JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 5843–5852 5843
DOI:10.1021/jf900625r

Identification and Quantification of Phenolic Compounds from the Forage Legume Sainfoin (*Onobrychis viciifolia*)

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Phenolic compounds of sainfoin (*Onobrychis viciifolia*) variety Cotswold Common are assumed to contribute to its nutritive value and bioactive properties. A purified acetone/water extract was separated by Sephadex LH-20 gel chromatography. Sixty-three phenolic and other aromatic compounds were identified by means of chemical, chromatographic, and spectroscopic methods. Reverse phase HPLC with diode array and chemical reaction detection was used to investigate the phenolic composition of different plant organs. All plant parts showed specific phenolic profiles. Moreover, there were considerable variations in the phenolic content among individual plants of the same variety. The three most abundant phenolic compounds were found to be arbutin [predominant in petiols, 17.7 mg/g of dry weight (DW)], rutin (predominant in leaves, 19.9 mg/g of DW), and catechin (predominant flavanol in petiols, 3.5 mg/g of DW). The present study reveals that the phenolic profile of sainfoin is even more complex than hitherto assumed.

KEYWORDS: Sainfoin (*Onobrychis viciifolia*); polyphenols; identification; LC-ESI-MS/MS; HPLC-DAD

INTRODUCTION

Sainfoin (Fabaceae: subfamily Papilionoideae: tribe Hedysareae) is a traditional fodder legume with high palatability and excellent nutritional and veterinary properties. It is a drought-resistant perennial plant and has an early growth habit, sprouting earlier than lucerne in spring to give good forage yields. Compared to other legumes, sainfoin is superior in terms of protein and energy value for ruminants and had promising health and environmental benefits, namely, prevention of bloat, controlling nematode parasitism in ruminants such as cattle and sheep, and lower methane and nitrogen emissions (*1*−*3*). These beneficial effects are attributed to particular tannin structures of sainfoin. As other legumes, *Onobrychis viciifolia* produces a wide range of diverse phenolic compounds, which may also be bioactive candidates. A recent study evaluating anthelmintic effects of sainfoin extracts (*4*) conclusively demonstrated that besides condensed tannins, other phenolic compounds such as flavonols may be involved in this beneficial property. The objectives of this study were to identify the soluble non-tannin phenolics and to investigate their composition in different parts of the sainfoin plant in order to lay a cornerstone for their physiological evaluation.

MATERIALS AND METHODS

Plant Material. For qualitative analysis, *O. viciifolia* (variety Cotswold Common) plants, ca. 50 cm high, were harvested at the bud stage by Ian Wilkinson, Cotswold Seeds Ltd., U.K. on May 31, 2006, air-dried at room temperature, and ground (< 1 mm).

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For the investigation of the phenolic concentrations from different aerial parts (new leaves, stems, flower stalks, and flower buds), six sainfoin plants (var. Cotswold Common) were grown at Freising-Weihenstephan and harvested at the flower bud stage. The new leaves (with still folded leaflets) were divided in young leaflets and petioles. The material was frozen in liquid nitrogen immediately after sampling and lyophilized.

Sources of Reference Compounds. L-Tryptophan and vanillic acid were purchased from Merck (Darmstadt, Germany); arbutin, ellagic acid, *p*-hydroxybenzoic acid, protocatechuic acid, gallic acid, *p*-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, dihydroquercetin, vitexin, catechin, epicatechin, quercitrin, rutin, and cyanidin 3-glucoside were available from Roth (Karlsruhe, Germany); dihydrokaempferol was obtained from TransMIT (Marburg, Germany), and isovitexin, orientin, epigallocatechin, nicotiflorin, and narcissin were from Extrasynthese (Genay, France). Gallocatechin and procyanidins B3 and B4 were purchased from Leuven Bioproducts (Heverlee, Belgium), and procyanidins B2, B5, E-B5, and C1 were previously isolated from buckwheat (*5*) and fruit shells of *Aesculus hippocastanum* (*6*). Quercetin 3-glucuronide was previously isolated from strawberries (*7*). Astragalin, kaempferol 3-glucoside-7-rhamnoside, kaempferol, and quercetin 3-rutinoside-7-rhamnoside, isoquercitrin, and quercetin 3-arabinoside were provided by Hans Geiger. Rhamnose, glucose, and galactose were purchased from Merck.

Extraction and Preparative Fractionation of Phenolic Compounds. The plant material (50 g) was exhaustively extracted with 70% ice-cold aqueous acetone (5 × 250 mL) in an ultrasonic water bath at 7 °C for 30 min. After centrifugation (15000 rcf, 15 min), the clear supernatants were combined and the acetone was removed under reduced pressure (< 30 °C). The remaining aqueous phase was diluted with H_2O and extracted successively with ice-cold chloroform (4.5 L in total) and EtOAc (2.8 L in total). The aqueous residue remaining after EtOAc extraction was evaporated until there was no smell of EtOAc and then diluted with H_2O, yielding a red-brown powder after freeze-drying (13.4 g, 26.8% of total leaf and stem material). Cellulose plates sprayed with *p*-dimethylaminocinnamic aldehyde reagent (DMACA) showed that this fraction had a high

 Published on Web 05/20/2009

concentration of condensed tannins. The EtOAc extracts were combined and concentrated under reduced pressure, yielding a yellow-brown powder after freeze-drying (1.5 g, 3% of total leaf and stem material). Extracts as well as the various residues obtained during the extraction process were tested by TLC and HPLC for their chemical composition. The EtOAc extract, redissolved in 10% aqueous MeOH, was chromatographed on a Sephadex LH-20 column (300 mm × 30 mm internal diameter) with different volumes of aqueous MeOH (10−100% in increments of 10%). The eluate was collected in 20 mL portions in 107 test tubes, which were combined according to their phenolic composition on the basis of their TLC behavior to afford 45 fractions (**Table 1**). Each fraction was characterized by HPLC, TLC, and LC-MS/MS. Later, the fractions were purified by HPLC and TLC.

Quantitative Extraction of Phenolic Compounds from Different Plant Parts. Freeze-dried material was ground in a mortar or a ball mill, depending on the available amount. The extraction was performed by adding 500 μL of 80% aqueous methanol to 100 mg of powder for 30 min in a cooled ultrasound water bath (7 °C). After centrifugation (10000 rcf, 10 min, 4 °C), the clear supernatant was transferred to an Eppendorf tube and the residue was washed twice, each time with 250 μL of 80% aqueous MeOH. After centrifugation, the corresponding supernatants were combined, the solvent was evaporated, and the residue was redissolved in 100 μL of MeOH. A 10 μL sample of the extract was injected for HPLC analysis.

Thin Layer Chromatography (TLC). Analytical TLC and preparative TLC were carried out on Merck cellulose plates and on polyamide plates from Macherey-Nagel (Düren, Germany) with *n*-BuOH/HOAc/H_2O (4:1:5) (BAW) and H_2O/EtOH/ethyl methyl ketone/acetylacetone (65:15:15:5) (WEEA), respectively. The chromatograms were evaluated under UV light both with and without spraying with Naturstoffreagenz A (diphenylboric acid 2-aminoethyl ester) and DMACA.

HPLC Analysis of Phenolic Compounds. The phenolic compounds from sainfoin were analyzed and purified from the fractions F1−F45 with an HPLC system consisting of two pumps (model 422, Kontron Instruments, Germany), an automatic sample injector (model 231, Gilson Abimed Systems, Germany), and a diode array detector (Kontron 540, Kontron Instruments). For postcolumn derivatization a further Gynkotek analytical HPLC pump (model 300 C, Germering, Germany) and a Vis detector (640 nm, Kontron Detector 432, Kontron Instruments) were used. Sainfoin compounds were separated on a Nucleosil column (250 × 4 mm, Macherey-Nagel) and eluted with a mixture of H_2O containing 5% HCO_2H (solvent A) and MeOH (solvent B). The following gradient was applied using a flow rate of 0.5 mL/min: 0−5 min, 5% B; 5−10 min, 5−10% B; 10−15 min, 10% B; 15−35 min, 10−15% B; 35−55 min, 15% B; 55−70 min, 15−20% B; 70−80 min, 20% B; 80−95 min, 20−25% B; 95−125 min, 25−30% B; 125−145 min, 30−40% B; 145−160 min, 40−50% B; 160−175 min, 50−90% B; 175−195 min, 90% B.

