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Chemical characterisation of low molecular weight phenolic compounds from the forage legume sainfoin (*Onobrychis viciifolia*)

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CONTENTS

1. INTRODUCTION

In recent years, tanniferous forage legumes gained in importance and are becoming a key component of future livestock farming systems with high environmental and economical performance (ROCHON et al., 2004; DEWHURST et al., 2009).

Sainfoin (*Onobrychis viciifolia* Scop.; Leguminosae subfamily Papilionoidae) has attracted renewed interest because of its positive nutritional, animal-health related and environmental attributes (HAYOT CARBONERO et al., 2011, 2012; MUELLER-HARVEY, 2009). In particular, protection of ruminants against legume bloat and protein metabolism is facilitated by its content of condensed tannins. Besides condensed tannins, flavanols and flavonols can have implication in sainfoin's anthelmintic activity which has been confirmed *in vitro* and *in vivo* (MANOLARAKI et al., 2010). Sainfoin also produces a number of isoflavonoid compounds which are known to act as phytoalexins (INGHAM, 1978).

Investigation on the effects of sainfoin on ruminant performance and health are inconsistent in their outcomes. The contradictory findings were repeatedly attributed to variations in polyphenols concentration and composition but also the analytical techniques may not have been appropriate (MANOLARAKI, 2011).

Throughout Europe, the cultivation of sainfoin has declined over the last 40 years due to the advent of cheap fertilizers. Furthermore, agronomic limitations have also contributed to its decline, including low productivity and problems with establishment, compared to *Medicago sativa* (BORREANI et al., 2003). In the meantime, various breeding programs have successfully improved the agronomic performance of lucerne and clovers. As a result, in 2010, only 19 varieties of *O.viciifolia* were registered on the European common catalogue, compared with 300+ cultivars of lucerne (European Commision, 2008).

However, recently the potential of sainfoin for sustainable farming approaches has attracted attention and a pre-breeding project network (HEALTHY HAY) had evaluated agronomic, genetic, nutritional and veterinary properties from a unique and extensive

germplasm collection. As anticipated, there were significant differences between sainfoin types and therefore, identification of suitable germplasm resources with a known polyphenol spectrum is a promising approach for future breading of sainfoin varieties with improved agronomic performance whilst maintaining the beneficial effects.

The aim of this work was to characterize the soluble phenolic 'metabolome' of sainfoin.

In this way, new acylated flavonol glycosides structures were investigated, which together with flavanols and flavonols, in addition to condensed tannins may be responsible for the nutritional and veterinary benefits attributed to sainfoin.

In the same time a high-performance liquid chromatography method with diode array detection and post-column derivatization with p-dimethylaminocinnamicaldehyde was validated for the analysis of sainfoin plant extracts and was used to examine changes in the content and composition of phenolic compounds in various organs at different developmental stages of sainfoin.

In order to identify interesting candidates for further breeding programs, the variation in phenolic content and composition of 37 sainfoin accession was studied.

2.1. SAINFOIN GENERAL DESCRIPTION AND USE

2.1.1. Botanical description

Sainfoin (*Figure 1*) belongs together with the 'big three' cultivated crops: white clover (*Trifolim repens*), lucerne (*Medicago sativa*) and red clover (*Trifolium pratense*) to the family Leguminosae. It is found in the literature under several Latin names: *Hedysarum onobrychis* L., *Onobrychis sativa* Lam., *Onobrychis viciaefolia* Scop. and *Onobrychis viciifolia* Scop., and is the most widespread of the about hundred species of genus *Onobrychis* (CELIKTAŞ et al., 2006).

Figure 1. ***Onobrychis viciifolia*** **picture**

The 40 to 80 cm tall sainfoin plant has many erect or sub-erect hollow stems arising from basal buds on a branched root stock. Its pinnate leaves comprise 5 - 14 pairs of leaflets and a terminal leaflet. The stipules are broad and finely pointed. The inflorescence is a densely flowering raceme carried on a long axillary peducle. As many as 80 pinkish red melliferous flowers open from the base of the raceme upwards and are pollinated by honey-bees. Each flower produces a 4 - 6 mm single plattened, kindney-shaped seed contained in a brown, indehiscent pod. Seed color ranges from olive to brown or black. The root system consists of deep tap root with a few main branches and numerous fine lateral roots bearing most of the nitrogen-fixing nodules (FRAME et al., 1998).

Two types of sainfoin were characterised previously via their growth habit and persistence, the common and giant type. The common type, also known as single-cut sainfoin (*Onobrychis sativa* var. *communis)* flowers in the year after establishment and the stands can persist for 20 - 30 years. Peak hay or silage yields are reached in the second or third harvest year. The giant or double-cut sainfoin (*Onobrychis sativa* var. *bifera* Hort) flowers in the establishment year and thereafter for only 2 - 3 years. It attains peak yield the year after sowing and two conservation cuts may be performed annually. The giant type has erect, longer stems with more internodes per stem and significantly more leaflets per leaf than common sainfoin (THOMSON, 1951a; DELGADO, 2008). There are no differences in seed weigh and color between giant and common type (THOMSON, 1951b).

The best known landraces of the common type are Cotswold Common, Hampshire Common and Sambourne, and of the giant type are Hampshire Giant and English Giant (HAYOT CARBONERO et al., 2011). Nevertheless, new sainfoin cultivars derivating from the common and giant types and Russian landraces were developed: Melrose and Nova (in Canada), Eski, Remont and Remunex (in the USA), Vala and Zeus (in Italy), Perly (in Switzerland), Fakir (in France) and Emyr (in Hungary), (KOIVISTO and LANE, 2001). The existing sainfoin germplasm show differences in their morphological characters, winter-hardiness, growth habit, maturity, persistence, yield potential and many other factors (HAYOT CARBONERO et al., 2011, 2012; DELGADO et al., 2008).

2.1.2. Geographical distribution and agricultural history

Indigenous to southern Europe and the more temperate regions of western Asia (CLARK and MALTE, 1913), sainfoin has a long history as a cultivated crop. It was introduced into central Europe in the 15th century (BURTON and CURLEY, 1968) and was first cultivated in France at the beginning of the 16th century (CHORLEY, 1981) where, 'They name it in French Saint Foin...' (LYTE, 1578, cited by CHORLEY, 1981). The cultivation spread slowly into other European countries especially Italy, England and Switzerland.

In Germany, where it is commonly called Esparsette, the cultivation of sainfoin can be traced back to the 16th century (RUPRECHT, 2005) and became here an important forage crop as early as 1716 (PIPER, 1975).

Its very high quality hay was used to feed the heavy working horses and as noted by GENBERLLCH (1836) promotes the milk production in sheep and the growth in young lambs: "Hat man in Winter nicht fortwährend Esparsetteheu zu füttern, so spart man dies bis zur Lammzeit auf, weil es den Milchertrag und das Gedeihen der jungen Lämmer besonders fördert". Frequent escape from cultivation, sainfoin could be found naturalized in calcareous grasslands and on roadsides. *O. viciifolia* was introduced to North America from Europe in the early 1900s, but its success as a forage crop did not occur until the 1960s, when improved varieties allowed wider cultivation in adapted areas.

Nowadays, *O. viciifolia* is still being cropped mainly in limestone areas of France, in S Russia and E Europe, in Italy, Spain, Iran and Turkey and gained importance in the USA and Canada.

2.1.3. Culture and production

O. viciifolia prefers to grow on light or medium soils with pH 6 or above without waterlogging and is adapted to soils low in phosphorus (MILLER and HOVELAND, 1995).

Agronomically, some advantages of sainfoin are:

- It is adapted to calcareous soils and led to the profitable cultivation of much dry calcareous land (PIPER, 1924),
- It is very resistant to drought 'When the other grasses and plants are destroyed by the parching heat of the sun…this flourished very much, heaving very great root and deep in the ground, and not easily exsiccated.' (CHORLEY, 1981),
- It is also grows well under irrigated conditions and is winter hardy (PEEL et al., 2004; MEYER and BADARUDDIN, 2001),
- It is resistant to serious insect pests such as *Medicago sativa* weevil (*Hypera postica*) and peas aphids (*Acrythosyphon pisum*) (MORILL et al., 1998) showing potential as alternative to *Medicago sativa*.

Some of the disadvantages of sainfoin:

- It is susceptible to crown and root disease caused by clover rot (*Sclerotinia trifoliorum*) and different *Fusarium* species (HVANG, et al., 1992; EKEN et al., 2004),
- It has less efficient nitrogen fixation system (WALSH et al., 1983),
- It has lower leaf-area index (LAI) and a less erect canopy structure than lucerne and therefore has less efficient utilization of incident irradiance than lucerne (SHEEHY and POPPLE, 1981),
- It has a slow growth in the establishment phase, and
- It has low stand persistence with lower yields in the 4th and 5th years on dryland and 3rd and 4th years under irrigation (CARLTON et al., 1968; SMOLIAK and HANNA, 1974).

Sainfoin can be sown in monoculture, but the presence of a non-competitive grass such as timothy (*Phleum pretense*) or meadow fescue (*Festuca pratensis*) improves sward density and reduces the degree of weed ingress (LIU et al., 2008). According to COOPER (1972) which evaluated the hay production of two sainfoin cultivars seeded alone and with low-growing grasses and legumes on irrigated land over a 4-year period; mixtures containing

either birdsfoot trefoil (*Lotus corniculatus* L.) or black medic (*Medicago lupulina* L.) were the most productive.

It is a forage and fodder plant. Its forage production is variable and largely determined by growing condition. The dry matter (DM) yields may range between 7 and 15 t DM ha-1 and are about 20% lower than those of lucerne. This was due to a lower leaf area index, a more prostrate canopy structure and less efficient nitrogen fixation (FRAME et al., 1998).

