LC-MSMS Profiling of Flavonoid Conjugates in Wild Mexican Lupine, *Lupinus reflexus*

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Profiles of flavonoid conjugates present in the root and leaf tissues of the Mexican wild lupine, *Lupinus reflexus*, were established using two LC-MSMS systems in the positive and negative ion modes. The ion trap mass spectrometer and quadrupole time-of flight instrument provided sequential MS^n spectra and MSMS spectra with accurate *m*/*z* values of $[M + H]^+$ and $[M - H]^-$ ions, respectively. Sixty-two flavone and isoflavone glycoconjugates were found and tentatively identified. Numerous isomeric or isobaric compounds with the same molecular mass could be differentiated. Isomeric di- and mono glucosides of biochanin A, genistein, 2′-hydroxygenistein, luteone, and 2,3-didehydrokievitone were distinguished on the basis of relative abundances of product ions. The studied flavonoid glycoconjugates were acylated with dicarboxylic aliphatic acids and their methyl esters at either the aglycone or glycosidic moiety.

Plants constitute an important group of living organisms used as food and feed sources that are rich in biologically active low molecular weight compounds. They also represent a valuable source of industrial raw materials, e.g., for construction, in the paper industry, as biofuels, and for biotechnological or pharmaceutical purposes. Thus research in various areas of plant sciences is rapidly growing.[1] Many of the practical applications of plant material depend on the presence of specific primary and/or secondary metabolites. It is commonly understood that a total qualitative and quantitative analysis of primary and secondary metabolites of a chosen plant species is a difficult task due to the presence of a large number of compounds. For example, it is estimated that about 5000 metabolites are present in *Arabidopsis thaliana*, and metabolomes of plant species with more developed genomes may contain more than 10 000 natural products.[2]

Flavonoids and their derivatives comprise a large group (ca. 7000 compounds) of secondary metabolites that are, most probably, present in all terrestrial plant species.[3] The structure of many of these compounds was characterized for pure entities using different physicochemical methods. However, flavonoids and their derivatives are often isomeric or isobaric compounds with different substitution patterns but very similar chromatographic properties that results in co-elution of numerous metabolites. Analysis of plant tissue extracts is challenging due to the large number of compounds present, differences in contents of individual compounds, and the low dynamic range of the detectors used in the chromatographic systems. Application of LC-MS has been frequently reported for the analysis of this group of secondary metabolites, and several reviews of this topic have been published.[3–8] The structural information available from the analyses performed using the HPLC-MSMS systems is rather limited, and for this reason HPLC-NMR systems have been applied in studies of flavonoids.[9–13] However, the sensitivity of NMR spectrometers is much lower than that of MS, and the analysis of co-eluting compounds is more difficult in this case. HPLC-MS, despite its limitations, has become an analytical technique frequently chosen for the analysis of plant flavonoids and different physiological and biochemical problems, as well as methodical issues.[14–22] Nevertheless, efficient analytical methods for separation and structural elucidation of unknown flavonoid compounds present in complex mixtures in plant tissues are still needed.

Lupinus is an evolutionary unique plant genus because it evolved independently in the Mediterranean area and in South and North America, forming species differing in many features.[23,24] Profiles of flavonoids synthesized in tissues of the Old World lupines as well as the glycosylation and malonylation pattern of these compounds have been thoroughly investigated using HPLC-MS techniques.[20,21,25–28] However, knowledge of the presence of these compounds in the New World lupine species is rather limited, and a systematic analysis of flavonoid glycocconjugates has been reported for only two species originating from North America (*L. exaltatus* and *L. hartwegii*, synonym of *L. mexicanus*).[29,30] Large differences in the flavonoid content and profiles between American and European lupines could be anticipated due to the divergence of alkaloids in these plant species.[31] A well-established protocol for targeted profiling of these compounds substituted with novel groups on either sugar or aglycone moieties is presented in this article.

Flavonoids of *L. reflexus* were analyzed using two different LC-MS systems. The structures of 62 compounds are proposed on the basis of the recorded tandem mass spectra and accurate masses of $[M + H]^+$ ions. For most compounds full sets of data (exact masses, CID MSMS spectra recorded in both negative and positive ion modes, and sequential MS^n spectra) were obtained. However, in some cases the data were insufficient to establish specific structural features such as the aglycone glycosylation pattern.

Results and Discussion

Preparation of the wild Mexican lupine, *L. reflexus*, extract samples for LC-MSMS profiling included a purification step by solid-phase extraction (SPE). Columns with a strong cation exchanger (SCX) were used for removal of quinolizidine alkaloids abundant in the analyzed plant material. Otherwise the alkaloids were strongly retained on the C-18 HPLC columns and modified their performance, causing tailing and change of retention time registered for consecutive flavonoid metabolites. Two different systems were used for the LC-MS analyses of extracts from *L. reflexus* roots and leaves. The first one consisted of a standard HPLC pump and an ion trap MS analyzer operating with low resolution; in the second one an RRLC (rapid resolution liquid chromatography) instrument was hyphenated with a high-resolution MS analyzer (QToF). The LC-MS analyses were performed using either positive or negative ionization in separate runs.