For their quantification, sainfoin compounds were grouped into 10 categories based on the maximum UV−vis absorption and were monitored and analyzed at 280 nm (amino compounds, simple phenolic acids, hydroxybenzoic acids, and dihydroflavonols), 320 nm (hydroxycinnamic acids, flavones), 350 nm (flavonols), and 540 nm (anthocyanins). Additionally, postcolumn derivatization with DMACA was used for selective detection of flavanols at 640 nm (*8*). Quantification was performed as follows: L-tryptophan, arbutin, ellagic acid, catechin, epicatechin, procyanidin B2, rutin, and cyanidin 3-glucoside were available as standards, hypaphorine was calculated as L-tryptophan, and 8-β-glucopyranosyloxycinnamic acid was calculated as cinnamic acid. Hydroxybenzoic acids were calculated as *p*-hydroxybenzoic acid, hydroxycinnamic acids as chlorogenic acid, dihydroflavonols as dihydroquercetin, and the flavones as vitexin. From the flavanols, gallocatechin and epigallocatechin were calculated as epicatechin and the oligomers as procyanidin B2. The flavonols were calculated as rutin.

HPLC Analysis of Sugars. The HPLC apparatus consisted of a gradient pump (GP50, Dionex, Idstein, Germany), an interface (Advanced Computer Interface, Dionex), a manual injector, and an electrochemical detector (ED40, Dionex). The sugars were separated on a CarboPac PA-100 column (250 × 4 mm, Dionex) with a mixture of 100 mM NaOH (B) and deionized water (C). The flow rate was 1 mL/min, the injected sample volume 25 μL, and the gradient as follows: 0−5 min, 10% B; 5−10 min, 10−15% B; 10−20 min, 15% B.

HPLC-ESI-MS/MS. HPLC-ESI-MS/MS analysis was performed with a Shimadzu LC-10A series liquid chromatograph (Shimadzu, Hannover, Germany) followed by an API 3000 triple-quadrupole mass spectrometer (Applied Biosystem, Darmstadt, Germany) fitted with an electrospray ionization source. The HPLC system consisted of an SCL-10Avp system controller, two LC-10ADvp pumps, an SIL-HTC refrigerated autosampler, a DGU-14A degasser, and a CTO-10ASvp column oven. The autosampler temperature was set at 4 °C and the column oven at 25 °C. The chromatographic separation was performed using a Phenomenex Synergy Fusion-RP 18 column (50 × 2 mm, Phenomenex, Aschaffenburg, Germany), and the flow rate was maintained at 0.2 mL/min. Solvent A was H_2O and solvent B was MeOH, both containing 0.1% HCO_2H, and the gradient protocol was as follows: 0−16 min, 6−17% B; 16−18 min, 17−20% B; 18−25 min, 20−40% B; 25−35 min, 40−100% B. The MS/MS detector was operated in the following conditions: polarity, negative; ion spray voltage, −4200 V; heater gas temperature, 350 °C; entrance potential, −10 V; focusing potential, −330 V; declustering potential, −50 V; and collision energy, −52 V. Nitrogen served as nebulizer, curtain, drying, and collision gas. The acquisition was performed in full-scan mode (*m*/*z* 50−2000 amu) using Analyst 1.4.1 (Applied Biosystem, Darmstadt, Germany) as software.

UV−Vis Spectroscopy. UV−vis spectra of flavonol glycosides and bathochromic shifts after addition of shift reagents were recorded with an Uvikon 931 UV−vis double-beam spectrometer (Kontron, Germany) according to the method of Mabry et al. (*9*).

Acid and Enzymatic Hydrolysis. Acid hydrolysis was performed by heating 100 μL samples with 100 μL of 0.1 M aqueous HCl in a boiling water bath for 15 min. The aglycones were extracted from this solution with EtOAc (3 × 300 μL). For the enzymatic hydrolysis a solution containing the unknown compound (0.2−1 mg) was evaporated to dryness under vacuum. The residue was further diluted with 300 μL of 0.1 M NaOAc buffer (pH 4.6) and incubated with 2 mg of enzyme (sulfatase and glucosidase from Sigma-Aldrich, Deisenhofen, Germany; tannase, Braunschweiger Biotechnologie, Germany) in a water bath at 50 °C for 3 h. The aglycones were extracted with EtOAc (3 × 500 μL), and the sugars were analyzed in the remaining aqueous phase.

RESULTS AND DISCUSSION

Identification of Phenolic Compounds from Sainfoin (*O. viciifolia*). The acetone extract of a mixed sample of sainfoin whole plants was dominated by low molecular weight secondary plant metabolites consisting of simple phenolic acids, hydroxybenzoic and hydroxycinnamic acids, dihydroflavonols, flavones, flavanols, and flavonols (see **Figure 1B−H** for chemical structures). Additionally, amino compounds were also identified (**Figure 1 A**). Sephadex LH-20 gel chromatography using a stepwise water/methanol gradient as shown in **Table 1** proved to be very effective to enrich and separate sainfoin compounds by different families in 45 fractions. Fraction components were further purified for the subsequent characterization of these structures through various techniques. TLC on polyamide plates was found to be a useful technique for the purification of flavonols with different degrees of glycosylation. The structures of the isolated compounds were established by means of acid and enzymatic hydrolysis, spectrometric, and chromatographic methods: UV with shift reagents (NaOMe, NaOAc, H_3BO_3, $AlCl_3$, and HCl), the LC-ESI-MS/MS technique, TLC, and HPLC. The spectral data obtained were confirmed by comparison with those previously reported. When standards were available, HPLC co-injection was performed. The 63 compounds identified in sainfoin are listed in **Table 2** together with their acronyms (**A1−P3**) and the chromatographic, UV, and mass spectral characteristics.

Amino Compounds. In addition to the amino acid L-tryptophan (**A1**), previously reported by Marais et al. (*10*), hypaphorine (**A2**), an α-*N*,*N*,*N*-trimethyltryptophan betaine, was isolated for the first time from sainfoin. The UV spectrum of **A2** showed a maximum at 279 nm with shoulders at 273 and 287 nm. The

Table 1. Characterization of Sainfoin Fractions Purified by Sephadex LH-20 Using a Methanol/Water Gradient Elution, Percentage of Methanol, Eluate Volume, Fraction Yields, and Their Main Components