Whilst various breeding programs have successfully improved the agronomic performance of lucerne and Trifolium species, little research been directed towards improving *O. viciifolia* varieties. Nevertheless, new sainfoin cultivars such as Melrose, Nova, Eski, Remont and Remunex have shown good yields, and in order to develop an economically viable forage legume current research is aimed to improve their agronomical performance (CASH et al., 1993).

Sainfoin is also appreciated for producing excellent yields of high quality honey (DUBBS, 1967) and is an excellent food source for bees and other pollinators (HAYOT CARBONERO et al., 2011).

2.1.4. Why sainfoin cultivation has declined?

The cultivation of *O. viciifolia* Scop. on vast area of agricultural land in Europe was abandoned after the World War II. While, for example in the United Kingdom, more than 150 tones seeds were sold every year in the late 1950s, enough for 2,500 hectares, in the late 1970s only approximately 150 hectares were cropped, and this number continued to decrease again after (HILL, 1998). The cause of this decline might have been due

- to the farmer support payments toward intensive production using cheap inorganic fertilizers (ROCHON et al., 2004),
- to the expansion and the dominance of autumn cereal cropping (HILL, 1998),
- to the agricultural structural changes and the gradual disappearance of livestock farms in hilly areas (BORREANI et al., 2003), and in main part,

- to the high establishment costs; the large seed, high seeding rate and high seed prices often reduce interest in using this species, and
- to the agronomic problems that sainfoin meets such as low yield and persistence, and poor regrowth after the first cut, when compared to *Medicago sativa* or *Trifolium* species (BORREANI et al., 2003).

2.2. QUALITIES OF SAINFOIN

From the nutritional, veterinary and environmental point of view *O. viciifolia* seems to be the most desirable of all forage legume plants.

In fact, the englisch term sainfoin is derived from the French 'sain foin'[1] which means 'healthy hay', while the Latin name, *Onobrychis* means 'donkey's favorite fodder' in Greek.

2.2.1. Condensed tannins (CTs)

The well-known feature of sainfoin is the presence of condensed tannins.

Condensed tannins (CTs, also known as proanthocyanidins) occur as dimmers, trimmers, oligomers and more complex polymers of flavan-3-ol units derived from the flavonoid pathway (HASLAM, 1996; STAFFORD, 1988). They play a protective role within the plant and have widespread effects on human health. Moreover, CTs can also produce important benefits in ruminant production (REED, 1995; MUELLER-HARVEY, 2006).

An important property of the CTs is their ability to form complexes with proteins and these is the basis of many biological effects of tannins. This property is attributed to the fact that the CTs are multidentate ligands, able to bind simultaneously at more than one site on a protein surface via either hydrophobic or hydrogen bonds or via a combination of these two distinctly different binding mechanisms (MUELLER-HARVEY, 2006). Complexation is pH dependent and therefore reversible. The capacity to bind to protein is influenced by factors

[1] The name has sometimes been erroneously written Saint Foin and has thus led to the misconception that it means 'Holy hay', (CLARK and MALTE, 1913).

such as CTs content and biological activity (astringency, mean degree of polymerization, prodelphinidin and cis content (FRAZIER et al., 2003; McALLISTER et al., 2005)).

Several studies have shown that CTs structure and concentration changed during plant tissue maturation (HAGERMAN and BUTLER, 1991; KOUPAI-ABIAZANY et al., 1993) and varies among the phenological stages (THEODORIDOU et al., 2011a, b). A correlation between high concentrations of lignin and CTs has also been found (BARRY and MANLEY, 1986).

CTs were first noted in sainfoin leaves by bloat researchers in the early 1970's (JONES and LYTTLETON, 1971). In a next study, SARKAR et al. (1976) found that the leaves and the stems of sainfoin contained polymers whose acid degradation products were cyanidin and delphinidin. Also flower petals and seed coat were found to contain CTs by GOPLEN et al. (1980).

Sainfoin CTs are highly complex mixtures which differ greatly between accessions in structural composition and content. Two recent studies (GEA et al., 2011 and STRINGANO et al., 2012) described tannins with mean degree of polymerization from 12 to 84; the proportion of prodelphinidin tannins from 53% to 95% and the proportion of trans flavanol units from 12% to 34%. Their contents varied from 0.57 to 2.80g /100 g freeze dried sainfoin.

According to THEODORIDOU et al. (2011a) the CTs concentration in sainfoin plants, the proportion of prodelphinidins and the mean degree of polymerization increased with the phenological stage (or plant maturity).

KOUPAY-ABYAZANI et al. (1993) have examined the CTs content and composition of sainfoin leaves at five stages of development. They showed that catechin, epicatechin, gallocatechin (GC) and epigallocatechin (EGC) were present as terminal units at all stages, while GC and EGC were the predominant extension units with lesser amounts of epicatechin incorporated at early stage. Catechin was not incorporated as an extension unit. The degree of polymerization and the proportion of trihydroxylated B-rings increased with leaf development whereas the composition of cis isomers decreased from 83 to 48%.

2.2.2. Nutritional properties

2.2.2.1. Palatability

Despite the fact that sainfoin is coarse and stemmy, and has a high amount of tannins, livestock producers repeatedly report that animals prefer sainfoin over other forage species, and that sainfoin was palatable. Both grazed and conserved sainfoin and sainfoin rich forages are highly accepted. As a result, it has high voluntary intakes by cattle, sheep and horses. (PARKER and MOSS, 1981; GRIGGS and MATCHES, 1991; WAGHORN, 1990; KARNEZOS et al., 1994; SCHARENBERG, 2005; NEUHOFF and BÜCKING, 2006).

2.2.2.2. Nutritional value

Sainfoin could be ranked among the best protein sources with crude protein (CP) concentrations between 13 and 19% (PARKER and MOSS, 1981; BAL et al., 2006) and a balanced essential amino acid composition. KALDY et al. (1979) compared the amino acid composition of sainfoin to that of lucerne and found that, the protein score, an estimation of protein quality for non-ruminants, was 68 for sainfoin forage and 71 for lucerne forage compared to 100 for an ‘ideal protein’. The protein scores, based on the distribution of each essential amino acid compared to the total essential amino acids and related to ideal (egg) protein, indicate that methionine is the first limiting amino acid, for both legumes and isoleucine and valine are the second and third limiting amino acids in both sainfoin and lucerne.

The CP concentration decreased with increasing maturity of the plant because stems, with their lower protein concentration, make up a larger portion of the herbage in more mature forage (BUXTON, 1996).

Compared to other forages, sainfoin has a better utilization of dietary protein by ruminants due to the condensed tannins. These led not only to higher N-retention but also to less environmental N pollution (THOMSON et al., 1971; EGAN and ULYATT, 1980; WAGHORN et al., 1990).

The water-soluble carbohydrate content in sainfoin ranged from 41 to 101 g/kg with sucrose as the main metabolite. The inositol (+) pinitol was also identified (MARAIS et al., 2000).

The neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) contents ranged from 46.14 to 55.71%, 33.40 to 40.15% and 7.10 to 11.10%. The NDF value is an estimate of most cell wall components which limit digestibility in forages (cellulose + hemicellulose + lignin), ADF quantified cellulose and lignin and ADL determined the lignin content. The levels of these three determinations increased with advanced maturity (BAL et al., 2006). In addition, some studies have linked decreased forage digestibility to increased syringil/guayacil (S/G) ratio in the lignin composition as a function of increased plant maturity (BUXTON and RUSSELL, 1988; GRABBER et al., 1997).

As reported by BAL et al. (2006), ash contents of sainfoin (total mineral content) ranged from 7.18 to 8.31% and falls with increasing plant maturity.

In comparison with grasses sainfoin is rich in mineral, except its calcium and sodium contents, 13.8 – 15.7 g kg^{-1} and 0.18 – 0.19 g kg^{-1}, which are generally lower than in other forage legumes (GERVAIS, 2000; FRAME, 2006). Besides the stage of growth, soil moisture level has also a variable effect on mineral concentrations in the forage of sainfoin (KIDAMBI et al., 1990).

2.2.3. Animal health and welfare

2.2.3.1. Bloat prevention

Sainfoin benefits ruminant health by preventing bloat, a worldwide problem which causes serious financial losses due to animal deaths.

Bloat is a digestive disorder caused by the formation of stable protein foam in the rumen. It occurs mainly when animals are allowed to graze a number of the most valuable and productive young lush forages such as clover or alfalfa (MAJAK et al., 1995, McMAHON, et

al., 2000; ROCHFORD et al., 2008). Bloat is normally controlled with twice daily oral administration of detergents, which disperse the foam.

The bloat inhibiting nature of sainfoin is attributed to its condensed tannins which can act as protein precipitants to preclude foam formation. The precise mechanism by which tannins prevent bloat is still unclear (McMAHON et al., 2000).

Animal deaths from bloat were also reduced by the substitution of a small amount (approximately 10%) of ingested alfalfa DM by sainfoin, which amounted to 2-5 g condensed tannins /kg in the diet (McMAHON et al., 1999, 2000).

2.2.3.2. Control of internal parasites

From the animal health and welfare aspect another interest in *O. viciifolia* lie in the parasite control potential.

Gastrointestinal parasites represent a major threat in the breeding of grazing ruminants. The control of these parasites is generally based on the use of chemical anthelmintics[2], but the development of resistance has been reported in almost all species of domestic animals (JABBAR et al., 2006).