Complementary results were obtained from both LC-MS systems used. The most important advantage of the high-resolution system was the possibility of elemental composition confirmation for the

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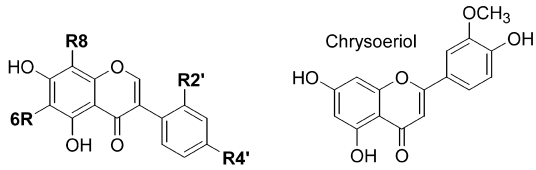

Isoflavone	R2`	R4`	R6	R8
Genistein	H	OH	H	H
2`-Hydroxygenistein	OH	OH	H	H
Biochanin A	H	OCH_3	H	H
Luteone	OH	OH	$-CH_2-CH=C(CH_3)_2$	H
2,3-didehydrokievitone	OH	OH	H	$-CH_2-CH=C(CH_3)_2$

$-CO-CH_2-COOR$ $-CO-CH_2-CHOH-COOR$

Acyl	R	Acyl	R
Malonic acid	H	Malic acid	H
Methyl malonate	CH_3	Methyl malate	CH_3

Figure 1. Structures of flavonoid aglycones recognized in glycosides identified in *L. reflexus* and structures of acyl substituents.

protonated molecules, $[M + H]^+$. For more than half of the analytes (35 compounds) the error of the *m/z* values was below 2 ppm, and for five compounds it exceeded 5 ppm. On the other hand, flavonoid aglycones present in the studied glycoconjugates could be unambiguously identified on the basis of the MS^n (in some cases up to MS^5) product ion spectra obtained in the ion trap and quasi MS^3 spectra in QToF in comparison with the spectra registered for the standards. Glycoconjugated flavonoids are present in plant tissues in numerous isomeric (derivatives of the same aglycone differing in the substitution pattern) and isobaric (compounds with the same molecular weight that are derivatives of different aglycones) forms. This results in a complex profile of compounds with various aglycones substituted with different sugars and acylated with different acids. Structural differences between these moieties are based on the presence or absence of certain hydroxy, methyl, or methoxy groups located in distinct parts of the molecule. There are several sources of the structural variability among the 62 flavonoid compounds recognized in *L. reflexus* (Figure 1; Table 1): (1) existence of flavone and isoflavone aglycones; (2) various hydroxylation and methoxylation of the isoflavone core (presence of genistein, 2′-hydroxygenistein, and biochanin A); (3) prenylation of isoflavones at different positions (luteone and 2,3-didehydrokievitone); (4) glycosylation of the aglycones at different hydroxy groups (*O*-glycosides) or carbon atoms (*C*-glycosides); (5) occurrence of diglycosides in the form of *O*-glycosylglucoside, di-*O*-glucoside, or *C,O*-diglucosides; (6) acylation of either the sugar or aglycone moiety with malonic acid, malic acid, or their methyl esters.

Six different aglycones, i.e., one flavone (chrysoeriol) and five isoflavones (genistein, 2′-hydroxygenistein, biochanin A, luteone, and 2,3-didehydrokievitone) (Figure 1), were identified among the phenolic secondary metabolites from the leaves and roots of *L. reflexus*. Only two of these compounds, genistein and 2′-hydroxygenistein, were detected as free aglycones in minute amounts. Fragmentation of the aglycones was observed in the product ion mass spectra registered in MS^n experiments in ion trap or in "quasi MS^3" spectra obtained with a QToF analyzer in which a high ionization potential was applied to achieve high in-source fragmentation (data not presented). Confirmation of the aglycone identity was achieved after comparison of the CID MSMS spectra of the analyzed compounds with those of standards. Four of the isoflavonoid diglycosides (**8**, **12**, **28**, **39**; MW = 678) contain the prenylated aglycones 2,3-didehydrokievitone and luteone, which differ only by the prenyl substitution site (Figure 1). 2,3-Didehydrokievitone and luteone diglycosides could be distinguished by differences of their retention times during HPLC and relative intensities of product ions in the respective mass spectra (Figure 2). However, only luteone was available as a standard. Additionally, biochanin A was identified as the aglycone present in nine glycoconjugates (Table 1). 2,3-Didehydrokievitone and luteone were isolated as free aglycones from the roots of *L. luteus* and *L. albus*, respectively,[32,33] but neither of these compounds nor biochanin A was found in glycosylated forms in European lupines.[20,25–27] Luteone and wighteone were accumulated as free aglycones in the leaves of *L. angustifolius* infected by *Colletotrichum lupini*, a pathogenic fungus causing anthracnose of lupines,[21] which is consistent with findings of Ingham et al.,[32] who showed the phytoalexin activity of these isoflavones.

The glycosidic part of the European lupine (*L. albus, L. angustifolius*, and *L. luteus*) flavonoid glycoconjugates may consist of one or two moieties attached to hydroxy groups at 4′-O or 7-O or directly to C-6 or C-8.[20,27,28] These sugar units are mono- or diglycosides comprising glucose, rhamnose, and xylose. However, none of the mass spectra recorded for *L. reflexus* samples contained neutral loss fragments corresponding to pentose and deoxyhexose units (132 and 146 amu, respectively) or diglycosides containing these sugars. It was therefore concluded that all the flavonoid glycoconjugates detected in *L. reflexus* were mono- and diglucosides (Table 1). Isomeric diglucosides of genistein and 2′-hydroxygenistein with different substitution pattern of the aglycones were detected and could be distinguished by differences of the CID product ions or by the relative intensities of the fragment ions (Figure 3).

Malonylation is an important structural feature of European lupine flavonoids.[20,27,28] Sugar moieties of these glycoconjugates may be esterified by one but seldom two malonic acid (Figure 1) molecules at different positions, giving rise to several isomeric compounds. Five different malonylated xylosyldiglucosides of chrysoeriol from the leaves of *L. angustifolius* could be chromatographically separated[28] and distinguished on the basis of mass spectra.[20] There is a noteworthy difference in the fragmentation mechanisms of malonylated compounds in the positive and negative ion modes. Whereas the malonyl group is released from the $[M + H]^+$ ion, its stepwise fragmentation is observed in the negative ion mode (Figure 4). The free carboxylic moiety of malonic acid is readily eliminated, and frequently $[M - CO_2 - H]^-$ ions instead of the respective deprotonated molecules $[M - H]^-$ are observed in the MS spectrum. The rest of the substituent is released as the ketene moiety (42 amu) or remains on the glucose moiety, giving a neutral loss of 204 amu.