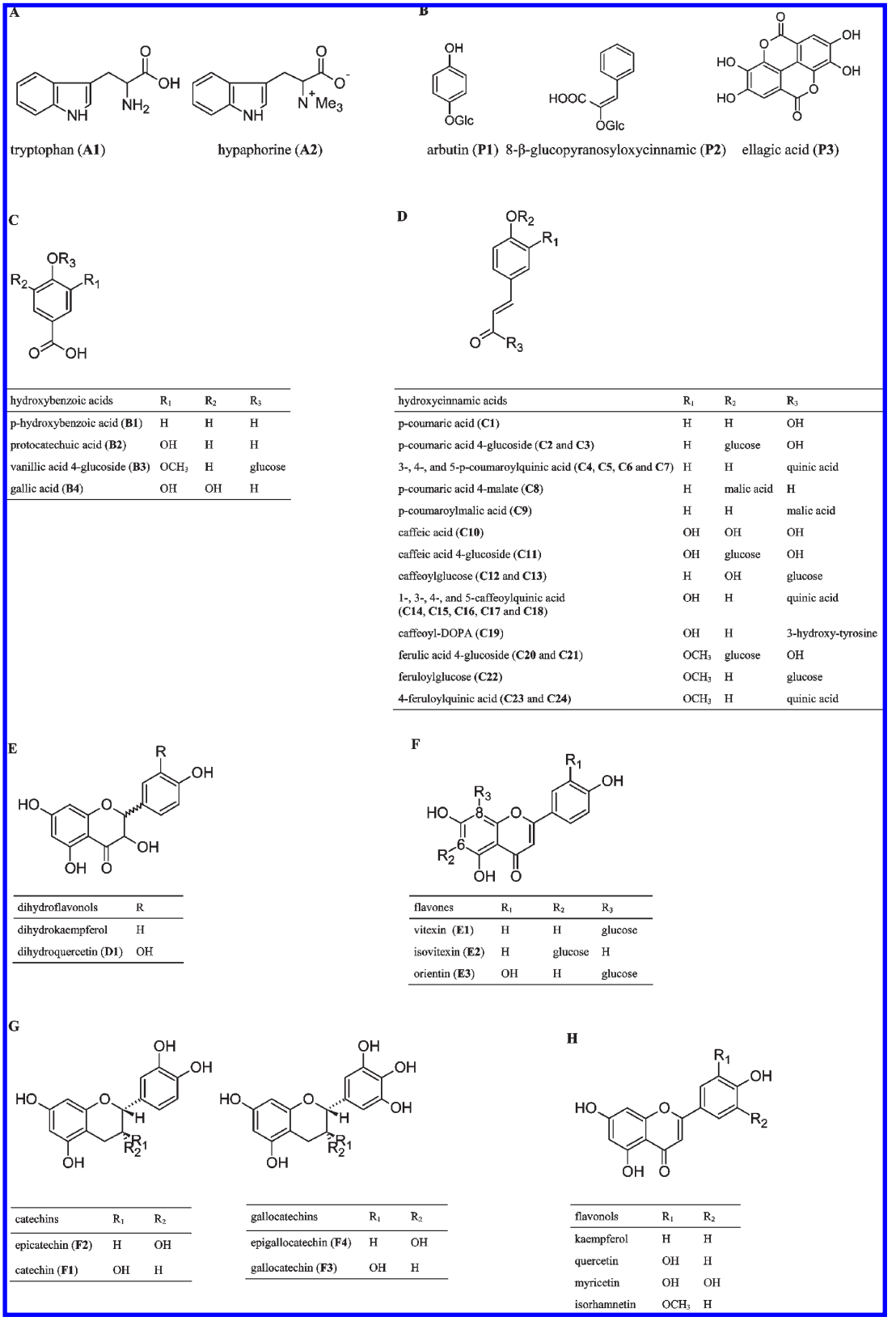
fraction	eluate (% MeOH in water)	glass[a]	eluate vol (mL)	fraction wt (mg)	main components[b]
1	10	1−3a	60	133.00	
2	10	4a	20	191.00	
3	10	5a	20	116.00	P1
4	10	6a	20	48.00	A1, C2, C20,
5	10	7a	20	59.00	P2, B4
6	10	8a	20	20.00	C4, C12, C15
7	10	9a	20	11.01	C21
8	10	10a	20	21.00	C3, C24
9	20	11b	20	17.00	C5
10	20	12b	20	21.00	C13, C16, C22
11	20	13b	20	20.00	A2
12	20	14b	20	20.00	C6, C7, C8, C23, G5, G14
13	20	15b	20	17.00	C11, D3, G9
14	20	16b	20	16.00	C14
15	20	17b	20	12.00	C17, D2, G4
16	20	18b	20	13.00	
17	20	19−20b	40	40.00	G13
18	30	21−23c	60	49.00	B1, B2, C9, C18, G2
19	30	24−25c	40	29.67	B3
20	30	26−27c	40	21.75	G8, G12
21	30	28c	40	8.84	
22	30−40	29−30c and 31−32d	40	24.09	G3
23	40	33d	40	5.14	C1, C10, E1, E2, F2
24	40	34−36d	60	19.09	C19, G10
25	40	37−39d	60	17.25	E3, F1, F4
26	40	40d	20	6.71	F3
27	50	41−43e	60	15.33	
28	50	44e	20	7.61	F5, G1
29	50	45−47e	60	34.00	F6, G7
30	50	48−50e	60	32.93	D1, F7, G6, G11
31	60	51f	20	8.58	
32	60	52f	20	7.70	
33	60	53−54f	40	14.55	P3
34	60	55−56f	40	69.15	
35	60	57f	20	11.90	
36	60	58f	20	9.89	
37	60	59−60f	40	18.98	
38	70	61 g	20	19.14	
39	70	62−63 g	40	16.85	
40	70	64−66 g	60	30.45	F8, F9, F10
41	70	67 g	20	8.94	
42	70	68 g	20	7.05	
43	70−80	69−70 g and 71−72 h	80	34.85	
44	80−90	73−80 h and 81−83i	220	66.68	
45	90−100	84−94i and 95−107j	480	66.05	

[a] a−j, volume of MeOH in water (from 10 to 100%). [b] A1−P3, identified compounds (see **Table 2** for acronym explanation).

LC-MS analysis gave the $[M - H]^-$ peak at m/z 245 and MS/MS fragments at m/z 74, 116, 130, 142, 156, and 167, which were consistent with the proposed chemical structure of hypaphorine and with spectral data published by Janzen et al. (*11*). On a TLC plate **A2** was visualized by spraying with DMACA, giving a deep purple color, whereas no color was observed after treatment with ninhydrin, presumably as a result of nitrogen methylation. Hypaphorine was previously isolated from the genera *Erythrina* (Fabaceae) (*12*−*15*), and *Abrus* (*16*) and regarded as a convulsive poison (*17*). However, Bel-Kassaoui et al. (*18*) isolated this betaine from *Astragalus lusitanicus*, a highly toxic plant for lambs and goats, and showed it to be nontoxic for goats even at high dose of 2 g/kg by oral administration.

Simple Phenolic Acids. Examination of fraction 3 by HPLC revealed one main polar component, which was identified as arbutin (**P1**) by comparison of its spectral data and chromatographic behavior (HPLC) with an authentic sample. Compound **P2** was identified as 8-β-glucopyranosyloxycinnamic acid by comparison of its spectral data with those reported by Lu et al. (*19*) both before and after hydrolysis with β-glucosidase. These two compounds (**P1** and **P2**) accounted for a considerable proportion of the phenolic constituents in sainfoin. They have been previously identified in sainfoin (*10*, *19*). Ellagic acid (**P3**) was identified for the first time in sainfoin by cochromatography with a reference substance and LC-MS analysis.

Hydroxybenzoic Acids. To our knowledge hydroxybenzoic acids have not yet been reported in *O. viciifolia* up to now. 4-Hydroxybenzoic acid (**B1**), protocatechuic acid (**B2**), and gallic acid (**B3**) were identified by comparison of t_R and UV and mass spectra with those of reference substances. Furthermore, compound **B4**, which gave a $[M - H]^-$ ion at m/z 329, and the ion of vanillic acid at m/z 167 in the MS/MS spectra was identified as vanillic acid 4-*O*-glucoside. This was confirmed by sulfatase hydrolysis of **B4** which gave vanillic acid and glucose. The UV−vis absorption spectrum of **B4** showed a hypsochromic shift as compared to vanillic acid (from 259 and 291 nm for vanillic



hydroxybenzoic acids	R_1	R_2	R_3
p-hydroxybenzoic acid (**B1**)	H	H	H
protocatechuic acid (**B2**)	OH	H	H
vanillic acid 4-glucoside (**B3**)	OCH_3	H	glucose
gallic acid (**B4**)	OH	OH	H