Feeding of conserved sainfoin reduced adult *Haemonchus contortus* population (47% in the case of hay; 49% in the case of silage) and faecal egg counts on a dry matter basis, (58% in the sainfoin hay group and 48% in the silage group) in lambs (HECKENDORN et al., 2006). Sainfoin hay consumption has been associated with a reduction in parasitism with gastrointestinal nematodes in goats (PAOLINI et al., 2003; HOSTE et al., 2005). In addition, in vitro experiments have demonstrated that sainfoin extracts have an inhibitory effect on the mobility of third stage larvae of different nematode species (MOLAN et al., 2000; BARAU et al., 2005; BRUNET et al., 2007).

In most of the publications the anthelmintic properties of sainfoin have mainly been related to its condensed tannins content. In the work of BARRAU et al. (2005) also the role of

[2] Anthelmintics are drugs of varying biochemical structure, used to kill helminthes and eliminate the worms from the hosts

flavonol glycosides such as rutin, nicotiflorin and narcissin in these effects has been substantiated through bioassays of biochemical fractions.

3. STRUCTURAL IDENTIFICATION OF PHENOLIC COMPOUNDS

The traditional fodder legume sainfoin (*Onobrychis viciifolia* Scop., Leguminosae) was attributed excellent nutritional, environmental and veterinary qualities, to which its phenolic constituents contribute. These natural products occur in several forms in plants:

- as soluble compounds extractable with water or with methanol and aqueous acetone and
- in non-extractable forms. After extraction they may remain in the residue due to their large molecular weight, or because they are bonded to, or in complexes with, other plant constituents (STRACK, 1997).

In the present work we have examined the soluble low molecular phenolic compounds of sainfoin. The obtained data together with the materials and method used were published in two articles (REGOS et al., 2009; VEITCH et al., 2011).

This chapter first describes briefly the isolation and purification work. The characterisation of isoflavones and several other compounds that could not completely be identified because only small amounts were available is than presented before the two original publications will conclude this chapter.

Isolation procedure. The phenolic compounds from 50 g mixed sample of sainfoin whole plants dried at room temperature (var. Cotswold Common), were isolated and identified according to the procedure outlined in *Figure 2*.

As extraction solvent we used a mixture of acetone/water 70/30 (v/v).

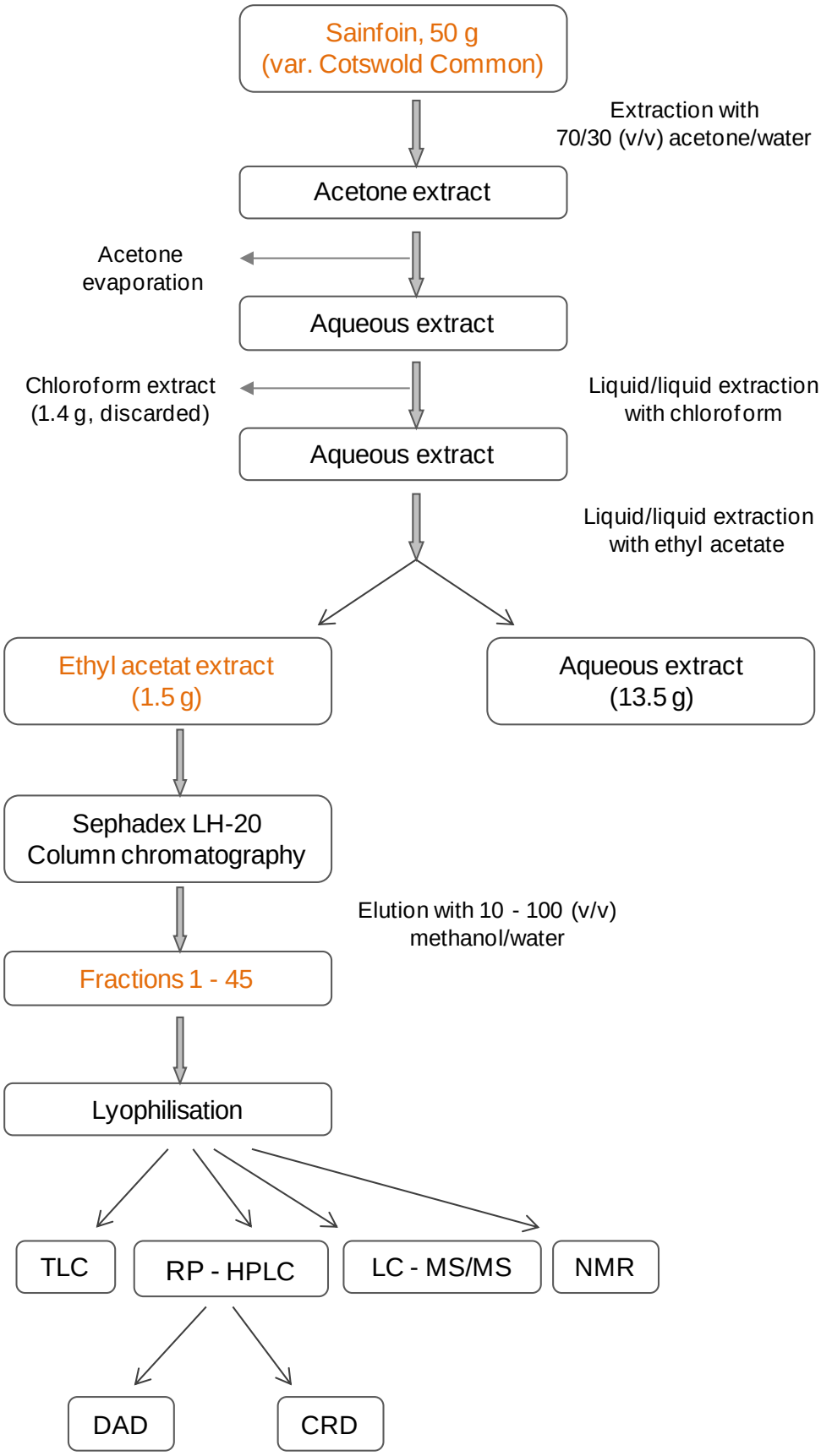

Figure 2. **Scheme for isolation and characterisation of phenolic and other aromatic compounds from sainfoin.** Abreviation used: TLC, thin layer chromatography; RP-HPLC, reverse-phase high- performance liquid chromatography; DAD, diode array detection; CRD, chemical reaction detection; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NMR, nuclear magnetic resonance.

The acetone was removed under low pressure by rotary evaporator and the remaining water extract was further defatted with chloroform. A second partition step with ethyl acetate was performed to remove the polar constituents (i.e. tannins, sugars).

The freeze dried purified ethyl acetate extract was redissolved in 10% aqueous methanol and fractionated by a Sephadex LH-20 column. A gradient elution was then performed with different volumes of aqueous methanol (10 - 100% with 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 behaviour to afford 45 fractions. Finally the fractions were lyophilised.

Each fraction was further submitted to LC-MS/MS, HPLC-DAD, and applied to TLC in order to obtain an overview of the phenolic compounds classes. The main constituents in the Sephadex LH-20 fractions are showed in Table 1.

Table 1. The main constituents in the Sephadex LH-20 fractions from sainfoin aq. acetonic extract

Fraction	*Eluent*	*Main constituents*
1 - 20	10 - 30 % aq. Methanol	Sugars, arbutin, amino compounds, benzoic and hydroxycinnamic acids
21 - 23	30 % aq. Methanol	Flavonols
24 - 26	40 % aq. Methanol	Flavanols and flavonols
27 - 45	50 - 100 % aq. Methanol	Flavanols, procyanidins, flavonols, flavones

Identification. Most of compounds were identified for the first time in sainfoin; eight acylated flavonol glycosides were new examples. Table 1A (Appendix 1) summaries the structure and UV spectra of phenolic and other natural compounds identified in plant material of *Onobrychis viciifolia* during the present work and from past phytochemical studies.

Their identification was performed by combining chemical, chromatographic, and spectroscopic methods (*Figure 3 - 5*).

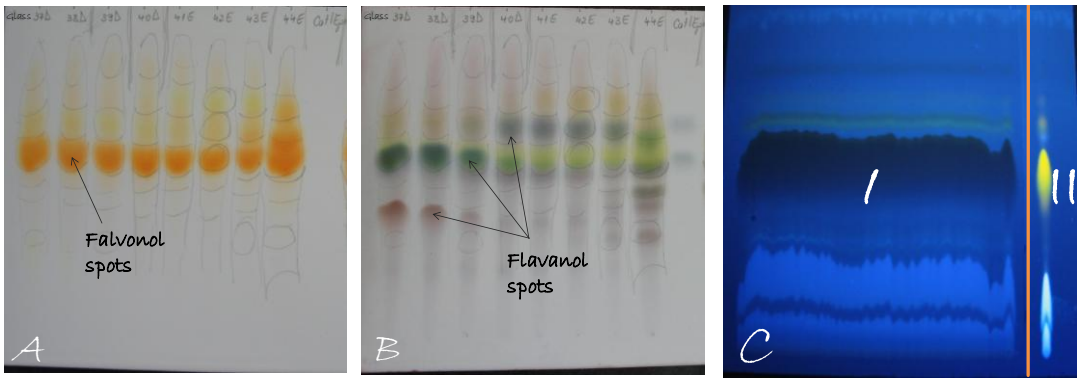

Figure 3. **Thin layer chromatography (TLC) plates of Sephadex LH-20 eluates.**

(A) – (B) Cellulose plates used for the characterisation of 40 and 50% aqueous methanol eluates on the Sephadex LH-20 gel (glasses 37D - 44E). The eluates showing similar spots on TLC were combined (37 - 39D, 41 – 43E). The orange spots are the flavonols after spraying with Naturstoffreagenz. (A)
The flavanol spots brawn, green and violet could be seen on the same TLC plate after spraying with 4-p-dimethylaminocinnamaldehyde (B).