The most remarkable difference between flavonoid glycoconjugates from *L. reflexus* and those from previously studied European lupines is the presence of substituents other than malonic acid. The presence of three different acyl groups is suggested from the MSMS spectra, in which elimination of neutral fragments of 100, 116, and 130 amu was observed (Figures 5 and 6). According to the accurate mass measurements, these fragments correspond to $C_4H_4O_3$, $C_4H_4O_4$, and $C_5H_6O_4$, respectively. In several MSMS spectra further fragmentation of the $C_4H_4O_3$ was observed due to elimination of CH_3OH (32 amu, Figure 6), leading to the conclusion that the substituent present in compounds **18**, **22**, **26**, **36**, **38**, **43**, **46**, **49**, **52**, **56**, and **60** was the methyl ester of malonic acid. The data also suggest that the two remaining substituents include malic acid present in compounds **5**, **9**, **15**, **16**, **31**, **34**, **37**, **42**, **54**, and **57** and its methyl ester in compounds **17**, **35**, **48**, and **62** (Table 1, Figure 1). In several cases the MSMS spectra provided evidence of the attachment of the mentioned substituents directly to the aglycone and not to a sugar moiety (Figures 5, 6).

It should be noted that some of the flavonoid glycosides were acylated with two malonic acid moieties (**20**, **27**, **41**, **45**, and **59**) or two different acyls (**38** and **42**). Malic acid has been reported as an acyl substituent of several anthocyanins[3] as well as apigenin and kaempferol glycosides of carnation (*Dianthus caryophyllus*).[34]

Table 1. Flavonoid Glycoconjugates Identified in Roots and Leaves of *Lupinus reflexus*

