FLAVONOID GLYCOCONJUGATES PROFILING IN LEAVES OF BLUE LUPINE *(Lupinus angustifolius)* USING LC/MS^n AND UPLC/MS SYSTEMS

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INTRODUCTION

Flavonoids and isoflavonoids are an important class of plant phenolic secondary metabolites. They play an important role in various biochemical and physiological processes connected with plant response to biotic and abiotic stresses.

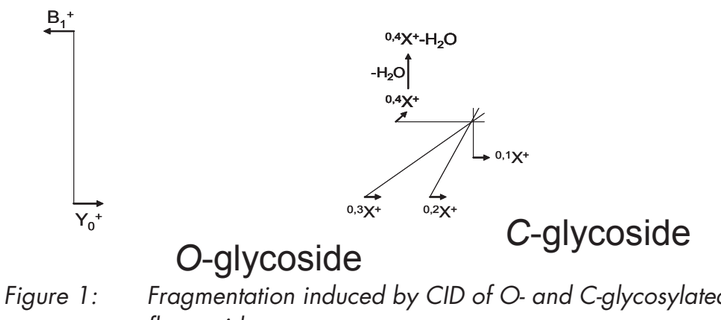

Figure 1: Fragmentation induced by CID of O- and C-glycosylated flavonoids

Flavonoids are usually accumulated in tissues as glycosides, frequently acylated with organic acids (e.g. malonic acid), with a large number of isomeric conjugates differing in the hydroxylation pattern of the aglycone as well as position and pattern of glycosilation and acylation. Identification of different derivatives may be achieved using tandem LC/MS techniques.

In addition, tandem MS can be used to distinguish between *O*– and *C*– glycosylated flavonoids. Fragmentation induced by CID gives rise to Y_0 and B_0 ions for *O*-glycosides but result in cross ring cleavages (X ions) for *C*-glycosides (Fig. 1)

We report here the results of profiling of flavonoid conjugates in leaves of blue lupine (*Lupinus angustifolius*). Phenolic secondary metabolites were extracted from tissues with 80% methanol, SPE was performed on RP C18 columns. Samples (5 µL) were analyzed using LC (RP C18 column)-IT MS (Esquire 3000, Bruker Daltonics, Germany)

UPLC (RP C18 column)-TOF MS (LCT Premier, Waters, UK) analysis was performed on the extracts (1 µL) to investigate the potential of UPLC-MS technology for natural product investigations. Data were acquired simultaneously at low and high source voltages (aperture 1 voltage) to generate both molecular weight and fragment information in a single analysis.

CONCLUSIONS

- **Using traditional LC-IT MS^n analysis of the plant extract 26 different flavonoid and isoflavonoid conjugates (Table 1) were separated and identified in about 45 min. Typical single ion chromatograms are shown in Fig. 2.**
- **Isomeric conjugates with the same molecular mass but different aglycones and/or glycosilation pattern were distinguished (Fig. 3).**
- **The use of UPLC instead of HPLC provides increased resolution and much shorter analysis times; UPLC separation performed in just 15 min! Typical single ion chromatograms are shown in Fig. 4**
- **Preliminary UPLC-Tof pseudo MS/MS experiments (using aperture 1 switching) were used to generate structurally diagnostic fragmentation data (Fig. 5b).**
- **Exact mass measurement and i-FIT can be used to identify the correct chemical formula of flavonoid and isoflavonoid conjugates (Fig. 6)**