(C) Fraction 12 on preparative polyamid plate under UV light.

(I) The dark band is the compound to be isolated, the kaempferol 3-O rhamnosylrutinoside.

(II) Fraction 12 was spotted on the same plate as a control, and sprayed with Naturstoffreagenz. The flavonol of interest is the yellow spot.

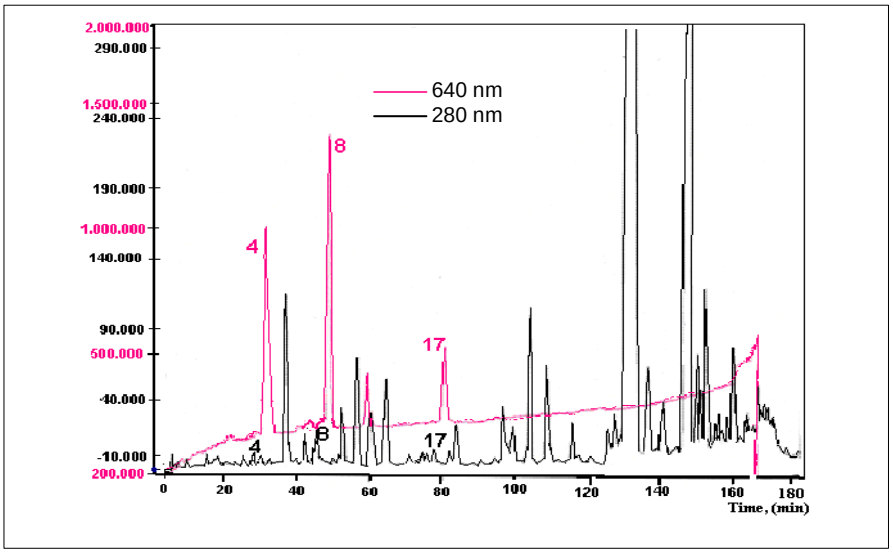

Figure 4. **High-performance liquid chromatography (HPLC) chromatogram overlay (640/280 nm) of fraction number 12.** Here can be seen how intensive are the flavanols peaks at 640 nm (4, 8 and 17) after post column derivatization with 4-p-dimethylaminocinnamaldehyde (DMACA) in comparison with the 280 nm measurement.

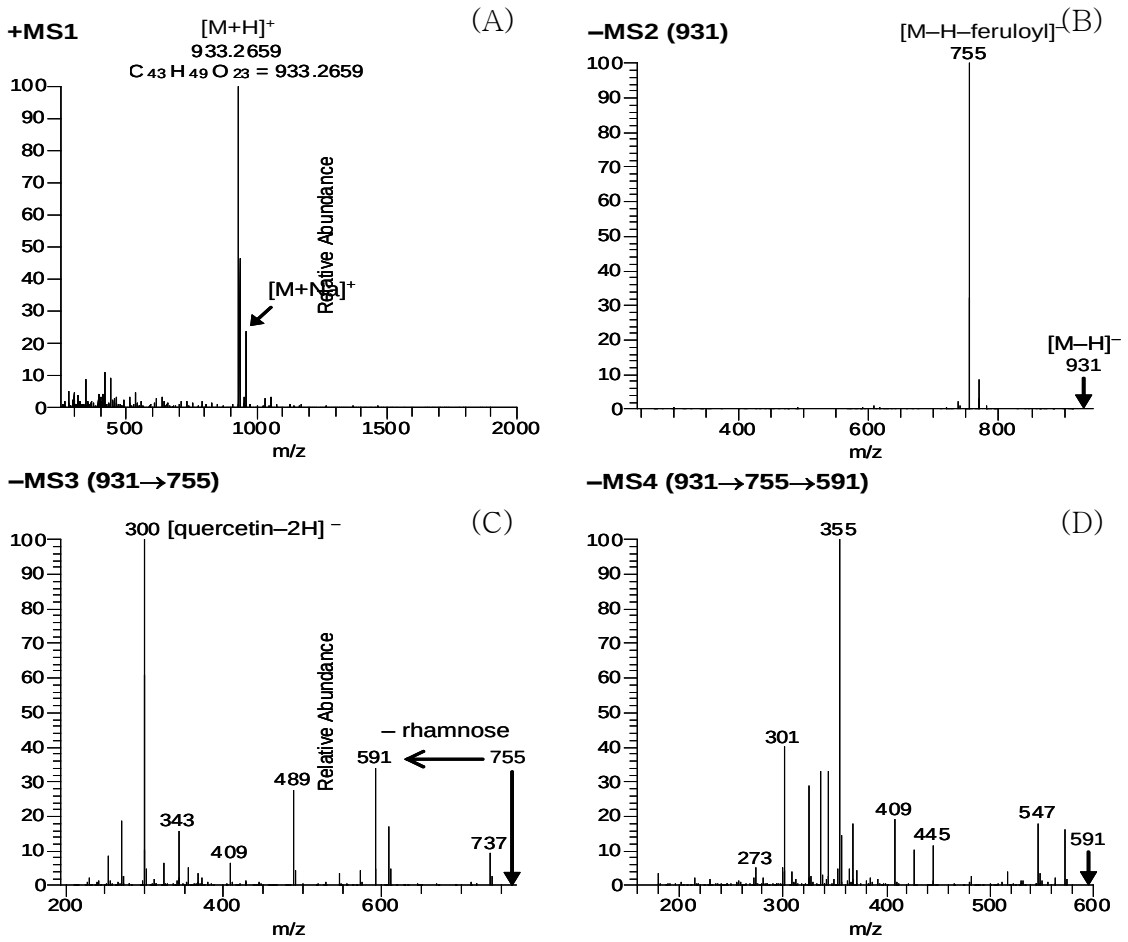

Figure 5. **Positive high-resolution electrospray-ionization (HRESIMS) and negative multistage tandem (MSn) mass spectra of quercetin 3-O-α-rhamnopyranosyl-(1→2)[(3-O-E-feruloyl)-α-rhamnopyranosyl-(1→6)]-β-galactopyranoside showing:**

(A) the accurate mass of the compound,

(B) the loss of feruloyl from the deprotonated molecule following MS2,

(C) the CID spectrum of $[M–H–feruloyl]^-$ in MS3 analysis typical of deprotonated quercetin 3-O-rhamnosyl(1→2)[rhamnosyl(1→6)]-hexosides, and

(D) MS4 analysis of the rhamnose-loss ion identifying the hexose sugar as galactose.

Isoflavones. The analysis of the mass and UV spectra combined with the information obtained from acid hydrolysis (Table 2) led to the identification of five isoflavones: the 7-O-(malonylglucoside) of the isoflavones afrormosin (**I1**), formononetin (**I3**) and fujikinetin (**I4**); the pratensein-glucoside (**I5**) and afrormosin-hexoside (**I2**). These compounds are reported for the first time in sainfoin. The formononetin and afrormosin aglycons were isolated from sainfoin leaves by INGHAM, 1987. Analysing the MS/MS spectra of **I1**, **I3** and **I4**, ions of $[M-H-162-86]^-$ at *m/z* 297, 267 and 311 corresponding to the aglycons afrormosin, formononetin and fujikinetin after the cleavage of malonylglucose were observed. The UV spectra of **I1**, **I3** and **I4**

and the results obtained by acid hydrolysis which gave glucose and afrormosin (**I1**), formononetin (**I3**) and fujikinetin (**I4)** are consistent with the proposed chemical structures. The MS/MS fragmentation of **I5** ($[M-H]^-$ at *m/z* 461) produced the pratensein pseudomolecular ion $[M-H-162]^-$ at *m/z* 299. Acid hydrolysis released glucose and pratensein. The determination of sugar position was not possible. On the basis of these data **I5** was characterized as pratensein-glucoside. Similarly, compound **I2** was characterized as afrormosine-hexoside by MS and acid hydrolysis. The fragmentation of the $[M-H]^-$ ion at *m/z* 505 generate the ion at *m/z* 297 of the afrormosin aglycon after the cleavage of a hexose moiety (162 Da).

Hydroxycinnamic acids. Several peaks from the purified fractions were grouped into derivatives of coumaric (**C25**-**C30**), caffeic (**C31**-**C34**) and ferulic acid (**C35**) based on LC-MS analysis, UV spectra) and acid or sulfatase hydrolysis (Table 2). The exact identity of the individual compounds could not be determined.

Dihydroflavonols. Compounds **D2**, **D3** and **D4** were characterized as dihydrokaempferol-glucoside, dihydroquercetin-glucoside and dihydroquercetin-hexoside (Table 2). Hydrolysis with sulfatase released glucose and the aglycons dihydrokaempferol (**D2**) and diydroquercetin (**D3**). The MS/MS fragmentation of **D2** ($[M-H]^-$ at *m/z* 449) produced the dihydrokaempferol pseudomolecular ion $[M-H-162]^-$ at *m/z* 287. The fragmentation of $[M-H]^-$ of **D3** and **D4** at *m/z* 465 gave the ion *m/z* 285 of dihydroquercetin after elimination of a hexose moiety (162 Da). The determination of the sugar positions of these dihydroflavonols-glycosides and the hydrolysis of **D4** was not possible because of the low amount available.