no.	t_R [min][a]	compound	root/leaf[b]	MW	elemental composition of $[M + H]^+$ ion	exact mass of $[M + H]^+$ ion calcd	exact mass of $[M + H]^+$ ion obsd	error [ppm]	fragmentation pathway (product ions obtained from precursor ions[c]) positive ions $[M + H]^+$ [*m*/*z*]	fragmentation pathway (product ions obtained from precursor ions[c]) negative ions $[M - H]^-$ [*m*/*z*]
1	2.75	2′-hydroxygenistein 4′,7-*O*-diglucoside	b/a	610	$C_{27}H_{31}O_{16}$	611.1607	611.1612	−0.8	611, 449, 287	609, 447, 285
2	3.63	2′-hydroxygenistein 7-*O*-glucosylglucoside	a/a	610	$C_{27}H_{31}O_{16}$	611.1607	611.1602	0.8	611, 449, 287	nr
3	4.02	genistein 4′,7-*O*-diglucoside	b/a	594	$C_{27}H_{31}O_{15}$	595.1657	595.1650	1.2	595, 433, 271	593, 431, 269/268
4	4.33	2′-hydroxygenistein *C,O*-diglucoside	a/a	610	$C_{27}H_{31}O_{16}$	611.1607	611.1617	−1.6	611, 449, 431, 413	609, 447, 327
5	4.47	2′-hydroxygenistein 4′,7-*O*-diglucoside malylated	a/a	726	$C_{31}H_{35}O_{20}$	727.1716	727.1725	−1.2	727, 565, 449, 287	725, 609, 489
6	4.58	genistein 6,8-*C*-diglucoside	a/nd	594	$C_{27}H_{31}O_{15}$	595.1657	595.1656	0.2	595, 457, 409, 379	593, 503, 473, 383, 353
7	4.68	2′-hydroxygenistein 4′,7-*O*-diglucoside malonylated (I)	a/a	696	$C_{30}H_{33}O_{19}$	697.1611	697.1611	0.0	697, 535, 449, 287	651, 489
8	4.75	luteone 4′,7-*O*-diglucoside	b/nd	678	$C_{32}H_{39}O_{16}$	679.2233	679.2242	−1.3	679, 517, 461, 355, 299	nr
9	4.89	genistein 4′,7-*O*-diglucoside malylated (I)	a/a	710	$C_{31}H_{35}O_{19}$	711.1767	711.1760	1.0	711, 549, 433, 271	709, 431
10	5.05	2′-hydroxygenistein 7-*O*-glucoside	b/a	448	$C_{21}H_{21}O_{11}$	449.1078	449.1083	−1.1	449, 287	447, 285/284
11	5.12	2′-hydroxygenistein 6-*C*-glucoside	a/c	448	$C_{21}H_{21}O_{11}$	449.1078	449.1095	−3.8	449, 431, 413, 395, 353, 329	447, 327, 297
12	5.27	luteone 7-*O*-glucosylglucoside	b/nd	678	$C_{32}H_{39}O_{16}$	679.2233	679.2237	−0.6	679, 517, 461, 355, 299	nr
13	5.32	2′-hydroxygenistein 4′,7-*O*-diglucoside malonylated (II)	b/a	696	$C_{30}H_{33}O_{19}$	697.1611	697.1630	−2.7	697, 535, 449, 287	695, 533, 371, 285
14	5.44	genistein 4′,7-*O*-diglucoside malonylated	b/a	680	$C_{30}H_{33}O_{18}$	681.1661	681.1660	0.2	681, 519, 433, 287	nr
15	5.56	2′-hydroxygenistein 6-*C*-glucoside malylated	nd/b	564	$C_{25}H_{25}O_{15}$	565.1188	565.1174	2.4	565, 547, 529, 431, 413, 395, 353, 329	563, 447, 357, 327
16	5.61	2′-hydroxygenistein 7-*O*-glucoside malylated	nd/a	564	$C_{25}H_{25}O_{15}$	565.1188	565.1200	−2.1	565, 287	563, 447, 285
17	5.72	genistein 4′,7-*O*-diglucoside methylmalylated	b/nd	724	$C_{32}H_{37}O_{19}$	725.1924	725.1948	− 3.3	725, 563, 433, 271	723, 517, 431, 269/268
18	5.79	2′-hydroxygenistein 4′,7-*O*-diglucoside methylmalonylated (I)	a/b	710	$C_{31}H_{35}O_{19}$	711.1767	711.1767	0.0	711, 549, 449, 287	709, 447, 285
19	5.82	genistein *C*-glucoside	b/b	432	$C_{21}H_{21}O_{10}$	433.1129	433.1136	−1.6	433, 415, 397, 379, 313	431, 311, 283
20	5.85	2′-hydroxygenistein 4′,7-*O*-diglucoside dimalonylated	a/a	782	$C_{33}H_{35}O_{22}$	783.1614	783.1671	−7.3	783, 535, 449, 287	781, 285
21	5.93	genistein 7-*O*-glucoside	b/b	432	$C_{21}H_{21}O_{10}$	433.1129	433.1132	−0.7	433, 271	431, 269/268
22	5.98	2′-hydroxygenistein 4′,7-*O*-diglucoside methylmalonylated (II)	a/nd	710	$C_{31}H_{35}O_{19}$	711.1767	711.1803	−5.1	711, 287	709, 447, 285
23	6.04	genistein 4′-*O*-glucoside	a/nd	432	$C_{21}H_{21}O_{10}$	433.1129	433.1163	−7.9	433, 271	431, 269/268
24	6.11	2′-hydroxygenistein *C*-glucoside malonylated	nd/a	534	$C_{24}H_{23}O_{14}$	535.1082	535.1111	−5.4	535, 517, 499, 473, 431, 413, 395, 329	533, 429, 357, 327, 297
25	6.19	genistein 7-*O*-glucosylglucoside	a/a	594	$C_{27}H_{31}O_{15}$	595.1657	595.1680	−3.9	595, 433, 271	593, 431, 269
26	6.27	genistein 4′,7-*O*-diglucoside methylmalonylated	b/b	694	$C_{31}H_{35}O_{18}$	695.1818	695.1825	−1.0	695, 533, 433, 271	693, 431, 268
27	6.33	genistein 4′,7-*O*-diglucoside dimalonylated	a/a	766	$C_{33}H_{35}O_{21}$	767.1665	767.1646	−2.5	767, 681, 519, 433, 271	765, 473, 431, 269/268
28	6.39	2,3-didehydrokievitone 4′,7-*O*-diglucoside	a/nd	678	$C_{32}H_{39}O_{16}$	679.2233	679.2269	−5.3	679, 517, 461, 355, 299	677, 515, 353/352, 297
29	6.44	2′-hydroxygenistein 7-*O*-glucoside malonylated	b/a	534	$C_{24}H_{23}O_{14}$	535.1082	535.1097	−2.8	535, 449, 287	nr
30	6.56	genistein *C*-glucoside malonylated (I)	nd/b	518	$C_{24}H_{23}O_{13}$	519.1133	519.1143	−1.9	519, 501, 439, 397, 379, 313	473, 413, 311, 283
31	6.62	genistein 7-*O*-glucoside malylated (I)	a/a	548	$C_{25}H_{25}O_{14}$	549.1239	549.1249	−1.8	549, 271	547, 269
32	6.67	genistein 7-*O*-glucoside malonylated (I)	c/nd	518	$C_{24}H_{23}O_{13}$	519.1133	519.1139	−1.2	519, 271	473, 269/268
33	6.72	chrysoeriol diglucoside	nd/a	624	$C_{28}H_{33}O_{16}$	625.1763	625.1771	−1.3	625, 463, 301	623, 299
34	6.81	genistein 4′,7-*O*-diglucoside malylated (II)	a/nd	710	$C_{31}H_{35}O_{19}$	711.1767	711.1793	−3.7	711, 433, 271	nr
35	6.89	chrysoeriol diglucoside methylmalylated	nd/c	740	$C_{32}H_{37}O_{20}$	741.1873	741.1870	0.4	741, 301	nr
36	7.05	chrysoeriol diglucoside methylmalonylated	nd/c	710	$C_{31}H_{35}O_{19}$	711.1767	711.1779	−1.7	711, 465/464, 301	709, 299
37	7.11	genistein 7-*O*-glucoside malylated (II)	a/a	548	$C_{25}H_{25}O_{14}$	549.1239	549.1239	0.0	549, 271	547, 269
38	7.27	genistein 4′,7-*O*-diglucoside malonylated and methylmalonylated	a/nd	780	$C_{34}H_{37}O_{21}$	781.1822	781.1847	−3.2	781, 585, 519, 433, 271	nr
39	7.33	2,3-didehydrokievitone 7-*O*-glucosylglucoside	a/nd	678	$C_{32}H_{39}O_{16}$	679.2233	679.2224	1.3	679, 517, 461, 355, 299	677, 515, 353
40	7.41	genistein 7-*O*-glucoside malonylated (II)	b/a	518	$C_{24}H_{23}O_{13}$	519.1133	519.1139	−1.2	519, 433, 271	473, 269
41	7.53	chrysoeriol *O*-diglucoside dimalonylated (I)	nd/b	796	$C_{34}H_{37}O_{22}$	797.1771	797.1796	−3.1	797, 711, 549, 463, 301	nr
42	7.56	chrysoeriol diglucoside malonylated and malylated	nd/a	826	$C_{35}H_{39}O_{23}$	827.1877	827.1872	0.6	827, 301	825, 781, 299/298
43	7.61	2′-hydroxygenistein *C*-glucoside methylmalonylated	c/c	548	$C_{25}H_{25}O_{14}$	549.1239	549.1259	−3.6	549, 513, 481, 395, 329	547, 269
44	7.72	chrysoeriol *O*-glucoside	nd/a	462	$C_{22}H_{23}O_{11}$	463.1235	463.1244	−1.9	463, 301	461, 298
45	7.79	chrysoeriol *O*-diglucoside dimalonylated (II)	a/a	796	$C_{34}H_{37}O_{22}$	797.1771	797.1768	0.4	797, 711, 549, 463, 301	795, 665, 623, 299, 284
46	7.85	2′-hydroxygenistein 7-*O*-glucoside methylmalonylated	a/a	548	$C_{25}H_{25}O_{14}$	549.1239	549.1267	−5.1	549, 449, 287	547, 285

