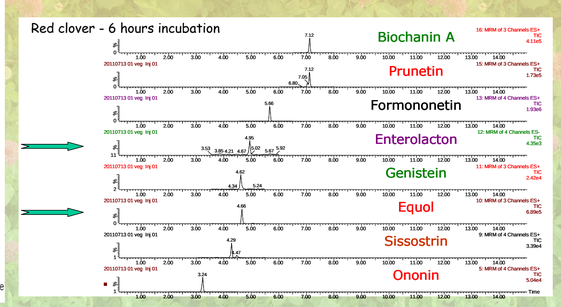
µg/g	Daughter ions	Herbe silage Lyophilised	Corn silage	Lucerne Lyophilised	Clover Dried
Daidzin	417.38>137			1,3	36,0
	417.38>199			0,7	34,6
	417.38>227			1,0	34,1
	417.38>255			1,0	33,5
Ononin	431.40>136			86,6	149,3
	431.40>213.5			63,0	116,9
	431.40>253.50			77,7	141,6
Naringenin	273.25>147	4,2	3,0	0,9	5,1
	273.25>153	3,2	2,2	1,2	3,9
Genistein	271.24>91	3,9		2,9	76,4
	271.24>153	6,9		20,2	69,5
	271.24>215	2,2			52,1
Formononetin	269.26>197	1,7		10,1	130,1
	269.26>213	1,9		11,7	141,4
	269.26>237	1,7		11,1	123,2
	269.26>254	1,8		11,7	132,8
Biochanin A	285.26>213	9,1		7,9	117,1
	285.26>242	8,5		7,8	146,4
	285.26>269	8,9		8,4	121,4



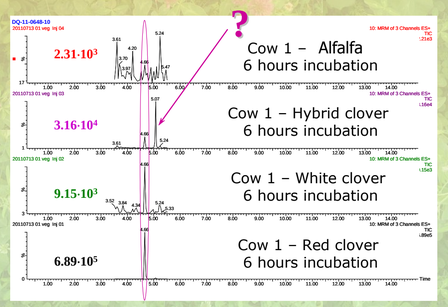


Relation between differents compounds - Analysis of 108 Red clover samples and 35 alfalfa

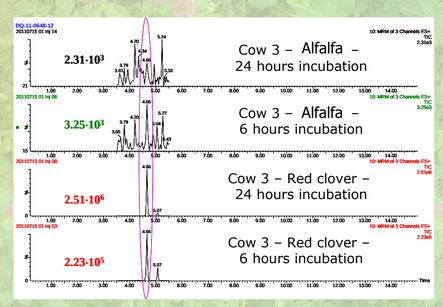
Rumen analysis



Apparition of equol and enterolacton after 6 hours incubation



Production of equol : comparison of 4 plants (3 clovers and alfalfa)



Production of equol : influence of time incubation

UPLC-MSMS separation : mix of 18 molecules

Conclusions

The developed analytical protocol (ultrasonic methanolic extraction and UPLC-MSMS) makes it possible to follow some phyto-oestrogens present in plants throughout their metabolization (in the rumen) and to evaluate their transfer in a product like milk.

18 molecules are separated : 6 phyto-oestrogens in glycosyled form, 8 in a-glycon form, 3 bacterial metabolites (equol, enterolacton and enterodiol) and coumestrol. In order to keep a simple elution gradient, the use of the mass spectrometry makes it possible to solve the bad separation between the prunetin and the biochanin A. The limits of detection on solution are about 1 µg·L^{-1} and the calibration curves obtained between 1 ppb and 1.000 ppb present a R^2 higher than 0,995.

The analysis of 108 samples of clover and 35 samples of alfalfa made it possible to highlight a relation between formononetin and biochanin A (R^2 = 0.95) on the one hand, and between sissotrin and ononin (R^2 = 0.89) on the other hand.

The presence of equol and enterolacton could be highlighted after 6 hours of incubation

The red clover appears as the plant leading to the most important production of equol. The alfalfa practically does not produce equol.

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