hydroxycinnamic acids	R_1	R_2	R_3
p-coumaric acid (**C1**)	H	H	OH
p-coumaric acid 4-glucoside (**C2** and **C3**)	H	glucose	OH
3-, 4-, and 5-p-coumaroylquinic acid (**C4**, **C5**, **C6** and **C7**)	H	H	quinic acid
p-coumaric acid 4-malate (**C8**)	H	malic acid	H
p-coumaroylmalic acid (**C9**)	H	H	malic acid
caffeic acid (**C10**)	OH	OH	OH
caffeic acid 4-glucoside (**C11**)	OH	glucose	OH
caffeoylglucose (**C12** and **C13**)	H	OH	glucose
1-, 3-, 4-, and 5-caffeoylquinic acid (**C14**, **C15**, **C16**, **C17** and **C18**)	OH	H	quinic acid
caffeoyl-DOPA (**C19**)	OH	H	3-hydroxy-tyrosine
ferulic acid 4-glucoside (**C20** and **C21**)	OCH_3	glucose	OH
feruloylglucose (**C22**)	OCH_3	H	glucose
4-feruloylquinic acid (**C23** and **C24**)	OCH_3	H	quinic acid

dihydroflavonols	R
dihydrokaempferol	H
dihydroquercetin (**D1**)	OH

flavones	R_1	R_2	R_3
vitexin (**E1**)	H	H	glucose
isovitexin (**E2**)	H	glucose	H
orientin (**E3**)	OH	H	glucose

catechins	R_1	R_2
epicatechin (**F2**)	H	OH
catechin (**F1**)	OH	H

gallocatechins	R_1	R_2
epigallocatechin (**F4**)	H	OH
gallocatechin (**F3**)	OH	H

flavonols	R_1	R_2
kaempferol	H	H
quercetin	OH	H
myricetin	OH	OH
isorhamnetin	OCH_3	H

Figure 1. Structures of phenolic and other aromatic compounds found in sainfoin: (**A**) amino compounds; (**B**) simple phenolic acids; (**C**) hydroxybenzoic acids; (**D**) hydroxycinnamic acids; (**E**) dihydroflavonols; (**F**) flavones; (**G**) flavanols; (**H**) flavonols.

acid to 254 and 289 nm for **B4**) caused by the glucosylation of the hydroxyl group in position 4 of vanillic acid. These data are consistent with the literature (*20*).

Hydroxycinnamic Acids. Compound **C1** was identified as free *trans-p*-coumaric acid by comparison with an authentic standard. Its $[M - H]^-$ ion at m/z 163 was found in the fragmentation pattern of compounds **C2** and **C3** after elimination of a glucose moiety (162 Da), and as fragment of **C4**, **C5**, **C6**, and **C7** after elimination of dehydrated quinic acid, and, furthermore, of **C8** and **C9** after elimination of dehydrated malic acid. Compounds **C2** and **C3** exhibited a $[M - H]^-$ ion at m/z 325. Both fractions released glucose after β-glucosidase hydrolysis and a mixture of

Table 2. Chromatographic, UV, and Mass Spectral Characteristics of Phenolic and Other Natural Compounds Isolated from the Sainfoin Acetone Extract

compd	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m*/*z*)	MS/MS of $[M-H]^-$ [a] (*m*/*z*)	identification[b]
				Amino Compounds	
A1	31.5	273sh, 277, 287sh	203	**116**	L-tryptophan
A2	54.1	273sh, 278, 287sh	245	**74**, 116, 130, 142, 156, 167	hypaphorine = *N*,*N*,*N*-trimethyltryptophan betaine
				Simple Phenolic Acids	
P1	6.9	281	271	71, **108**, 109	arbutin = 4-(β-D-glucopyranosyloxy)phenol
P2	54.5	279	325	91, 101, 117, **119**	8-β-glucopyranosyloxycinnamic acid
P3	138.9	254, 366	301		ellagic acid
				Hydroxybenzoic Acids	
B1	23.5	254	137	65, 75, **93**	*p*-hydroxybenzoic acid
B2	15.3	258, 293	153	65, 81, 91, **108**, 109	protocatechuic acid
B3	9.8	270	169	51, **79**, 124	gallic acid
B4	19.4	254, 289	329	**108**, 123,152, 167	vanillic acid 4-*O*-glucoside
				Hydroxycinnamic Acids	
C1	56.7	309	163	117, **119**	*trans*-*p*-coumaric acid
C2	29.3	284	325	93, **119**, 163	*cis*-*p*-coumaric acid 4-*O*-glucoside
C3	29.5	294	325	93, 117, **119**, 163	*trans*-*p*-coumaric acid 4-*O*-glucoside
C4	22.5	305	337	85, 93, **119**, 163, 173, 191	*cis*-3-*p*-coumaroylquinic acid
C5	25.3	310	337	85, 93, **119**, 163, 173	*trans*-3-*p*-coumaroylquinic acid
C6	24.1	313	337	85, 93, **119**, 163, 191	*trans*-4-*p*-coumaroylquinic acid
C7	46.0	311	337	**93**, 119, 163, 173, 191	*trans*-5-*p*-coumaroylquinic acid
C8	68.6	308	279	**71**, 93, 115, 117, 133, 119, 163	*trans*-*p*-coumaric acid 4-*O*-malate
C9	80.0	313	279	71, 93, 117, 119, **163**	*trans*-*p*-coumaroylmalic acid
C10	36.7	322	179	117, **134,** 135	*trans*-caffeic acid
C11	34.9	291, 313sh	341	**135**, 179, 117, 107, 79	*trans*-caffeic acid 4-*O*-glucoside
C12	33.8	301	341	**135**, 179	*cis*-caffeoylglucose
C13	36.0	314	341	**135**, 179	*trans*-caffeoylglucose
C14	17.1	250sh, 327	353	**135**, 179, 191	*trans* 1-caffeoylquinic acid
C15	15.4	316	353	**135**, 179, 191	*cis*-3-caffeoylquinic acid
C16	18.2	251sh, 324	353	**135**, 179, 191	*trans*-3-caffeoylquinic acid
C17	36.5	248sh, 326	353	93, **135**, 173, 191	*trans*-4-caffeoylquinic acid
C18	43.8	248sh, 326	353	85, 93, **191**	*trans*-5-caffeoylquinic acid
C19	64.7	288, 320	358	**135**, 161, 178, 196	caffeoyl-DOPA
C20	44.6	286	355	**134**, 178, 193	*cis*-ferulic acid 4-*O*-glucoside
C21	43.2	291, 315	355	**134**, 178, 193	*trans* ferulic acid 4-*O*-glucoside
C22	44.2	328	355	134, **160**, 175, 178	*trans*-feruloylglucose
C23	64.5	317	367	93, **134**, 178, 193	*cis*-4-feruloylquinic acid
C24	35.7	323	367		*trans*-4-feruloylquinic acid
				Dihydroflavonols	
D1	76.4	286	303	**125**, 285	dihydroquercetin
D2	43.1	293	449	**125**, 269, 287	dihydrokaempferol-glucoside
D3	27.4	287, 325	465	**125**, 285, 303	dihydroquercetin-glucoside
				Flavones	
E1	104.6	267, 333	431	268, **283**, 311, 341	vitexin = apigenin 8-*C*-glucoside
E2	126.3	269, 335	431	269, **283**, 311, 341, 413	isovitexin = apigenin 6-*C*-glucoside
E3	99.6	266, 341	447	284, **299**, 327, 357	orientin = luteolin 8-*C*-glucoside
				Flavanols	
F1	26.8	277	289	**109**, 123	catechin (cat)
F2	45.4	277	289	**109**, 123	epicatechin (epi)
F3	14.6	269	305	**125**, 137	gallocatechin
F4	29.1	269	305	**109**, 125, 137	epigallocatechin
F5	37.8	278	577	**125**, 245, 289, 407	procyanidin B2 = epi-(4β→8)-epi
F6	19.5	278	577	**125**, 245, 289, 407	procyanidin B3 = cat-(4α→8)-cat
F7	28.1	277	577	**125**, 245, 289, 407	procyanidin B4 = cat-(4α→8)-epi
F8	115.4	277	577	**125**, 245, 289, 407, 425	procyanidin B5 = epi-(4β→6)-epi
F9	66.8	277	865		procyanidin C1 = epi-(4β→8)-epi-(4β→8)-epi
F10	134.1	277	865		procyanidin E-B5 = epi-(4β→6)-epi-(4β→6)-epi
				Flavonols	
G1	147.9	264, 346	447	227, **255**, 284	astragalin = kaempferol 3-*O*-glucoside
G2	144.4	264, 346	593	151, 179, 227, 255, **284**	kaempferol 3-*O*-rhamnogalactoside
G3	150.5	264, 346	593	151, 179, 255, 284, **285**	nicotiflorin = kaempferol 3-*O*-rhamnoglucoside
G4	144.4	265, 345	593	151, 255, 284, **285**, 430, 447	kaempferol 3-*O*-glucoside-7-rhamnoside
G5	137.3	264, 346	739	151, 227, **284**, 593	kaempferol 3-*O*-rhamnosylrutinoside
G6	155.3	256, 348	447	271, **300**	quercitrin = quercetin 3-*O*-rhamnoside
G7	131.3	256, 353	463	151, 255, 271, **300**	isoquercitrin = quercetin 3-*O*-glucoside
G8	136.6	255, 353	609	151, 179, **300**, 463	rutin = quercetin 3-*O*-rhamnoglucoside
G9	122.4	259, 353	755	151, 179, **300**, 609	quercetin 3-*O*-rhamnosylrutinoside