Table 1 Continued

no.	t_R [min][a]	compound	root/leaf[b]	MW	elemental composition of $[M + H]^+$ ion	exact mass of $[M + H]^+$ ion		error [ppm]	fragmentation pathway (product ions obtained from precursor ions[c])	
						calcd	obsd		positive ions $[M + H]^+$ [*m/z*]	negative ions $[M - H]^-$ [*m/z*]
47	7.89	genistein *O*-glucoside malylated (III)	b/c	548	$C_{25}H_{25}O_{14}$	549.1239	549.1264	−4.6	549, 271	547, 269
48	8.12	genistein *O*-glucoside methylmalylated	a/nd	562	$C_{26}H_{27}O_{14}$	563.1395	563.1407	−2.1	563, 401, 271	561, 431, 269
49	8.19	genistein *C*-glucoside methylmalonylated	nd/a	532	$C_{25}H_{25}O_{13}$	533.1290	533.1297	−1.3	533, 453, 411, 393, 311	nr
50	8.35	chrysoeriol *O*-glucoside malonylated	a/b	548	$C_{25}H_{25}O_{14}$	549.1239	549.1218	3.8	549, 463, 301	547, 299
51	8.53	biochanin A diglucoside malonylated (I)	nd/b	694	$C_{31}H_{35}O_{18}$	695.1818	695.1827	−1.3	695, 447, 285	693, 649, 607, 589, 529, 283
52	8.65	genistein 7-*O*-glucoside methylmalonylated (I)	b/nd	532	$C_{25}H_{25}O_{13}$	533.1290	533.1314	−4.5	533, 271	531, 431, 269
53	8.70	biochanin A diglucoside	nd/a	608	$C_{28}H_{33}O_{15}$	609.1814	609.1817	−0.5	609, 285	607, 283
54	8.84	biochanin A diglucoside malonylated malylated	nd/b	810	$C_{35}H_{39}O_{22}$	811.1927	811.1927	0.0	811, 563, 285	809, 283
55	9.27	biochanin A diglucoside malonylated (II)	nd/b	694	$C_{31}H_{35}O_{18}$	695.1818	695.1835	−2.5	695, 447, 285	693, 649, 607, 589, 529, 283
56	9.42	genistein 7-*O*-glucoside methylmalonylated (II)	b/nd	532	$C_{25}H_{25}O_{13}$	533.1290	533.1300	−1.9	533, 433, 271	531, 499, 431, 269
57	9.48	biochanin A *O*-glucoside malylated	nd/b	562	$C_{26}H_{27}O_{14}$	563.1395	563.1411	−2.8	563, 285	561, 283, 268
58	9.69	biochanin A *O*-glucoside	nd/c	446	$C_{22}H_{23}O_{10}$	447.1286	447.1299	−2.9	447, 285	491(+HCOOH), 283
59	10.02	biochanin A *O*-diglucoside dimalonylated	nd/b	780	$C_{34}H_{37}O_{21}$	781.1822	781.1830	−1.0	781, 695, 533, 447, 285	779, 283
60	11.3	chrysoeriol *O*-glucoside methylmalonylated	a/nd	562	$C_{26}H_{27}O_{14}$	563.1395	563.1428	−5.9	563, 463, 301	Nr
61	11.7	biochanin A *O*-glucoside malonylated	nd/c	532	$C_{25}H_{25}O_{13}$	533.1290	533.1302	−2.2	533, 285	487, 283, 268
62	12.3	biochanin A *O*-glucoside methylmalylated	nd/b	576	$C_{27}H_{29}O_{14}$	577.1552	577.1568	−2.8	577, 285	575, 283, 268

[a] Retention times in the rapid resolution HPLC system. [b] Abundances of ions in the MS spectra from the QToF instrument: a = abundance over 1×10^5; b = abundance in the range 1×10^4 to 1×10^5; c = abundance below 1×10^4; nd = not detected. [c] Major ions observed in both IT and QToF instruments.

However, this is the first report of the presence of malonic and malic acid methyl ester moieties as substituents of flavonoid glycosides. It should be noted that the above structural identifications based on the mass spectral analysis must be regarded as tentative. Nevertheless, there was no possibility to perform more detailed analysis of the studied compounds, e.g., using HPLC-NMR techniques. The analyzed 62 compounds were poorly resolved on the HPLC column, and some of them were present in minute amounts. Overlapping NMR spectra of co-eluting compounds did not give useful information.

Results of analysis of flavonoid glycoconjugates obtained using two LC systems hyphenated with ion trap and QToF mass spectrometers permitted the tentative identification of 62 different compounds. MS provided information sufficient for structural elucidation of flavonoids in extracts obtained from tissues of *Lupinus reflexus*, a plant species not yet investigated. These metabolites were different from those previously identified in lupine species originating from the Mediterranean area (*L. albus, L. angustifolius*, and *L. luteus*); that is, different aglycones were found in *L. reflexus* (biochanin A, luteone, and 2,3-didehydrokievitone), while the only sugar present in the glycosides was glucose and unique acyl substituents (malonic and malic acid and their methyl esters) attached either to the aglycone or the sugar moieties. However, a complete structure characterization of target metabolites was not possible without profound NMR analyses. Attempts to use LC-NMR were not efficient due to co-elution of numerous compounds, some of which occurred in minute amounts. LC-MS analysis of flavonoid glycoconjugate profiles in other Mexican lupines and their chemotaxonomic comparison with European species will continue.