RESULTS

No	Compound identified	MW	Rt [min]
1	genistein 7,4'-O-diglucoside	594	24.1
2	genistein 7-O-glucosyl-glucoside	594	26.5
3	genistein 7,4'-O-diglucoside malonylated – I	680	28.1
4	genistein 7,4'-O-diglucoside malonylated – II	680	28.8
5	methylluteolin glucoside-xylosylglucoside	756	29.7
6	quercetin 3-O- glucoside 7-O-rhamnoside	610	31.7
7	2-hydroxy genistein 7-O-glucoside	448	32.0
8	methylluteolin glucoside-xylosylglucoside malonylated	842	32.2
9	genistein 4',7-O-diglucoside-dimalonylated	766	32.3
10	genistein 7-O-glucoside	432	33.1
11	methylluteolin-xylosylglucoside malonylated	680	33.3
12	methylluteolin glucoside-xylosylglucoside-dimalonylated	928	33.8
13	methylluteolin diglucoside	624	34.0
14	genistein-xylosylglucoside	564	34.9
15	genistein 4'-O-glucoside	432	35.2
16	methylluteolin-xylosylglucoside	594	35.4
17	2-hydroxy genistein 7-O-glucoside malonylated	534	35.9
18	methylluteolin glucoside	462	37.2
19	genistein 7-O-glucoside malonylated – I	518	37.8
20	genistein-xylosylglucoside malonylated	650	38.0
21	methylluteolin-xylosylglucoside malonylated – I	680	38.3
22	genistein 7-O-glucoside malonylated – II	518	39.0
23	methylluteolin-xylosylglucoside malonylated – II	680	39.6
24	genistein glucoside malonylated	518	39.8
25	methylluteolin glucoside malonylated	548	40.0
26	methylluteolin-xylosylglucoside-dimalonylated	766	41.2

* only identified using negative mode

Table 1: Flavonoid and Isoflavonoids identified in extracts from Lupinus angustifolius at three different ages by LC-MS^n (Esquire 3000 Ion Trap)

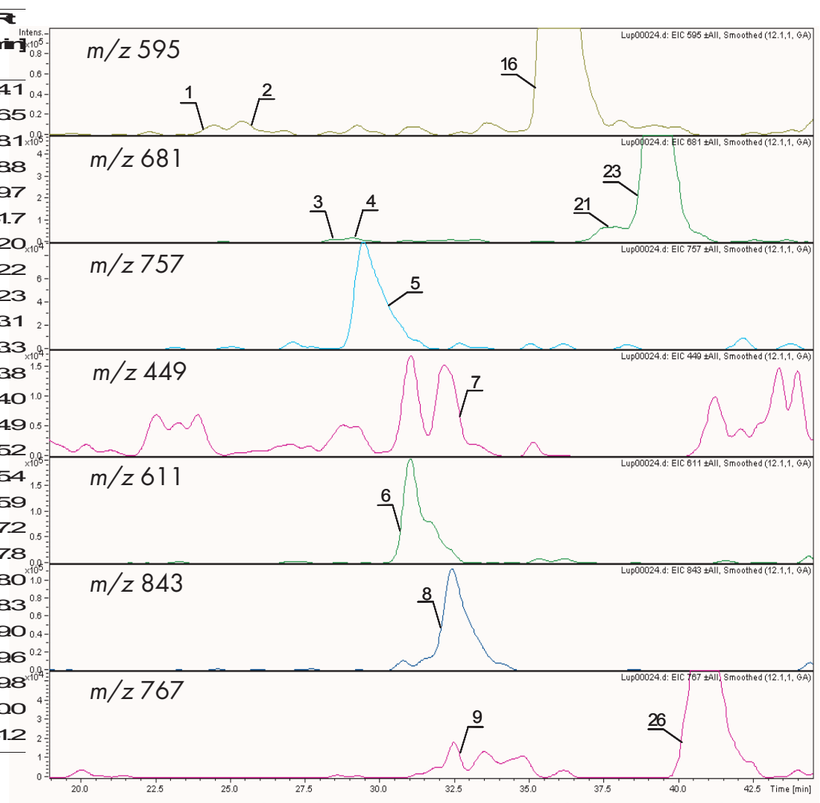

Figure 2: Typical single ion chromatograms of Flavonoid and Isoflavonoids identified in the Lupinus angustifolius extract obtained on LC-MS analysis (Esquire 3000 Ion Trap)