Table 2. Continued

compd	t_R (min)	λ_{max} (nm)	$[M - H]^-$ (*m*/*z*)	MS/MS of $[M - H]^-$ [a] (*m*/*z*)	identification [b]
G10	128.1	257, 352	477	151, 179, **30**	quercetin 3-glucuronide
G11	116.2	259, 349	463	179, 271, 287, **316**	myricitrin = myricetin 3-*O*-rhamnoside
G12	113.6	260, 355	625	179, 271, 287, **316**	myricetin 3-*O*-rhamnoglucoside
G13	155.8	255, 354	623	151, 179, 300, **315**	narcissin = isorhamnetin 3-*O*-rhamnoglucoside
G14	138.2	255, 349	769	151, 179, 299, **314**, 623	isorhamnetin 3-*O*-rhamnosylrutinoside

[a] In the MS/MS, the most abundant ion is shown in boldface. [b] Abreviation for flavan-3-ols: epi, epicatechin; cat, catechin.

cis- and *trans*-*p*-coumaric acid. The cis:trans ratio was 8:2 for **C2** and 1:9 for **C3**. Therefore, **C2** was assigned to be *cis*-*p*-coumaric acid 4-*O*-β-glucoside, whereas **C3** was proposed to be *trans*-*p*-coumaric acid 4-*O*-β-glucoside. Upon UV light exposure for 5 h, 20% from the cis isomer **C2** was converted to its trans isomer **C3**. The hypsochromic shifts observed in the UV absorption spectra of **C2** (λ_{max} = 284 nm) and **C3** (λ_{max} = 294 nm) as compared to cis (λ_{max} = 294 nm) and *trans*-*p*-coumaric acid (λ_{max} = 309 nm) and the disappearance of the typical spectral feature of the aglycone corroborated that the glucose is bound to the hydroxyl group in position 4, as also described by Määttä et al. (*21*).

In addition to the glucosides of *p*-coumaric acid, several isomeric quinic acid esters were detected. Compounds **C4**, **C5**, **C6**, and **C7** showed a $[M - H]^-$ ion at *m*/*z* 337 and the MS-MS spectra displayed distinct fragments corresponding to a *p*-coumaric acid residue (*m*/*z* 119, [*p*-coumaric acid $- H - CO_2]^-$) and quinic acid (*m*/*z* 191, [quinic acid $- H]^-$ and *m*/*z* 173, [quinic acid $- H - H_2O]^-$) (*22*). The fragmentation patterns were similar to those of 3-, 4-, and 5-*p*-coumaroylquinic acid isomers, respectively, even though the relative intensities of the fragments were different from those described by other authors (*23*−*25*). On the basis of the elution behavior on HPLC, compounds **C4** and **C5** were identified as *cis*- and *trans*-3-*p*-coumaroylquinic acid, **C6** as *trans*-4-*p*-coumaroylquinic acid, and **C7** as *trans*-5-*p*-coumaroylquinic acid. In addition, sulfatase hydrolysis gave a cis:trans ratio of 1:1 for **C4**, of 1:9 for **C5**, of 2:8 for **C6**, and of 1:9 for **C7**. The bathochromic shifts in the UV spectra as compared to the aglycone were an indication that the quinic acid was esterified to the carboxylic function of the *p*-coumaric acid as described by Baderschneider and Winterhalter (*26*). The occurrence of free *p*-coumaric acid (**C1**), *cis*-*p*-coumaric acid 4-*O*-β-glucoside (**C2**), *cis*- and *trans*-3-*p*-coumaroylquinic acid (**C4** and **C5**), and *trans*-4-*p*-coumaroylquinic acid (**C6**) in sainfoin has not been reported before. Compounds **C8** and **C9** showed molecular ions at *m*/*z* 279 and intensive fragments corresponding to *p*-coumaric acid residue (*m*/*z* 119, 163) as well as a small signal for malic acid (*m*/*z* 71, [malic acid $- H - CO_2 - H_2O]^-$), (*27*, *28*). After enzymatic hydrolysis, they released *p*-coumaric acid in a cis:trans ratio of 2:8 for **C8** and of 1:9 for **C9**. Therefore, both compounds are trans isomers of *p*-coumaric acid. The malic acid is bound to position 4 in **C8** and to the carboxyl group of **C9**. This can be deduced from their UV absorbance spectra showing a maximum at 308 nm for C8 and at 313 nm for **C9**. Thus, **C9** and **C8** were identified as trans-*p*-coumaroylmalic acid and *trans*-*p*-coumaric acid 4-*O*-malate, respectively. Phenolic compounds containing malic acid have not been reported from sainfoin up to now.

In accordance with previous studies, free caffeic acid (**C10**), detected in *Onobrychis tanaitica* Sprengel (*29*), and 5-caffeoylquinic acid (**C18**) (chlorogenic acid) were identified by comparison of t_R and UV and mass spectra with those of reference substances. Other caffeic acid derivatives as well as glycosides and esters of ferulic acid have not been reported before in the genus *Onobrychis*. The LC-MS spectra of compounds **C15**, **C16**, **C17**, and **C14** yielded a $[M - H]^-$ ion at *m*/*z* 353, which is characteristic for caffeoylquinic acid isomers. Compound **C16** was identified as *trans*-3-caffeoylquinic acid (neochlorogenic acid) by comparison of the spectroscopic and HPLC data with those of a reference substance. Compound **C15** was established as *cis*-3-caffeoylquinic acid by MS, showing a fragmentation pattern similar to that of **C16**. Its cis configuration was confirmed by UV light treatment for 4 h, when 74% was converted to its trans isomer (**C16**). Compound **C17** was identified as *trans*-4-caffeoylquinic acid (cryptochlorogenic acid). The observation of the *m*/*z* 173 [quinic acid $- H - H_2O]^-$, dehydrated quinic acid, as a distinct peak in the MS/MS spectrum was an indication that the caffeic acid is bound to the hydroxyl of C4 of quinic acid. Compound **C14**, which eluted before *trans*-3-caffeoylquinic acid, was tentatively identified as *trans*-1-caffeoylquinic acid isomer. The elution order of these isomers (**C14**, **C16**, and **C17**) matched those of reference compounds prepared by isomerization of 5-caffeoylquinic acid in a phosphate buffer solution, which are known to be 1-, 3-, and 4-caffeoylquinic acids (*30*). Compounds **C12**, **C13**, and **C11** were structurally similar as found by LC-MS/MS analysis, with a $[M - H]^-$ ion at *m*/*z* 345, which produced a small signal from caffeic acid at *m*/*z* 179 after elimination of a glucose moiety, and a base peak at *m*/*z* 135, after the subsequent decarboxylation of caffeic acid [caffeic acid $- H - CO_2]^-$. Hydrolysis with sulfatase corroborated the presence of caffeic acid in their structures, and sugar analysis indicated that the hexose was glucose. Compound **C11** was identified as *trans*-caffeic acid 4-*O*-glucoside on the basis of the hypsochromic shift observed in the UV spectra (from 323 to 291 nm) and the disappearance of the typical spectral feature of the aglycone. The similarity in the UV spectral features of **C12** and **C13** with that of free caffeic acid supported their identification as *cis*- and *trans*-caffeoylglucose. LC-MS analysis of compound **C19** with absorption maxima at 288 and 320 nm revealed a $[M - H]^-$ ion at *m*/*z* 358 and MS/MS fragments at *m*/*z* 135 and 196 corresponding to the decarboxylated caffeic acid ([caffeic acid $- H - CO_2]^-$) and to a deprotonated 3-hydroxytyrosine molecule, respectively. Hence, **C19** was an amide derivative of caffeic acid identified as *cis*-clovamide, previously reported in red clover (*31*, *32*).