Flavanols. The analysis of the fractions by direct injection into the LC-MS/MS system yielded dimeric (**F11**, **F12**) and trimeric (**F13**) flavanols ions (Table 2). **F11** with $[M-H]^-$ at *m/z* 593 is a heteropolymer containing both catechin/epicatechin (C/E) and gallocatechin/epigallocatechin (GC/EGC) units in his molecule. Based on the $[M-H]^-$ at

m/z 593, compound **F12** was characterized as pure prodelphinidin [2 X (GC/EGC)] and **F13** with *m/z* 865 as pure procyanidin homopolymer [3 X (C/E)].

Flavonols. The acylated flavonol glucosides, kaempferol-acetyl-glucoside (**G15**) and quercetin-malonyl-glucoside (**G16**) were identified based on their UV and MS spectra and acid hydrolysis (Table 2). The MS/MS fragmentation of **G15** ($[M-H]^-$ at *m/z* 489) produced the kaempferol pseudomolecular ion at *m/z* 284. The fragmentation of $[M-H]^-$ of **G16** at *m/z* 549 gave the ion of $[M-H-162-86]^-$ at *m/z* 301 corresponding to the aglycon quercetin after the cleavage of malonylglucose. These results are consistent with the UV spectra characteristic of a kaempferol (**G15**) and quercetin (**G16**) glucoside and the acid hydrolysis which released glucose and the aglycons kaempferol (**G15**) and quercetin (**G16**).

Table 2. Chromatographic, UV, and mass spectral characteristics of isoflavones and not completed identified compounds in purified fractions of ethyl acetate sainfoin extract.

Compound	*Fraction*	t_R *(min)*	λ_{max} *(nm)*	[a]*[M-H]⁻; ([2M-H]⁻)* *(m/z)*	[b]*MS/MS of [M-H]⁻* *(m/z)*	*Identification*
				Isoflavones		
I1	15	168.4	259, 319	545; (1091)	282, **297**	Afrormosin 7-O-(malonylglucoside)
I2	14	151.3	260, 322	505	**297**	Afrormosin-hexoside
I3	18	162.2	257, 300sh	515; (1031)	**267**	Formononetin 7-O-(malonylglucoside)
I4	18	164.1	261, 289sh, 318sh	559; (1119)	**311**	Fujikinetin 7-O-(malonylglucoside)
I5	24	150.8	260, 330sh	461	**299**	Pratensein-glucoside
				Hydroxycinnamic acids		
C25	12	124.7	314	337; **675**; 697	-	trans p-Coumaric acid derivative
C26	13	86.7	313	323	**113**, 119, 163	trans p-Coumaric acid derivative
C27	24	99.9	312	417	117, 119, **145**, 163	trans p-Coumaric acid derivative
C28	12	68.7	308	337; 675; **697**	-	trans p-Coumaric acid derivative
C29	12	115.8	310	337; 675; **697**	163, 191, 305, **697**	trans p-Coumaric acid derivative
C30	12	125.5	312	437	119, 133, **145**, 161, 163	trans p-Coumaroyl malic acid derivative
C31	23	99.9	255sh, 329	473	**135**, 149, 179, 293	trans Caffeic acid derivative
C32	24	52.4	326	335	**133**, 161	trans Caffeic acid with malic acid derivative
C33	20	66.3	255sh, 328	339	**113**, 135, 159, 179	trans Caffeic acid derivative
C34	20	102.2	318	-	-	trans Caffeic acid derivative
C35	13	103.6	327	158; 353; **707**	-	trans Ferulic acid derivative

(continued on next page)

Table 2. (continued)

Compound	Fraction	t_R (min)	λ_{max} (nm)	[a][M-H]$^-$; ([2M-H]$^-$) (m/z)	[b]MS/MS of [M-H]$^-$ (m/z)	Identification
				Dihydroflavonols		
D2	15	43.1	293	449	**125**, 269, 287	Dihydrokaempferol-glucoside
D3	13	27.4	287, 325	465	**125**, 285, 303	Dihydroquercetin-glucoside
D4	24	60.3	292, 325	465	125, **285**	Dihydroquercetin-hexoside
				[c]*Flavanols*		
F11	30	-	-	593	**289**	1 X (C/E) + 1 X (GC/EGC)
F12	30	-	-	609	**305**	2 X (GC/EGC)
F13	38	-	-	865	-	3 X (C/E)
				Flavonols		
G15	23	152.3	264, 345	489	227, 255, **284**	Kaempferol-acetyl-glucoside
G16	23	137.2	256, 353	549	151, 179, 271, **300**	Quercetin-malonyl-glucoside

[a] Some hydroxycinnamic acids shows three intensive [M-H]$^-$ ions; an intensive [2M-H]$^-$ ion was observed for isoflavones 7-O-(malonylglucoside). [b] In the MS/MS, the most abundant ion is shown in boldface. [c] Flavanols were identified by direct injection of the fractions into the MS; abbreviation used: C, catechin; E, epicatechin; GC, gallocatechin; EGC, epigallocatechin

Article 1 – Summary

Identification and quantification of phenolic compounds from the forage legume sainfoin (*Onobrychis viciifolia*)

REGOS, I., URBANELA, A., TREUTTER, D.

2009. Journal of Agriculture and Food Chemistry 57, 5843-5852.

Sainfoin (*Onobrychis viciifolia* Scop.; Leguminosae subfamily Papilionoideae) is a traditional forage legume with excellent nutritional and veterinary properties, namely prevention of bloat and controlling nematode parasitism in ruminants. These beneficial effects are thought to be due to the particular tannin structure, characteristic of sainfoin but also other phenolic compounds such as flavonols may be involved in this beneficial property. However, since the identification of interesting candidates for future breeding programmes is necessary, the purpose of this study was to isolate and identify the phenolic compounds from sainfoin and to investigate their distribution in the aerial plant parts with different ontogenetic stages. Aqueous acetone extracts of the aerial parts of sainfoin variety Cotswold Common yielded amino acids, alkaloids, phenolic acids, dihydroflavonols, flavone-C-glucosides, flavanols, flavonols and isoflavones. After isolation and purification, their structures were elucidated combining chemical, chromatographic, and spectroscopic methods. Most of these substances have not been described hitherto in this plant material. 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 on the same place. This indicates that the commonly used sainfoin varieties may not yet be homogeneous with respect to their content of bioactive secondary metabolites. Therefore, if the beneficiary 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.

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Article 1 – Authors' contributions

Identification and quantification of phenolic compounds from the forage legume sainfoin (*Onobrychis viciifolia*)

REGOS, I., URBANELA, A., TREUTTER, D.

2009. Journal of Agriculture and Food Chemistry 57, 5843-5852.

REGOS, I.: literature search, study design, experimental work, data collection, data analysis, data interpretation, writing.

URBANELA, A.: isolation of several flavonol glycosides by preparative TLC and investigation of their sugar position by spectral properties after addition of diagnostic reagents.

TREUTTER, D.: supervision and scientific conception, revision of the manuscript.

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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

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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

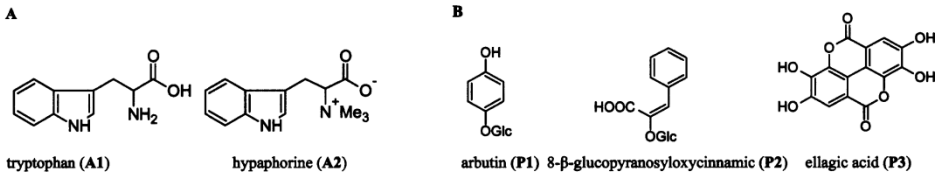
fraction	eluate (% MeOH in water)	glass[a]	eluate vol (mL)	fraction wt (mg)	main components[b]
1	10	1−3a	60	133.00	
2	10	4a	20	191.00	
3	10	5a	20	116.00	P1
4	10	6a	20	48.00	A1, C2, C20,
5	10	7a	20	59.00	P2, B4
6	10	8a	20	20.00	C4, C12, C15
7	10	9a	20	11.01	C21
8	10	10a	20	21.00	C3, C24
9	20	11b	20	17.00	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



C

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

D

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

E

dihydroflavonols	R
dihydrokaempferol	H
dihydroquercetin (D1)	OH

F

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

G

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

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, $[p\text{-coumaric acid} - H - CO_2]^-$) and quinic acid (*m/z* 191, [quinic acid − H]$^-$ and *m/z* 173, $[\text{quinic acid} - H - H_2O]^-$) (*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, $[\text{malic acid} - H - CO_2 - H_2O]^-$), (*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 $[\text{caffeic acid} - H - CO_2]^-$. 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 ($[\text{caffeic acid} - H - CO_2]^-$) 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 Goševac 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.

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Article 2 – Summary

Structural identification of new acylated flavonol glycosides from the forage legume sainfoin, (*Onobrychis viciifolia*)

VEITCH, N.C., REGOS, I., KITE, G.C., TREUTTER, D.

2011. Phytochemistry 72, 423-429.

The traditional fodder legume sainfoin (*Onobrychis viciifolia* Scop., Leguminosae) has excellent nutritional and palatability qualities for livestock, to which its phenolic constituents contribute. During characterisation of the phenolic metabolome of this species, ten acylated flavonol glycosides were isolated from water-acetone (3:7) extracts of aerial parts, and purified using a combination of column chromatography on Sephadex LH-20 and reverse phase HPLC coupled to diode array detection. Their UV spectra were typical of flavonol glycosides acylated with hydroxycinnamic acids. Structure determination was by LC-MS/MS and NMR (700 MHz + cryoprobe), revealing that the sainfoin flavonoids were feruloyl and sinapoyl derivatives of 3-*O*-di- and triglycosides of kaempferol and quercetin. Negative ion electrospray and serial MS were used to aid the identification of the primary 3-*O*-linked hexose residues as glucose or galactose. Eight of the acylated flavonol glycosides were new compounds, and the remaining two had previously been reported only from the legume, *Vicia amurensis*. The diglycosides were acylated at the primary Glc residue of O-α-Rhap(1→6)-β-Glcp (rutinose), whereas the triglycosides were acylated at the terminal Rha residues of the branched trisaccharides, O-α-Rhap(1→2)[α-Rhap(1→6)]-β-Galp or O-α-Rhap(1→2)[α-Rhap(1→6)]-β-Glcp. Analysis of UV and MS spectra of the acylated flavonol glycosides provided additional diagnostic features relevant to direct characterisation of these compounds in hyphenated analyses. Quantitative analysis of the acylated flavonol glycosides present in different aerial parts of sainfoin revealed that the highest concentrations were in mature leaflets.