Experimental Section

Reagents and Standards. HPLC solvents were MS grade from Sigma (Poznań, Poland); ultrapure water was obtained using a Millipore (Billerica, MA) model MiliQ Plus system. Standards of flavonoids chrysoeriol (3′-*O*-methylluteolin), apigenin, genistein, and biochanin A were from Extrasynthese (Genay, France); 2′-hydroxygenistein, luteone, and wighteone were a kind gift of Dr. S. Tahara from Sapporo University (structures shown in Figure 1).

Plant Material. Seeds of *Lupinus reflexus* Rose were collected in different locations in Jalisco, Mexico, and taxonomically characterized by Dr. Jacqueline Reynoso Dueńas from Guadalajara University. A plant sample was deposited under the voucher number 58867 in the Herbarium of the Institute of Botany of the University of Guadalajara (IBUG). Plants were grown in pots containing a mixture of sand and perlite (1:1) under controlled greenhouse conditions with an average temperature of 25 °C, 50% humidity, and 16 h photoperiod. Plants were watered every two days and supplemented with fertilizer NPK (6:3:6, 3 g/L, 25 mL per pot) once a week. Six-week-old plants were used for the isolation of flavonoids.

Isolation of Phenolic Secondary Metabolites from Plant Tissue. For the analyses of flavonoid glycosides, frozen leaves and roots (2 g fresh weight) were homogenized in 80% MeOH (12 mL), and the suspension was placed in an ultrasonic bath for 30 min. The extract was centrifuged, and the supernatant was transferred to a screw-capped tube. The solvent was evaporated in a Savant SPD 121P vacuum concentrator (Thermo Electron Corporation, Waltham, MA); during this procedure the tube was placed in a tray rotating under vacuum at room temperature. Dried extract samples were dissolved in 10% MeOH (2 mL), and alkaloids were removed using SPE on SCX (300 mg, Supelco) columns. The loaded SPE columns were washed with 2 mL of 10% MeOH and 2 mL of MeOH, and the eluate was evaporated and stored at −80 °C prior to the LC-MS analyses.

Liquid Chromatography with UV and MS Detection. Analyses of plant extracts were performed with two LC-UV-MS systems. The first consisted of an Agilent 1100 HPLC (Waldbronn, Germany) combined with an ion trap (IT) mass spectrometer, model Esquire 3000 (Bruker Daltonics, Bremen, Germany), and the second was an Agilent RR1200 SL system connected to a micrOTOF-Q spectrometer from Bruker. X-Bridge C-18 columns (150 × 2.1 mm; 3.5 μm grain diameter, Waters) and Zorbax Eclipse XDB-C18 columns (100 × 2.1 mm; 1.8

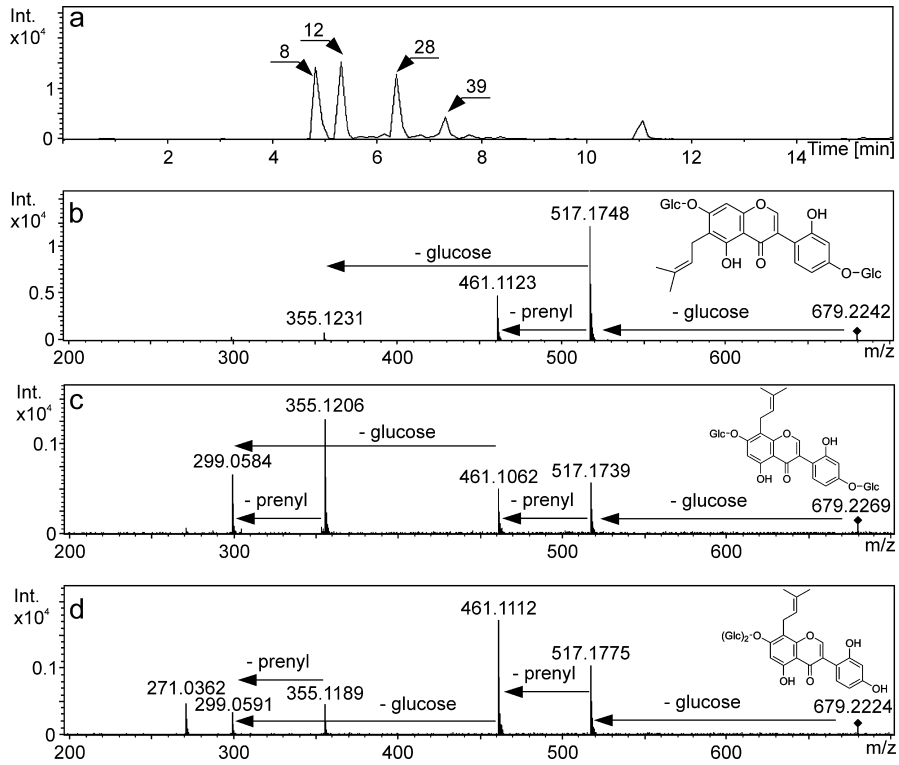

Figure 2. Chromatogram of ion at *m*/*z* 679 in positive ion mode (a) and CID MSMS spectra of luteone 4′,7-*O*-diglucoside (b); 2,3-didehydrokievitone 4′,7-*O*-diglucoside (c), and 2,3-didehydrokievitone 7-*O*-glucosylglucoside (d).