cis- and *trans*-ferulic acid 4-*O*-glucoside (**C20** and **C21**), and *trans*-feruloylglucose (**C22**), as well as *cis*- and *trans*-4-feruloylquinic acids (**C23** and **C24**) have not been previously reported in *O. viciifolia*. The spectroscopic and HPLC analyses agreed well with literature data on other plant species (*21*, *33*). Hydrolysis with sulfatase of **C20** and **C23** gave rise to about a 1:1 mixture of *cis*- and *trans*-ferulic acid in the EtOAc phase, whereas **C21** and **C24** released predominantly *trans*-ferulic acid. Additionally, the analysis of the aqueous phase of the hydrolysates from **C20** and **C21** indicated the presence of glucose. Compared to ferulic acid, hypsochromic shifts were observed in the UV spectra of **C20** and **C21**, whereas **C22**, **C23**, and **C24** showed bathochromic shifts. Aditionally, the disappearance of the typical spectral feature of the aglycone was observed in **C20**. On this basis they were classified as glycosides and esters of ferulic acid. Two distinct peaks at *m*/*z* 173 and 93 in the product ion scan of the quinic acid esters **C23** and **C24** allowed their identification as *cis*- and *trans*-4-feruloylquinic acid. The fragmentation pattern of **C22** was

Table 3. UV Spectral Characteristics of Flavonols from *Onobrychis viciifolia* in MeOH and after Addition of Shift Reagents

compound	MeOH λ_{max} (nm)	+ NaOMe λ_{max} (nm)	+ $AlCl_3$ λ_{max} (nm)	+ $AlCl_3$/HCl λ_{max} (nm)	+ NaOAc λ_{max} (nm)	+ NaOAc/H_3BO_3 λ_{max} (nm)
kaempferol 3-*O*-glucoside **(G1)**	266, 348	275, 402	274, 352, 396	273, 344, 389	274, 382	266, 351
kaempferol 3-*O*-rhamnogalactoside (**G2**)	266, 351	275, 402	274, 354, 399	274, 347, 397	275, 397	266, 353
kaempferol 3-*O*-rhamnoglucoside **(G3)**	266, 350	275, 402	274, 355, 399	274, 347, 396	274, 392	266, 349
kaempferol 3-*O*-glucoside- 7-rhamnoside (**G4**)	267, 347	283, 410	275, 344, 399	274, 341, 395	268, 395	266, 348
kaempferol 3-*O*-rhamnosylrutinoside (**G5**)	266, 347	273, 397	274, 350, 398	275, 347, 398	274, 380	267, 350
quercetin 3-*O*-glucoside (**G7**)	256, 358	272, 412	274, 435	265, 360, 397	272, 402	259, 376
quercetin 3-*O*-rhamnoglucoside (**G8**)	257, 359	272, 412	275, 433	269, 366, 401	273, 404	262, 379
quercetin 3-*O*-rhamnosylrutinoside (**G9**)	256, 355	271, 401	275, 435	270, 363, 402	272, 388	261, 372

different from that of the other compounds and the aglycone, which gave a predominant ion at *m*/*z* 134, representing the demethylated and decarboxylated ion of ferulic acid [ferulic acid − H − CH_3−CO_2]$^-$. **C22** showed an abundant peak at m/*z* 160 presumably corresponding to the demethylated and dehydrated ion of ferulic acid [ferulic acid − H − CH_3 − OH]$^-$. Thus, the specific fragmentation pattern may be a key in the identification of the sugar esters of ferulic acid.

Dihydroflavonols. Dihydroflavonols were identified for the first time in sainfoin. Dihydroquercetin (**D1**) was detected by cochromatography with a standard. Compound **D3** gave a [M − H]$^-$ ion at *m*/*z* 465. In the MS/MS experiments a [dihydroquercetin − H]$^-$ ion at *m*/*z* 303 and a [dihydroquercetin − H_2O]$^-$ ion at 285 were shown, suggesting the existence of dihydroquercetin in its structure. Hydrolysis with sulfatase released dihydroquercetin and glucose. On the basis of these data **D3** was characterized as dihydroquercetin-glucoside. Similarly, compound **D2** was characterized as dihydrokaempferol-glucoside by MS and hydrolysis with sulfatase. The [M − H]$^-$ ion at *m*/*z* 465 showed characteristic ions at *m*/*z* 287 [dihydrokaempferol − H]$^-$ and 269 [dihydrokaempferol − H_2O]$^-$, respectively. The determination of the sugar positions was not possible because of the low amount of these dihydroflavonol-glucosides.

Flavones. Three flavone *C*-glucosides, vitexin (apigenin 8-*C*-glucoside) (**E1**), isovitexin (apigenin 6-*C*-glucoside) (**E2**), and orientin (luteolin 8-*C*-glucoside) (**E3**), were identified for the first time in *O*. *viciifolia*. Vitexin was reported previously in the aerial parts of *O*. *montana* subsp. *scardica* by Gođevac et al. (*34*). Analyzing the MS/MS spectra of **E1**, **E2**, and **E3**, ions of [M − H − 18]$^-$, [M − H − 90]$^-$, and [M − H − 120]$^-$ were observed, which were demonstrated as characteristic ions of C-glycosidic flavonoids by Wu et al. (*35*). The presence of [M − H − 120]$^-$ and the simultaneous absence of [M − H − 60]$^-$ indicated a hexose as the sugar of C-glycosylation (*36*). The ion [M − H − 18]$^-$ was detected only in the MS/MS spectrum of **E2**, indicating that the hexose moiety was linked to C6. Compound **E1** showed the same [M − H]$^-$ ion at *m*/*z* 431 as **E2**, but its retention time in the HPLC system described was shorter. Therefore, compounds **E1** and **E2** were identified as vitexin and isovitexin, respectively. The MS analysis of **E3** gave the [M − H]$^-$ ion at *m*/*z* 447 corresponding to orientin. Additionally, cochromatography with reference substances supported the proposed chemical structures.

Flavanols. The cochromatography with standards and the application of mass spectrometry in comparison to Marais et al. (*10*) and other identification criteria as postcolumn derivatization with DMACA permitted the identification of the monomeric [catechin (**F1**), epicatechin (**F2**), gallocatechin (**F3**), epigallocatechin (**F4**)], dimeric [procyanidins B2 (**F5**), B3 (**F6**), B4 (**F7**), and B5(**F8**)], and trimeric flavanols [procyanidins C1 (**F9**) and E-B5 (**F10**)]. Only the monomers and procyanidin B2 had been reported by Koupai-Abyazani et al. (*37*). The other dimers and the trimers were identified for the first time in a sainfoin extract.