Article 2 – Authors' contributions

Structural identification of new acylated flavonol glycosides from the forage legume sainfoin, (*Onobrychis viciifolia*)

VEITCH, N.C., REGOS, I., KITE, G.C., TREUTTER, D.

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Article 2 rounds out the work performed in article 1 about the structure elucidation of sainfoin phenolics.

REGOS, I.: literature search, extraction, isolation, purification and identification of the compounds as synapoylated and feruloylated glycosides of quercetin and kaempferol by UV, MS, hydrolysis; quantitative analysis.

TREUTTER, D.: supervision and scientific conception, revision of the manuscript

VEITCH, N.C. and KITE, G.C. (from *Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey TW9 3DS, U.K*.): elucidation of the exact structure of the compounds by HRESIMS analysis, NMR analysis, interpretation and writing of data obtained.

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Acylated flavonol glycosides from the forage legume, *Onobrychis viciifolia* (sainfoin)

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ABSTRACT

Ten acylated flavonol glycosides were isolated from aqueous acetone extracts of the aerial parts of the forage legume, *Onobrychis viciifolia*, and their structures determined using spectroscopic methods. Among these were eight previously unreported examples which comprised either feruloylated or sinapoylated derivatives of 3-*O*-di- and 3-*O*-triglycosides of kaempferol (3,5,7,4′-tetrahydroxyflavone) or quercetin (3,5,7,3′,4′-pentahydroxyflavone). The diglycosides were acylated at the primary Glc residue of *O*-α-Rha*p*(1 → 6)-β-Glc*p* (rutinose), whereas the triglycosides were acylated at the terminal Rha residues of the branched trisaccharides, *O*-α-Rha*p*(1 → 2)[α-Rha*p*(1 → 6)]-β-Gal*p* or *O*-α-Rha*p*(1 → 2)[α-Rha*p*(1 → 6)]-β-Glc*p*. Identification of the primary 3-*O*-linked hexose residues as either Gal or Glc was carried out by negative ion electrospray and serial MS, and cryoprobe NMR spectroscopy. Analysis of UV and MS spectra of the acylated flavonol glycosides provided additional diagnostic features relevant to direct characterisation of these compounds in hyphenated analyses. Quantitative analysis of the acylated flavonol glycosides present in different aerial parts of sainfoin revealed that the highest concentrations were in mature leaflets.

1. Introduction

Sainfoin (*Onobrychis viciifolia* Scop.; Leguminosae subfamily Papilionoideae) is a high quality forage legume that has attracted recent interest because of its excellent nutritional and palatability properties. In particular, protein digestibility and protection of ruminants against legume bloat are facilitated by its content of polyphenols (Mueller-Harvey, 2006). It has also been shown that flavonol glycosides present in sainfoin contribute to its anthelmintic properties (Barrau et al., 2005). Previous phytochemical studies indicate that sainfoin contains a range of soluble phenolics, including hydroxycinnamic acids, flavonoids and isoflavonoids (Ingham, 1978; Lu et al., 2000; Marais et al., 2000; Regos et al., 2009). To further characterise the soluble phenolic 'metabolome' of sainfoin we have investigated acylated flavonol glycosides present in extracts of aerial parts of this species. This type of phenolic has not been recorded previously in sainfoin. Although the detection of acylated flavonol glycosides during LC–MS/MS analyses of plant extracts is relatively straightforward (Llorach et al., 2003), full characterisation requires isolation of individual constituents as a prerequisite for structure elucidation. In the present work, effective separation and purification of the complex matrix of acylated flavonol glycosides present in *O. viciifolia* was achieved using HPLC, although a relatively lengthy gradient method was necessary (Section 3.3). As such, the purified compounds were obtained only in submilligram amounts. We therefore used positive and negative ion electrospray and serial MS, together with cryoprobe NMR at 600 and 700 MHz, for structure determination. Of the ten acylated flavonol glycosides characterised from *O. viciifolia* by these methods eight new examples (**3**–**10**) were found (Fig. 1), whose structures are reported here.

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2. Results and discussion

2.1. UV and LC–MS/MS characterisation of acylated flavonol glycosides

Column chromatography of 70% aq. acetone extracts of the aerial parts of *O. viciifolia* followed by HPLC yielded **1**–**10** as pale yellow amorphous solids. Their UV spectra were typical of flavonol glycosides acylated by hydroxycinnamic acids (Llorach et al., 2003), with band I at 330–336 nm, and either band II at 254–256 nm with a shoulder at 266 nm (**1**, **2**, **4**, **5**, **7** and **8**), or band II at 265 nm (**3**, **6**, **9** and **10**), corresponding to quercetin and kaempferol glycosides, respectively. The identification of the flavonol aglycones of **1**–**10** was confirmed by serial MS (MSn) of protonated molecules $[M+H]^+$ in LC–MS/MS analyses, and additional LC–MSn experiments provided data on the glycans and acyl groups prior to NMR analysis (Sections 2.2–2.4).

In the MS2 spectra of $[M+Na]^+$, neutral losses of either quercetin (**1**, **2**, **4**, **5**, **7** and **8**) or kaempferol (**3**, **6**, **9** and **10**) were observed, corresponding to $[(M+Na)-\text{quercetin}]^+$ or $[(M+Na)-\text{kaempferol}]^+$,

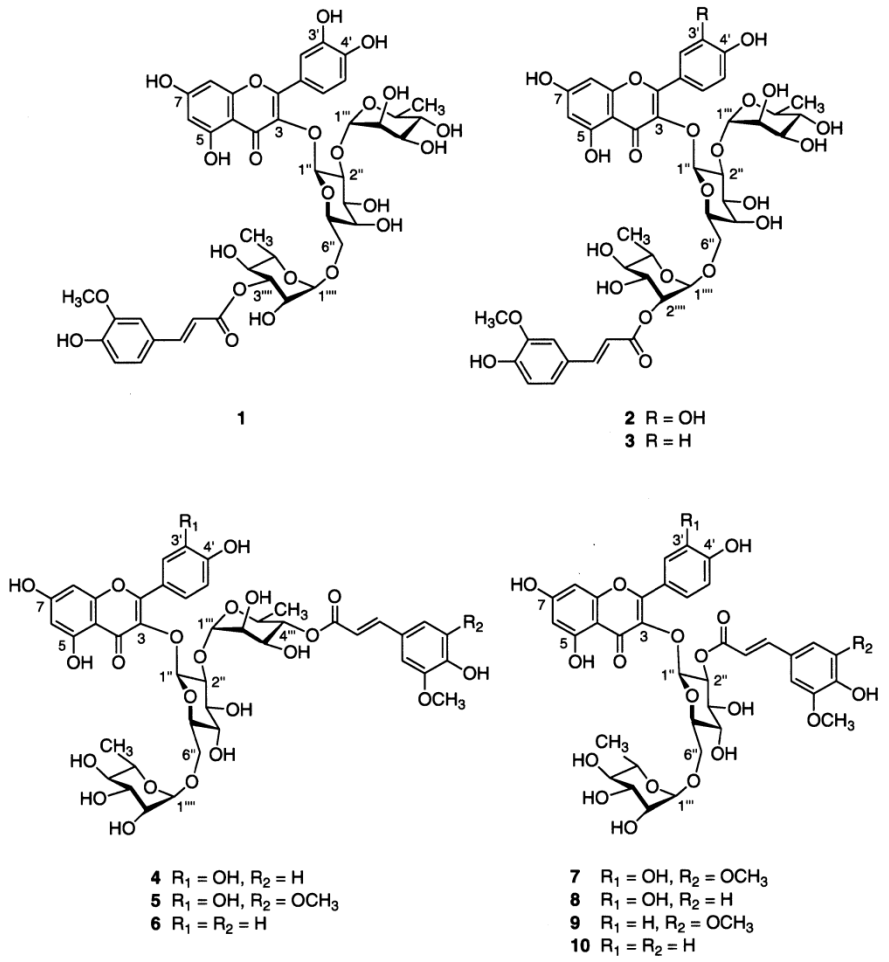

Fig. 1. Acylated flavonol glycosides from *Onobrychis viciifolia*.

respectively, suggesting that the flavonols were glycosylated at only one position. In negative mode, the MS2 spectra of deprotonated **1–6** showed ions at $[(M-H)-176]^-$ (**1–4** and **6**) or $[(M-H)-206]^-$ (**5**) resulting from neutral losses of feruloyl or sinapoyl residues, respectively. CID of the resulting product ions in MS3 analyses gave spectra typical of deprotonated quercetin or kaempferol 3-*O*-rhamnosyl(1‴ → 2″)[rhamnosyl(1⁗ → 6″)]-hexosides in which the primary hexose was assigned as glucose or galactose using the MSn experiments described by Kite and Veitch (2009). Thus **1–3** were acylated 3-*O*-rhamnosyl(1‴ → 2″)[rhamnosyl(1⁗ → 6″)]-galactosides, and **4–6**, acylated 3-*O*-rhamnosyl(1‴ → 2″)[rhamnosyl(1⁗ → 6″)]-glucosides. For **7–10**, negative mode MS2 spectra of $[M-H]^-$ showed ions at $[(M-H)-176]^-$ and $[(M-H)-194]^-$ (**8** and **10**), or $[(M-H)-206]^-$ and $[(M-H)-224]^-$ (**7** and **9**), corresponding to neutral losses of feruloyl and ferulic acid residues, or sinapoyl and sinapic acid residues, respectively. The MS3 spectra of the former ions were characteristic of 3-*O*-rhamnosyl(1‴ → 6″)-glucosides (Kite and Veitch, 2009). Compounds **7** and **8** were therefore sinapoyl and feruloyl derivatives, respectively, of quercetin 3-*O*-rhamnosyl(1‴ → 6″)-glucoside, and **9** and **10**, the corresponding kaempferol analogues. For the determination of specific sites of acylation, cryoprobe NMR spectroscopy was used. The molecular formulae of **1–10** were established from HRESIMS measurements (Sections 3.5–3.14).