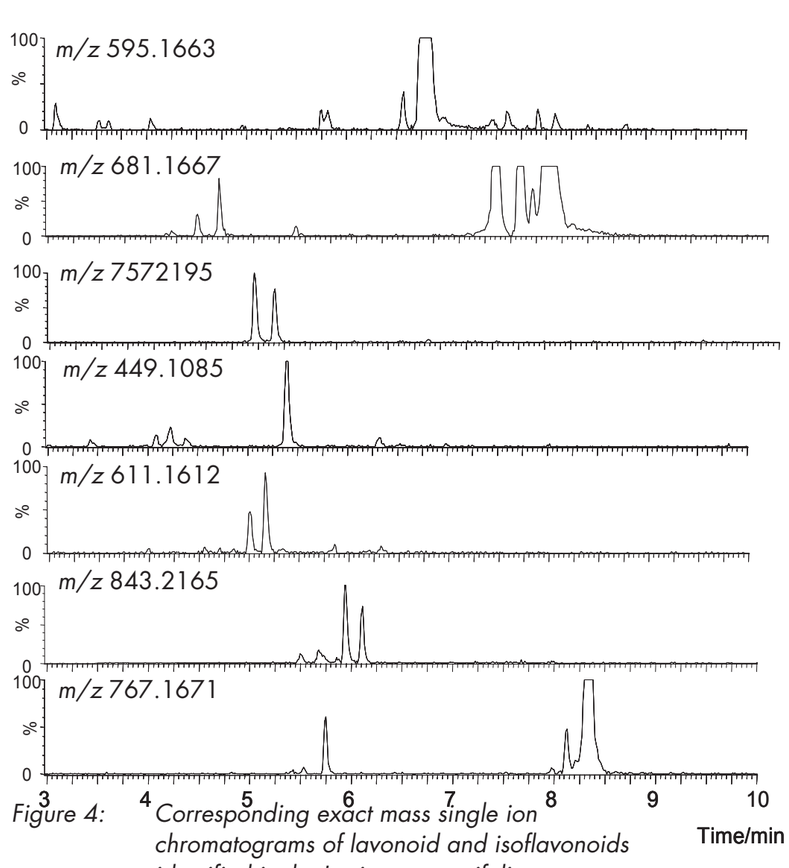

Figure 4: Corresponding exact mass single ion chromatograms of lavonoid and isoflavonoids identified in the Lupinus angustifolius extract (UPLC-MS (LCT Tof) analysis)

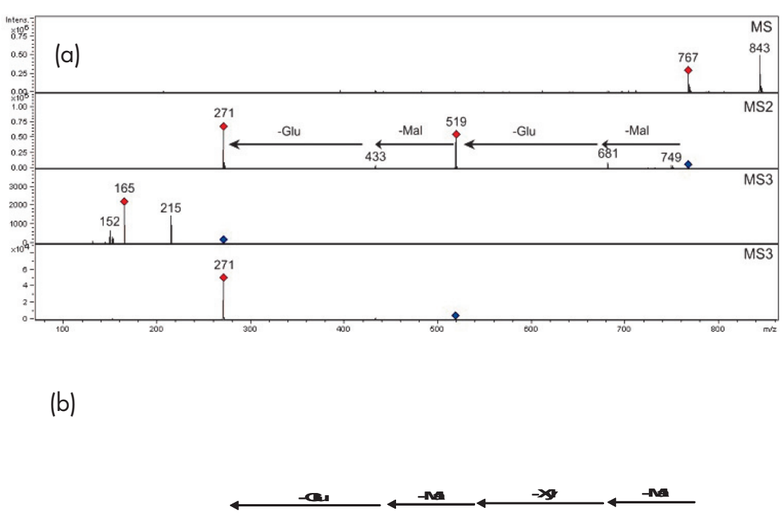

Figure 3: Example fragmentation data obtained on LC-MS^n (Esquire 3000 Ion Trap) analysis
(a) genistein 4',7-O-diglucoside dimalonylated
(b) methylluteolin xylosylglucoside dimalonylated