Flavonols. The analysis of the mass and UV spectroscopy data combined with the information obtained from acid hydrolysis, TLC, and HPLC (**Tables 2** and **3**) led to the recovery of three flavonol disaccharides, the 3-*O*-rutinosides of kaempferol (**G3**), quercetin (**G8**), and isorhamnetin (**G13**), and two trisaccharides, the branched 3-*O*-rhamnosylrutinosides of quercetin (**G9**) and kaempferol (**G5**), previously reported in sainfoin (*4*, *10*, *19*). Compound **G12** showed λ_{max} at 260 and 355 nm, similar with those of myricetin 3-*O*-rutinoside previously reported in sainfoin by Lu et al. (*19*). The most abundant ion on MS/MS fragmentation ([M − H]$^-$ at *m*/*z* 625) was *m*/*z* 316 [M − H − 146 − 162 − 1]$^-$ corresponding to myricetin after the cleavage of hexose and deoxyhexose moieties. This fragmentation pattern corresponded to those of other 3-O-glycosylated flavonols (**G9**, **G5**, **G8**, **G3**, and **G13**). The MS/MS spectra of the reference substances quercetin, kaempferol 3-*O*-rutinoside-7-rhamnoside, and kaempferol 3-*O*-glucoside-7-rhamnoside (**G4**) showed a distinct ion [M − H − 146]$^-$ that clearly indicates the removal of rhamnosyl moiety from the hydroxyl group of C7 and showed almost the same intensity as the aglycone. Previous studies (*38*, *39*) described the removal of the sugar residues from the hydroxyl in position 7 as being much more favored in ESI-MS than from position 3. Due to these findings (**G12**) was identified as myricetin 3-*O*-rutinoside.

Further flavonol glycosides reported here for the first time in *O*. *viciifolia* were grouped into monoglycosides (**G1**, **G6**, **G7**, and **G11**), 3- and 3,7-diglycosides (**G2** and **G4**), rhamnosylrutinosides (**G14**), and glucuronides (**G10**). Kaempferol 3-*O*-glucoside (**G1**), quercetin 3-*O*-rhamnoside (**G6**), quercetin 3-*O*-glucoside (**G7**), and myricetin 3-*O*-rhamnoside (**G11**) were identified by comparison with standards. LC-MS studies of compounds **G1** and **G7** revealed [M − H]$^-$ ions at *m*/*z* 447 and 463 and demonstrated the cleavage of a glucose moiety (162 Da) generating the ions *m*/*z* 284 and 300 of kaempferol and quercetin aglycone, respectively. Compounds **G11** and **G6** gave [M − H]$^-$ ions at *m*/*z* 463 and 447, respectively. Their fragmentation showed the ions *m*/*z* 316 and 301 of myricetin and quercetin aglycone after elimination of a rhamnose moiety (146 Da). Acid hydrolysis of **G1** and **G7** gave glucose as well as kaempferol and quercetin, respectively, whereas compound **G11** released myricetin and rhamnose. Rf values of **G1** and **G7** on polyamide (0.23) and color reactions (deep purple to yellow and brown to orange in UV + Naturstoffreagenz A) were consistent with the proposed chemical structures. The shifts in the λ_{max} of **G1** and **G7** after the addition of diagnostic reagents confirmed the presence of free hydroxyl groups at positions 5, 7, 3′, and 4’, suggesting that both compounds are glycosylated at the 3-position of the respective aglycone (**Table 3**) (*9*).

Furthermore, we detected kaempferol 3-*O*-rhamnogalactoside (**G2**) and kaempferol 3-*O*-glucoside-7-rhamnoside (**G4**). The MS/MS fragmentation of **G2** ([M − H]$^-$ at *m*/*z* 593) produced almost exclusively the aglycone pseudomolecular ion [M − H − 146 − 162 − 1]$^-$ at *m*/*z* 284 showing a similar fragmentation pattern observed for kaempferol 3-rutinoside (**G3**). Acid hydrolysis of **G2** released kaempferol and the sugars rhamnose and galactose in the

Table 4. Composition of Phenolic Compounds (Milligrams per Gram of Dry Weight) in Organs of Sainfoin Plants of the Variety Cotswold Common

label[a]	compound	young leaflets[b]	young petioles[b]	stems[c]	flower stalks[c]	flower buds[c]
			Simple Phenolic Acids			
P1	arbutin	–	17.67 ± 7.33	4.90 ± 1.03	8.71 ± 2.30	–
P2	8-β-glucopyranosyloxycinnamic acid	0.01 ± 0.01	1.94 ± 0.74	1.80 ± 0.93	2.03 ± 1.23	0.28 ± 0.15
	other simple phenolic acids	2.59 ± 1.12	3.31 ± 1.34	0.72 ± 0.13	1.26 ± 0.39	3.08 ± 0.92
	total simple phenolic acids	*2.61*	*22.92*	*7.42*	*12.00*	*3.36*
			Hydroxybenzoic Acids			
B4	vanillic acid 4-*O*-glucoside	–	–	0.07 ± 0.05	0.09 ± 0.09	0.07 ± 0.03
	total hydroxybenzoic acids	–	–	*0.07*	*0.09*	*0.07*
			Hydroxycinnamic Acids			
C2 + C3	*cis* + *trans*-*p*-coumaric acid 4-*O*-glucoside	0.38 ± 0.25	0.37 ± 0.15	0.11 ± 0.04	0.28 ± 0.14	0.34 ± 0.16
C5	*trans*-3-*p*-coumaroylquinic acid	0.04 ± 0.04	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.02
C13	*trans*-caffeoylglucose	0.08 ± 0.05	0.06 ± 0.05	0.03 ± 0.02	0.04 ± 0.02	0.09 ± 0.06
C16	*trans*-neochlorogenic acid	0.97 ± 0.64	0.37 ± 0.32	0.14 ± 0.12	0.23 ± 0.19	0.39 ± 0.22
C18	*trans*-chlorogenic acid	0.66 ± 0.43	0.25 ± 0.04	0.03 ± 0.02	0.08 ± 0.06	0.28 ± 0.32
	other hydroxycinnamic acids	1.32 ± 0.62	0.82 ± 0.39	0.25 ± 0.07	0.58 ± 0.29	1.25 ± 0.36
	total hydroxycinnamic acids	*3.45*	*1.87*	*0.58*	*1.22*	*2.39*
			Flavanols			
F1	catechin	0.59 ± 0.34	3.46 ± 1.15	0.53 ± 0.27	1.10 ± 0.49	0.55 ± 0.33
F2	epicatechin	0.22 ± 0.1	1.23 ± 0.83	0.29 ± 0.17	0.41 ± 0.28	0.07 ± 0.04
F3	gallocatechin	0.72 ± 0.36	0.28 ± 0.20	0.07 ± 0.05	0.05 ± 0.03	0.19 ± 0.08
F4	epigallocatechin	0.35 ± 0.13	0.36 ± 0.09	0.08 ± 0.06	0.09 ± 0.06	0.20 ± 0.09
F5	procyanidin B2	0.03 ± 0.002	0.30 ± 0.11	0.09 ± 0.05	0.10 ± 0.08	0.01 ± 0.004
F8	procyanidin B5	–	0.09 ± 0.05	0.02 ± 0.01	0.01 ± 0.01	–
F10	procyanidin E-B5	–	0.03 ± 0.01	0.01 ± 0.004	–	–
	other flavanols	1.59 ± 0.47	2.53 ± 0.84	0.54 ± 0.2	0.93 ± 0.65	0.57 ± 0.35
	total flavanols	*3.50*	*8.26*	*1.60*	*2.69*	*1.58*
			Flavones			
	flavone derivatives	1.22 ± 1.06	1.86 ± 2.27	0.53 ± 0.42	1.41 ± 1.45	1.14 ± 1.21
	total flavone derivatives	*1.22*	*1.87*	*0.53*	*1.41*	*1.14*
			Flavonols			
G3	nicotiflorin	2.82 ± 0.98	0.24 ± 0.04	0.06 ± 0.02	0.16 ± 0.08	1.31 ± 0.58
G7	isoquercitrin	0.57 ± 0.33	0.60 ± 0.49	0.17 ± 0.17	0.26 ± 0.13	0.44 ± 0.24
G8	rutin	19.94 ± 12.07	9.14 ± 10.46	2.57 ± 1.92	6.63 ± 5.09	5.78 ± 2.30
G9	quercetin 3-*O*-rhamnosylrutinoside	2.14 ± 2.00	1.52 ± 2.12	0.78 ± 0.76	1.19 ± 1.57	0.58 ± 0.44
G13	isorhamnetin 3-*O*-rutinoside	3.56 ± 3.08	3.56 ± 3.08	0.69 ± 0.84	1.48 ± 1.35	0.29 ± 0.25
1	quercetin 3-arabinoside	0.79 ± 0.69	0.72 ± 0.62	0.43 ± 0.64	–	0.10 ± 0.11
	other flavonols	3.53 ± 1.29	1.64 ± 0.88	0.68 ± 0.45	0.98 ± 0.50	1.41 ± 0.73
	total flavonols	*33.34*	*17.43*	*5.37*	*10.90*	*9.91*
			Anthocyanins			
2	cyanidin 3-*O*-glucoside	–	0.04 ± 0.04	0.18 ± 0.14	0.08 ± 0.04	0.04 ± 0.04
	other anthocyanins	–	–	0.05 ± 0.02	–	0.05 ± 0.03
	total anthocyanins	–	*0.04*	*0.23*	*0.08*	*0.09*