*2.2. Flavonol 3-O-triglycosides acylated at 6″-O-Rha (**1–3**)*

Compounds **1** and **2** were quercetin 3-*O*-α-rhamnopyranosyl (1‴ → 2″)[(3⁗-*O*-*E*-feruloyl)-α-rhamnopyranosyl(1⁗ → 6″)]-β-galactopyranoside (amurenoside A) and quercetin 3-*O*-α-rhamnopyranosyl(1‴ → 2″)[(2⁗-*O*-*E*-feruloyl)-α-rhamnopyranosyl(1⁗ → 6″)] -β-galactopyranoside (amurenoside B), respectively, according to comparisons of NMR spectroscopic data with literature values (Kang et al., 2000), and MS analysis (Section 2.1). These isomeric feruloylated quercetin triglycosides were obtained previously from MeOH extracts of the whole plant of the legume *Vicia amurensis* Oett. (Leguminosae). Both MS (Section 2.1) and NMR (Table 1) data indicated that **3** was the kaempferol analogue of **2**. As expected, the ^{1}H and ^{13}C NMR assignments of the terminal 2″-*O*-Rha and 6″-*O*-Rha residues of the 3-*O*-linked branched trisaccharide *O*-α-Rha*p*(1‴ → 2″)[α-Rha*p*(1⁗ → 6″)]-β-Gal*p* of **3** and **2** were almost identical (Table 1), including those of the downfield-shifted H-2⁗

Table 1
^{1}H and ^{13}C NMR spectroscopic data for 2‴-*O*-*E*-feruloylated flavonol 3-*O*-triglycosides **2** and **3** (CD_3OD, 30 °C).

Atom		**2**		**3**	
		δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
Aglycone	6	6.17 *d* (2.1)	99.8	6.16 *d* (2.0)	99.9
	8	6.34 *d* (2.1)	94.7	6.34 *d* (1.9)	94.9
	2'	7.71 *d* (2.2)	117.2	8.04 *d* (8.9)	132.0
	3'			6.86 *d* (8.9)	115.9
	5'	6.84 *d* (8.5)	115.9	6.86 *d* (8.9)	115.9
	6'	7.54 *dd* (8.6, 2.2)	122.8	8.04 *d* (8.9)	132.0
3-*O*-Gal	1″	5.64 *d* (7.6)	101.0	5.55 *d* (7.8)	100.9
	2″	3.96 *dd* (9.2, 7.7)	77.3	3.93 *dd* (9.5, 7.9)	77.3
	3″	3.73 *dd* (9.3, 3.4)	75.4	3.70 *m*	75.5
	4″	3.82 *m*	70.8	3.78 *br d* (3.4)	70.7
	5″	3.70 *br d* (5.7)	75.2	3.67 *m*	75.2
	6″	3.74 *m*	66.8	3.71 *m*	67.1
		3.57 *m*		3.53 *m*	
2″-*O*-Rha	1‴	5.21 *br d* (1.3)	102.4	5.22 *br s*	102.4
	2‴	4.00 *dd* (3.3, 1.4)	72.3	4.00 *dd* (3.3, 1.5)	72.2
	3‴	3.81 *m*	72.2	3.82 *dd* (9.5, 3.4)	72.3
	4‴	3.33 *t* (9.5)	74.0	3.34 *t* (9.7)	74.0
	5‴	4.06 *dd* (9.6, 6.2)	69.6	4.09 *dd* (9.7, 6.3)	69.6
	6‴	0.97 *d* (6.2)	17.3	1.00 *d* (6.2)	17.4
6″-*O*-Rha	1⁗	4.63 *d* (1.5)	99.0	4.60 *d* (1.5)	98.9
	2⁗	4.95 *dd* (3.4, 1.6)	73.4	4.94 *dd* (3.4, 1.6)	73.4
	3⁗	3.76 *dd* (9.5, 3.5)	70.5	3.75 *dd* (9.6, 3.4)	70.4
	4⁗	3.34 *t* (9.5)	74.0	3.34 *t* (9.7)	74.0
	5⁗	3.60 *dd* (9.6, 6.2)	69.7	3.58 *dd* (9.6, 6.2)	69.7
	6⁗	1.22 *d* (6.2)	17.9	1.21 *d* (6.2)	17.8
2‴-*O*-*E*-feruloyl	α	6.32 *d* (15.9)	115.2	6.33 *d* (15.9)	115.1
	β	7.58 *d* (15.9)	147.0	7.60 *d* (15.9)	147.1
	2	7.20 *d* (1.8)	111.6	7.21 *d* (1.8)	111.5
	5	6.82 *d* (8.2)	116.2	6.82 *d* (8.2)	116.3
	6	7.08 *dd* (8.3, 1.9)	124.0	7.09 *dd* (8.4, 1.9)	124.1
	3-OMe	3.91 *s*	56.3	3.91 *s*	56.3

of 6″-*O*-Rha (δ_H 4.95 and 4.94, respectively) corresponding to the site of acylation. Similarly, the chemical shift value of δ_H 3.93 for H-2″ of the primary hexose residue of **3**, and the $J_{3'',4''}$ coupling constant of 3.4 Hz were consistent with its assignment as Gal. Compound **3** was therefore kaempferol 3-*O*-α-rhamnopyranosyl (1‴ → 2″)[(2⁗-*O*-*E*-feruloyl)-α-rhamnopyranosyl(1⁗ → 6″)]-β-galactopyranoside. Support for the 2⁗-OH acylation site also came from comparative mass spectrometry, with the MS2 spectrum of protonated **3** showing an abundant ion at *m/z* 305 [feruloylRha−H_2O+H]$^+$ as in the MS2 spectrum of **2** (which was also acylated at 2⁗-OH), whereas this ion was absent from the MS2 spectrum of **1**, which was acylated at 3⁗-OH. This water-loss ion was also the base ion in **2** and **3** following fragmentation of [feruloylRha+H]$^+$ in MS3 experiments, but at 1% abundance in the corresponding spectrum of **1**. An additional acylated quercetin triglycoside eluting between **2** and **3** but obtained only in very low amounts (Section 3.3) was tentatively identified as the glucosyl analogue of **2** on the basis of LC–MS/MS data and partial assignment of its ^{1}H NMR spectrum (Supplementary Data).

2.3. Flavonol 3-O-triglycosides acylated at 2″-O-Rha (4–6)

The branched trisaccharides *O*-linked at 3-OH of **4–6** were identical to those of **1–3**, except that the primary sugar was Glc. For the terminal 2″-*O*-Rha residue, H-4‴ of each of **4–6** was shifted downfield in the ^{1}H NMR spectrum (δ_H 5.01–5.02); similarly C-4‴ was shifted downfield in the ^{13}C NMR spectrum, and both C-3‴ and C-5‴ experienced upfield shifts, suggesting that 4‴-OH was the site of acylation (Itoh et al., 2004). This was confirmed from the HMBC spectrum of **4**, in which a long range correlation from H-4‴ of 2″-*O*-Rha to the carbonyl carbon of the acyl group at δ_C 168.8 was detected. Thus in **4–6**, the site of acylation was at 4‴-OH of 2″-*O*-Rha, in contrast to either 2⁗-OH or 3⁗-OH of 6″-*O*-Rha, as found in **1–3**. With a common trisaccharide and acylation site, **4–6** differed only in aglycone structure and the nature of the acyl group, both of which were readily identified from MS (Section 2.1) and NMR data (Table 2). The acylated triglycosides were therefore quercetin 3-*O*-(4‴-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1‴ → 2″)[α-rhamnopyranosyl-(1⁗ → 6″)]-β-glucopyranoside (**4**), quercetin 3-*O*-(4‴-*O*-*E*-sinapoyl)-α-rhamnopyranosyl-(1‴ → 2″)[α-rhamnopyranosyl-(1⁗ → 6″)]-β-glucopyranoside (**5**), and kaempferol 3-*O*-(4‴-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1‴ → 2″)-[α-rhamnopyranosyl-(1⁗ → 6″)]-β-glucopyranoside (**6**).