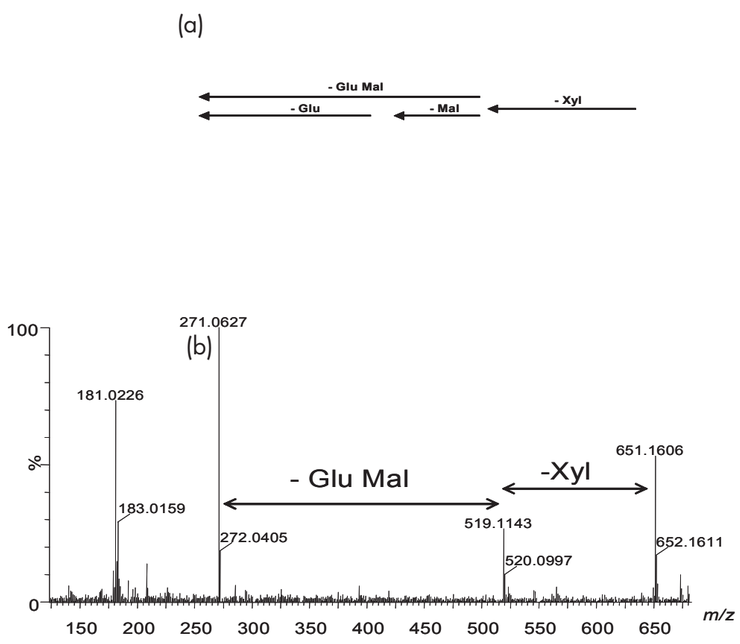

Figure 5: Comparison of fragmentation data obtained for genistein xylosylglucoside malonylated
(a) LC-MS^n (Esquire 3000 Ion Trap) analysis
(b) UPLC-Tof pseudo MS/MS experiments (ap 1 =40)

DISCUSSION

A profile of the flavonoid conjugates extracted from leaves of blue lupine (*Lupinus angustifolius*) using LC-MS^n has been performed. LC-MS^n technology enabled identification of 26 flavonoid glucoconjugates (Table 1).

Both isofalvone and flavone conjugates were identified. CID MS^n spectra permitted us to some extent to propose the glycosilation patterns (Fig 3,5a), it was possible to evaluate the degree of acylation of flavonoid conjugates with malonic acid. However, MS^n spectra registered for aglycone ions in the mass spectrum of minor compounds did not give structural information about flavone or isoflavone.

We have demonstrated that the UPLC can drastically reduce LC analysis times. The enhanced chromatographic resolution has in many cases enabled us to identify analytes that may be additional structural isomers (Table 1). For example, two flavonoid conjugates with *m/z* 767 have been identified and assigned using the data obtained on LC-MS^n analysis. However, the improved resolution obtained using UPLC (Fig. 4) enables us to identify an additional peak that may well be caused by another structural isomer.

Our preliminary studies to obtain fragmentation data using aperture 1 switching on the LCT have been successful. Fragmentation data was obtained for most major peaks (example data Fig. 5B and 6B). Potential compounds of interest could be screened by plotting exact mass chromatograms of diagnostic fragment ions from the high energy data. This technique however is limited if co-elution of two analytes occurs, particularly when common fragment ions are produced. Though the information generated provides a sound basis for deciding which targeted UPLC/MS/MS analysis to perform.

Using exact mass data (in most cases < 3 ppm) and the i-FIT software (Fig. 6) we have been able to propose the chemical formulae of many of the ions giving rise to peaks in the selected ion chromatograms.