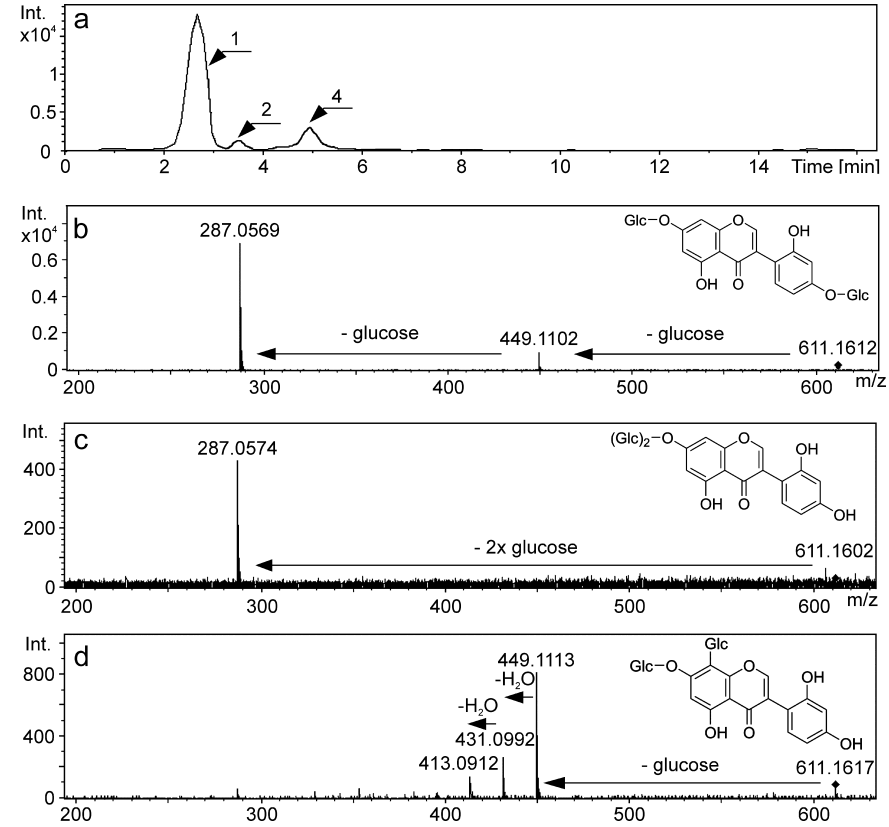

Figure 3. Chromatogram of ion at *m*/*z* 611 in positive ion mode for 2′-hydroxygenistein diglucosides (a); and CID MSMS spectra of 2′-hydroxygenistein 4′,7-*O*-diglucoside (b), 2′-hydroxygenistein 7-*O*-glucosylglucoside (c), and 2′-hydroxygenistein 7-*O*-glucoside-8-*C*-glucoside (d).

μm grain, Agilent) were used. Chromatographic runs on the Agilent 1200 system were performed at a 0.5 mL/min flow rate using mixtures of two solvents: A (99.5% H_2O, 0.5% HCOOH v/v) and B (99.5% MeCN, 0.5% HCOOH, v/v) with a split of the column effluent 3:2 so 0.2 mL/min was delivered to the ESI ion source. The elution steps were as follows: 0−8 min linear gradient from 5 to 30% of B, 8−10

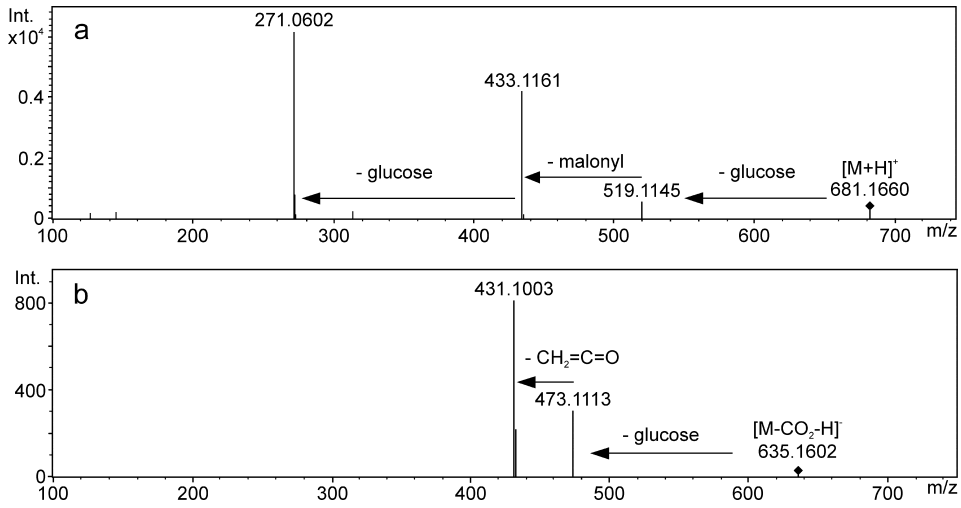

Figure 4. Mass spectra of malonylated genistein 4′,7-*O*-diglucoside recorded in (a) positive and (b) negative ion modes.