[a] Compounds previously identified in the acetone extract were labeled with letters and numbers referring to **Table 2** except labels 1 and 2, which indicate additionally identified compounds by cochromatography with standards. [b] Means and standard deviations of phenolic concentration from three plants. [c] Means and standard deviations of phenolic concentration from six plants

ratio 1:1, and the UV spectroscopic analysis using customary shift reagents indicated the presence of a 3-glycosylation (**Table 3**). On TLC polyamide, **G2** gave an Rf value of 0.39 and the color (UV, 366 nm) shifted from deep purple to yellow when sprayed with Naturstoffreagenz A. Compound **G4** with $[M - H]^-$ at m/z 593 produced the most abundant MS/MS fragment at m/z 285 $[M - H - 146 - 162]^-$ (aglycone). The cleavage products corresponding to rhamnose ($[M - H - 146]^-$ at m/z 447) and glucose ($[M - H - 162 - 1]^-$ at m/z 430) showed almost the same intensities. On acid hydrolysis of **G4**, kaempferol, glucose, and rhamnose (glucose/rhamnose, 1:1) were identified by HPLC. Treatment with β-glucosidase gave kaempferol 7-*O*-rhamnoside and glucose. These results agree with the UV study in methanol and after the addition of alkaline and metal reagents (**Table 3**), which indicated that **G4** was a kaempferol derivative, with the hydroxyls at the 7- and 3-positions blocked. Cochromatography with kaempferol 3-glucoside-7-rhamnoside standard was also performed. Compound **G14** showed a molecular ion peak at m/z 769 in the negative ESI mass spectrum. Its MS/MS fragmentation produced almost exclusively an ion at m/z 314 $[M - H - 146 - 162 - 146 - 1]^-$ corresponding to isorhamnetin after cleavage of two rhamnose and one glucose moieties, which was

consistent with the acid hydrolysis. The same fragmentation pattern was observed for the branched 3-rhamnosylrutinosides of kaempferol (**G5**) and quercetin (**G9**). In the case of linear as well as branched saccharides, the cleavage of one rhamnose molecule from compound **G14** gave a low signal at m/z 623 that corresponds to [isorhamnetin 3-rutinoside − H]$^-$. Cleavage of a second rhamnose would be possible only in the case of a linear structure and, as a result, the ion at m/z 477 coresponding to isorhamnetin 3-glucoside would occur. This mass did not appear because the cleavage of a second molecule from an already charged diglycoside would generate a second negative charge (*40*). Therefore, the absence of the isorhamnetin 3-glucoside anion mass proves the branched structure of **G14** which was identified as isorhamnetin 3-*O*-rhamnosylrutinoside. This assignment is also supported by the fact that the triglycosides of quercetin and kaempferol were bound at the C3 of the aglycone.

Flavonols containing glucuronic acid have not been reported from *O. viciifolia* up to now. Compound **G10** showed a negative molecular ion at m/z 477 in LC-MS, which fragmented to quercetin ion at m/z 301 (loss of a glucurone) and its typical fragments at m/z 151 and 179 in MS/MS. Thus, **G10** was identified as quercetin 3-glucuronide after cochromatography with an authentic standard from strawberries.

Quantitative Analysis of Phenolic Compounds in Different Aerial Parts of Sainfoin Plants. The methanolic extracts of young leaves, stems, flower stalks, and flower buds from *O. viciifolia* plants (variety Cotswold Common) at preflowering stages were analyzed for their phenolic composition. The contents of the identified single phenolic acids and flavonoids as well as their total amount were evaluated. In addition to the compounds identified in the acetone extract, the flavonol quercetin 3-arabinoside and the anthocyanin cyanidin 3-glucoside were also quantified. In sainfoin, all tested aerial parts contained phenolic compounds and showed organ-specific composition (**Table 4**). For example, arbutin occurred in young petioles (amounting to 17.7 mg/g of DW) as predominant compound, as well as in stems and flower stalks, but did not appear in leaves and flower buds. The other two most abundant polyphenols were rutin (predominant in young leaflets, 19.9 mg/g of DW) and catechin (predominant flavanol in young petiols, 3.5 mg/g of DW). The flavonols represent 75% of the phenolics in young leaflets and 53% in flower buds. Simple phenolic acids were dominant in young petioles (43% of all phenolics), in stems (47%), and in flower stalks (42%). HPLC with postcolumn derivatization provided a valuable tool for the quantification of flavan-3-ols and dimeric and trimeric proathocyanidins, which usually are overlapped by the phenolic acids in LC-DAD. The greatest quantity of flavanols was found in young petioles (15%).

A perusal of the results obtained for each individual plant revealed qualitative as well as quantitative differences, which can be deduced from the high standard deviations found in **Table 4**. For example, the young petioles of one plant (replicant 1) contained no quercetin 3-arabinoside or isorhamnetin 3-rutinoside, but produced higher quantities of rutin (21.1 mg/g of DW) as compared to another two plants (replicants 2 and 3). The latter accumulated in addition to rutin (1.9 mg/g of DW in plant 2 and 4.3 mg/g of DW in plant 3) the other two flavonol glycosides (1.1 mg/g of DW quercetin 3-arabinoside, 5.2 mg/g of DW isorhamnetin 3-rutinoside in plant 2, and 1.1 mg/g of DW quercetin 3-arabinoside, 5.4 mg/g of DW isorhamnetin 3-rutinoside in plant 3). In total, 63 compounds could be characterized in a sainfoin acetone extract, most of which have not been described hitherto in this plant material. Their identification and quantification will aid in understanding the health benefits of this plant as forage legume and constitute a new step in the preselection of interesting candidates for future breeding programs. The metabolomes of organs from different individuals show that the phenolic composition of plants within one variety differs not only quantitatively but also qualitatively even when grown at the same place. This indicates that the variety Cotswold Common is not homogeneous with respect to its content of bioactive secondary metabolites. If the beneficial effect of sainfoin for animal health will be a target for breeders and farmers, it will be necessary to select lines with a well-defined and more stable phenolic profile.

ABBBREVIATIONS USED

BAW, *n*-butanol/acetic acid/water; DMACA, *p*-dimethylaminocinnamic aldehyde; LC-ESI-MS/MS, liquid chromatography−electrospray ionization−tandem mass spectrometry; m/z, mass-to-charge ratio; Rf, retention factor; RP-HPLC-DAD, reversed phase high-performance liquid chromatography coupled with diode array detector; TLC, thin layer chromatography; UV−vis, ultraviolet−visible absorption spectroscopy; WEEA, water/ethanol/ethyl methyl ketone/acetylacetone.

ACKNOWLEDGMENT

We thank Hans Geiger for the gift of some reference substances and Hans Eichinger and Thomas Hofmann (ZIEL, Bioanalytik, Weihenstephan) for giving us the possibility to use the LC-MS/MS.

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Received February 23, 2009. Revised manuscript received April 22, 2009. These investigations were supported by the European Comission (Project MRTN-CT-2006-035805).