$C_{27}H_{31}O_{15}$ 595.1663	t_r (min)	3.089	6.56	6.75		
	measured *m/z*	595.1642	595.1653	595.1665		
	error (ppm)	3.5	1.7	1.3		
	i-FIT score	1.4	6	9.6		
$C_{30}H_{33}O_{18}$ 681.1667	t_r (min)	4.45	4.65	7.36	7.57	7.84
	measured *m/z*	681.1677	681.1677	681.172	681.1665	681.1671
	error (ppm)	1.5	1.3	7.8	0.3	0.1
	i-FIT score	1	0.5	2	2.8	24
$C_{33}H_{41}O_{20}$ 757.2191	t_r (min)	4.96	5.15			
	measured *m/z*	757.2180	757.2175			
	error (ppm)	1.5	2.1			
	i-FIT score	0.1	0			
$C_{21}H_{21}O_{11}$ 449.1085	t_r (min)	5.39				
	measured *m/z*	449.1073				
	error (ppm)	2.4				
	i-FIT score	0.2				
$C_{27}H_{31}O_{16}$ 611.1612	t_r (min)	5				
	measured *m/z*	611.1614				
	error (ppm)	0.3				
	i-FIT score	1				
$C_{36}H_{43}O_{23}$ 843.2195	t_r (min)	6.1	5.93	5.5	5.67	
	measured *m/z*	843.2200	843.2181	843.2164	843.2161	
	error (ppm)	0.6	1.7	3.7	4	
	i-FIT score	1.1	0.9	0.1	0.9	
$C_{33}H_{35}O_{21}$ 767.1671	t_r (min)	5.74	8.82	8.11		
	measured *m/z*	767.1664	767.1635	767.1682		
	error (ppm)	0.9	4.7	1.4		
	i-FIT score	5.74	8.82	8.11		

Table 2: Summary of ions detected with correct chemical formulae for selected flavonoid and isoflavonoids in the Lu pinus angustifolius extract (obtained on UPLC-MS (LCT Tof) analysis (single scan data)

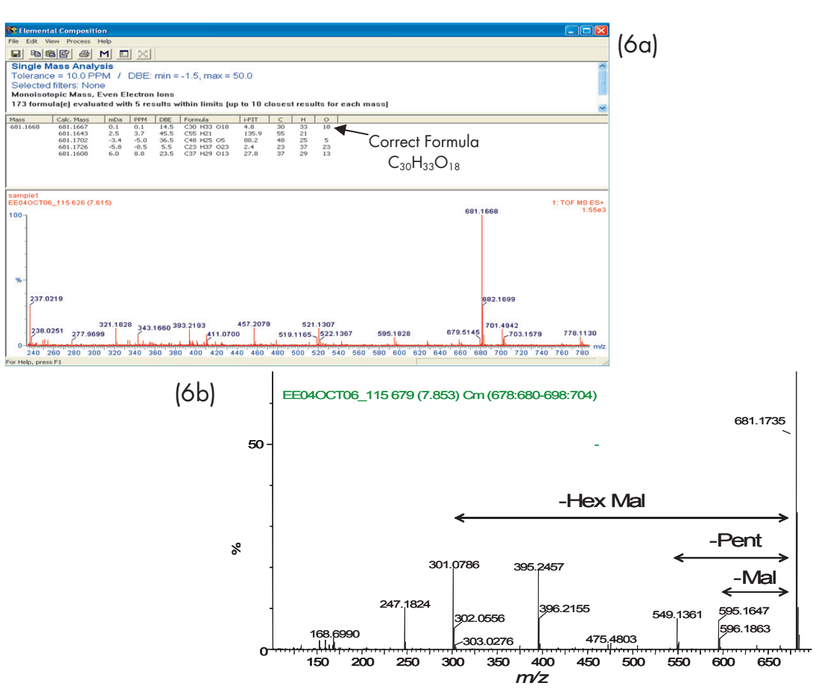

Figure 6: (a) Illustration of elemental composition analysis calculator with mass measurement errors and determined elemental composition for methylluteolin xylosylglucoside malonylated (b) psuedo MS/MS data

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