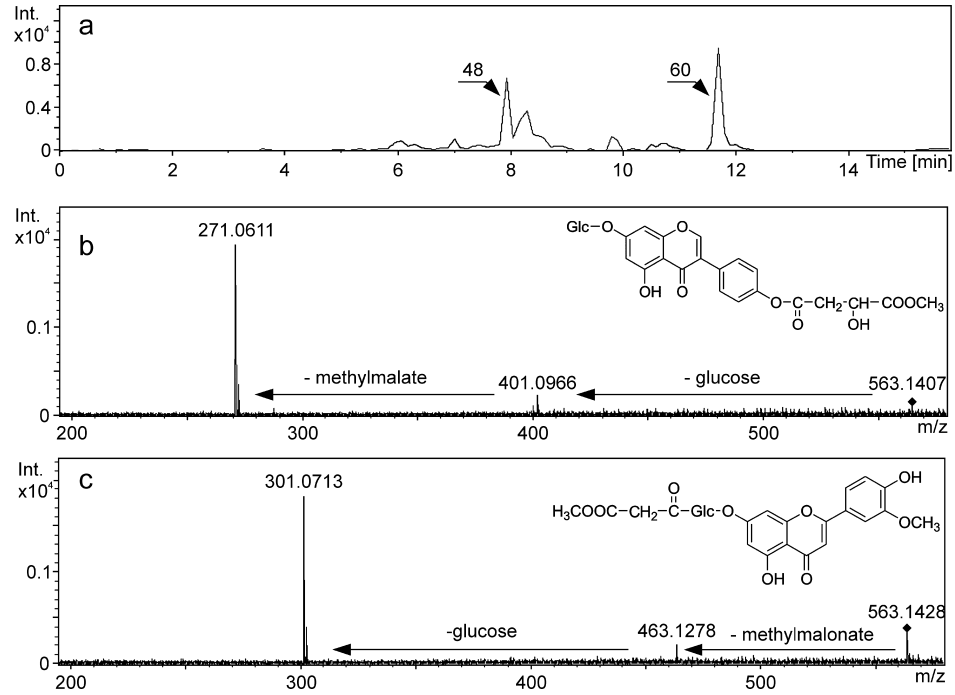

Figure 5. Chromatogram of ion at *m/z* 563 in positive ion mode (a) and CID MSMS spectra of isobaric compounds genistein 7-*O*-glucoside acylated with malic acid methyl ester at the aglycone moiety (b) and chrysoeriol 7-*O*-glucoside acylated with malonic acid methyl ester at the glucose moiety (c).

min linear gradient to 95% B, 10−12 min isocratic at 95% B; after returning to the initial conditions the equilibration was achieved after 3 min. The same solvents were used on the Agilent 1100 system at a 0.2 mL/min flow, and the gradient was 0−35 min linear at 5−30% of B, 35−45 min up to 95% B, 45−52 min isocratic at 95% B, returning to initial conditions an re-equilibration (until 60 min).

The settings of the IT mass spectrometer were as follows: ESI voltage +4 kV or −4 kV (depending on the ion mode), nebulization with N_2 at 1.7 bar, dry gas flow 7 L/min, gas temperature 310 °C, skimmer 1 voltage +12.4 or −11.2 V, collision energy set to 1 V and ramped within 40−200% of this value. The ion number accumulated in the trap was set to 10 000, and the maximum accumulation time was 200 ms. According to results of preliminary experiments, spectra were recorded in the targeted mode in the mass range *m/z* 50−1500. The instrument operated under EsquireControl version 5.1, and data were analyzed using the DataAnalysis version 3.1 package delivered by Bruker.

The micrOTOF-Q spectrometer consisted of ESI operating at a voltage of ±4.5 kV, nebulization with N_2 at 1.6 bar, and dry gas flow of 8.0 L/min at a temperature of 220 °C. The system was calibrated externally using the calibration mixture containing sodium formate clusters. Additional internal calibration was performed for every run by injection of the calibration mixture using the divert valve during the LC separation. All calculations were done with the HPC quadratic algorithm. Such a calibration gave at least 5 ppm accuracy. MSMS spectra were acquired with the frequency of 1 scan per second for ions chosen on the basis of preliminary experiments, and Ar was used as collision gas. Collision energy depends on molecular masses of compounds and was set between 15 and 30 eV in positive or 30 eV in negative ion mode. The instrument operated at a resolution higher than 10 000 (fwhm: full width at half-maximum) under the program micrOTOF Control ver. 2.3, and data were analyzed using the DataAnalysis ver. 4 package delivered by Bruker. Metabolite profiles were registered in positive and

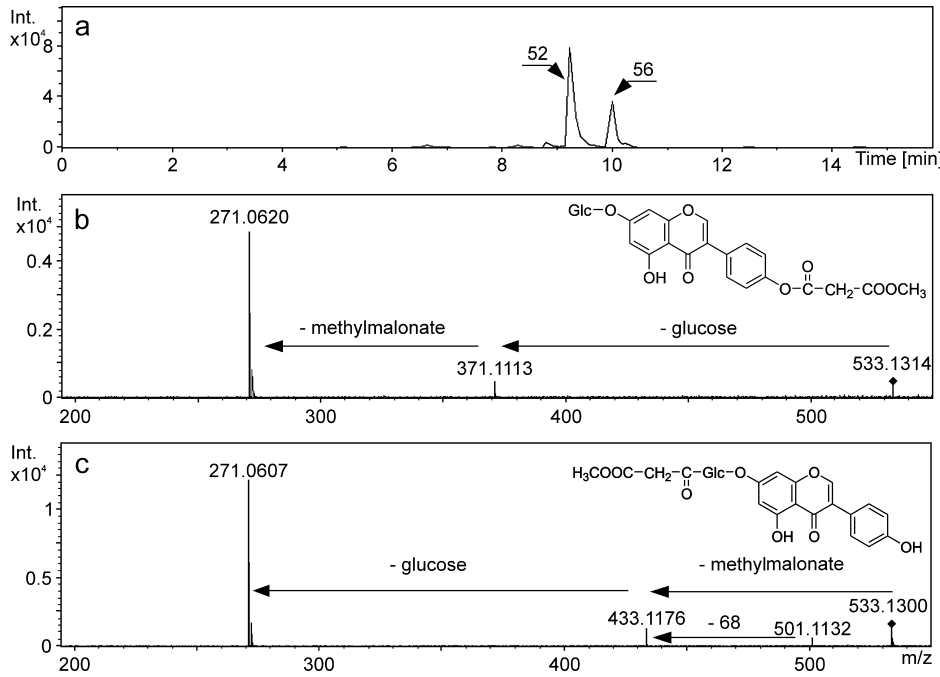

Figure 6. Chromatogram of ion at *m/z* 533 in positive ion mode for genistein glucosides acylated with malonic acid methyl ester (a) and CID MSMS spectra of compounds acylated at the aglycone (b) and glucose (c) moieties.

negative ion modes. For identification of compounds the instrument operated in the targeted MSMS mode, and single ion chromatograms for exact masses of $[M + H]^+$ and $[M - H]^-$ ions (±0.005 Da) were recorded.

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