2.4. Flavonol 3-O-rutinosides acylated at primary Glc (7–10)

The flavonol diglycosides **7–10** were identified as acylated 3-*O*-α-rhamnopyranosyl-(1‴ → 6″)-β-glucopyranosides (rutinosides) of either quercetin (**7** and **8**) or kaempferol (**9** and **10**) using MS (Section 2.1) and NMR (Table 3) data. Of these, **7** and **9** were *E*-sinapoyl derivatives, and **8** and **10** were *E*-feruloyl derivatives. The ^{1}H NMR assignments of the primary Glc residues of **7–10** suggested that the sites of acylation were at 2″-OH, since the resonances of H-2″ showed appreciable shifts downfield to either δ_H 5.03 (**7** and **8**) or δ_H 5.00 (**9** and **10**). Confirmation of 2″-OH as the common site of acylation was obtained from the HMBC spectrum of **8**, in which a long range correlation from H-2″ of Glc (δ_H 5.03) to the carbonyl carbon of a feruloyl group at δ_C 168.2 was detected. The same spectrum yielded the expected correlations from H-1″ of Glc to C-3 of the aglycone (δ_C 134.5), CH_2-6″ of Glc to C-1‴ of Rha, and H-1‴ of Rha to C-6″ of Glc. The acylated flavonol rutinosides were therefore quercetin 3-*O*-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-*O*-*E*-sinapoyl)-β-glucopyranoside] (**7**), quercetin 3-*O*-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-*O*-*E*-feruloyl)-β-glucopyranoside] (**8**), kaempferol 3-*O*-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-*O*-*E*-sinapoyl)-β-glucopyranoside] (**9**), and kaempferol

Table 2
^{1}H and ^{13}C NMR spectroscopic data for 4‴-*O*-*E*-acylated flavonol 3-*O*-triglycosides **4–6** (CD_3OD, 30 °C).

Atom		**4**		**5**		**6**	
		δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
Aglycone	6	6.17 *br d* (1.8)	100.0	6.15 *br s*	100.2	6.21 *d* (2.1)	99.8
	8	6.34 *br s*	94.9	6.33 *br s*	95.0	6.39 *d* (2.1)	94.7
	2'	7.60 *d* (2.1)	117.3	7.60 *d* (2.2)	117.3	8.01 *d* (8.9)	131.9
	3'					6.88 *d* (8.9)	115.9
	5'	6.86 *d* (8.4)	115.8	6.86 *d* (8.3)	115.9	6.88 *d* (8.9)	115.9
	6'	7.59 *dd* (8.5, 2.1)	123.2	7.59 *dd* (8.3, 2.2)	123.2	8.01 *d* (8.9)	131.9
3-*O*-Glc	1″	5.49 *d* (7.6)	100.9	5.46 *d* (7.8)	100.9	5.50 *d* (7.7)	100.9
	2″	3.67 *dd* (9.1, 7.7)	79.8	3.66 *dd* (9.1, 7.8)	80.0	3.62 *dd* (9.2, 7.7)	79.8
	3″	3.56 *t* (9.1)	78.7	3.55 *m*	78.8	3.55 *t* (9.1)	78.7
	4″	3.26 *t* (9.4)	71.7	3.25 *m*	71.7	3.22 *m*	71.8
	5″	3.33 *m*	76.8	3.33 *m*	76.9	3.34 *m*	76.8
	6″	3.83 *m*	68.4	3.83 *m*	68.4	3.83 *m*	68.5
		3.35 *m*		3.34 *m*		3.35 *m*	
2″-*O*-Rha	1‴	5.27 *br d* (1.7)	102.2	5.27 *br d* (1.4)	102.3	5.26 *d* (1.8)	102.2
	2‴	4.07 *br dd* (3.3, 1.7)	72.3	4.07 *m*	72.4	4.06 *dd* (3.2, 1.7)	72.4
	3‴	4.12 *dd* (9.7, 3.2)	70.3	4.11 *dd* (9.6, 3.4)	70.3	4.11 *dd* (9.7, 3.3)	70.3
	4‴	5.01 *t* (9.7)	75.5	5.02 *t* (9.9)	75.6	5.01 *t* (9.8)	75.5
	5‴	4.43 *dd* (9.8, 6.2)	67.7	4.44 *dd* (9.7, 6.4)	67.7	4.40 *dd* (9.7, 6.3)	67.6
	6‴	0.92 *d* (6.2)	17.2	0.94 *d* (6.3)	17.2	0.90 *d* (6.2)	17.2
6″-*O*-Rha	1⁗	4.49 *br d* (1.7)	102.2	4.49 *br d* (1.6)	102.2	4.49 *d* (1.8)	102.2
	2⁗	3.61 *br dd* (3.4, 1.7)	72.0	3.61 *br dd* (3.5, 1.7)	72.0	3.60 *dd* (3.2, 1.7)	71.9
	3⁗	3.51 *dd* (9.6, 3.3)	72.1	3.50 *dd* (9.7, 3.5)	72.1	3.49 *dd* (9.5, 3.4)	72.1
	4⁗	3.24 *t* (9.6)	73.7	3.24 *m*	73.8	3.25 *t* (9.4)	73.6
	5⁗	3.43 *dd* (9.5, 6.3)	69.5	3.42 *dd* (9.7, 6.2)	69.6	3.43 *dd* (9.5, 6.3)	69.6
	6⁗	1.09 *d* (6.2)	17.6	1.09 *d* (6.1)	17.7	1.09 *d* (6.3)	17.7
4‴-*O*-*E*-feruloyl	α	6.30 *d* (15.9)	115.5			6.30 *d* (15.9)	115.5
	β	7.50 *d* (16.0)	146.5			7.50 *d* (16.0)	146.3
	2	7.12 *d* (1.8)	111.3			7.12 *d* (2.0)	111.4
	5	6.76 *d* (8.2)	116.2			6.76 *d* (8.2)	116.2
	6	6.92 *dd* (8.2, 1.8)	124.2			6.91 *dd* (8.3, 2.0)	124.1
	3-OMe	3.86 *s*	56.3			3.86 *s*	56.3
4‴-*O*-*E*-sinapoyl	α			6.35 *d* (15.9)	116.1		
	β			7.53 *d* (15.9)	146.9		
	2/6			6.85 *s*	106.9		
	3/5-OMe			3.85 *s*	56.7		

Table 3
^{1}H and ^{13}C NMR spectroscopic data for 2″-*O*-*E*-acylated flavonol 3-*O*-diglycosides **7–10** (CD_3OD, 30 °C).

Atom		**7**		**8**		**9**		**10**	
		δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C	δ ^{1}H (*J* in Hz)	δ ^{13}C
Aglycone	6	6.11 *br s*	100.3	6.14 *d* (2.0)	99.9	6.13 *br s*	100.2	6.15 *d* (2.1)	99.9
	8	6.29 *br s*	94.8	6.31 *d* (2.0)	94.8	6.31 *br s*	94.9	6.33 *d* (2.0)	94.8
	2'	7.57 *d* (2.1)	117.0	7.57 *d* (2.1)	117.2	7.98 *d* (8.9)	132.1	7.99 *d* (9.0)	132.0
	3'					6.89 *d* (8.9)	116.1	6.89 *d* (8.9)	116.1
	5'	6.86 *d* (8.1)	116.1	6.87 *d* (8.2)	116.0	6.89 *d* (8.9)	116.1	6.89 *d* (8.9)	116.1
	6'	7.56 *dd* (8.1, 2.1)	123.2	7.56 *dd* (8.2, 2.1)	123.3	7.98 *d* (8.9)	132.1	7.99 *d* (9.0)	132.0
3-*O*-Glc	1″	5.50 *d* (8.0)	100.9	5.51 *d* (8.0)	100.8	5.54 *d* (8.0)	100.8	5.55 *d* (8.0)	100.7
	2″	5.03 *dd* (9.4, 8.0)	75.6	5.03 *dd* (9.5, 8.0)	75.7	5.00 *dd* (9.4, 8.0)	75.6	5.00 *dd* (9.5, 8.0)	75.6
	3″	3.60 *dd* (9.4, 8.7)	76.1	3.60 *dd* (9.5, 8.8)	76.2	3.60 *t* (9.2)	76.1	3.60 *dd* (9.5, 8.7)	76.1
	4″	3.37 *dd* (9.7, 8.7)	71.4	3.37 *dd* (9.7, 8.8)	71.6	3.34 *m*	71.6	3.34 *dd* (9.6, 8.8)	71.6
	5″	3.41 *m*	77.2	3.41 *ddd* (9.6, 6.4, 1.7)	77.3	3.42 *m*	77.2	3.41 *m*	77.3
	6″	3.89 *dd* (11.3, 1.8)	67.8	3.89 *dd* (11.2, 1.7)	68.1	3.88 *m*	68.2	3.88 *dd* (11.2, 1.7)	68.2
		3.46 *dd* (11.3, 6.4)		3.46 *dd* (11.1, 6.3)		3.43 *m*		3.44 *m*	
6″-*O*-Rha	1‴	4.57 *d* (1.8)	102.2	4.56 *d* (1.8)	102.2	4.55 *br d* (1.5)	102.2	4.55 *d* (1.8)	102.2
	2‴	3.67 *dd* (3.4, 1.7)	72.0	3.66 *dd* (3.5, 1.7)	72.0	3.65 *dd* (3.5, 1.5)	71.9	3.64 *dd* (3.4, 1.7)	72.0
	3‴	3.56 *dd* (9.6, 3.5)	72.0	3.55 *dd* (9.5, 3.5)	72.2	3.54 *dd* (9.5, 3.4)	72.2	3.53 *dd* (9.5, 3.5)	72.2
	4‴	3.29 *t* (9.6)	73.8	3.28 *t* (9.6)	73.8	3.28 *t* (9.5)	73.8	3.28 *t* (9.6)	73.7
	5‴	3.50 *dd* (9.5, 6.2)	69.5	3.49 *dd* (9.5, 6.2)	69.7	3.49 *dd* (9.6, 6.3)	69.7	3.48 *dd* (9.5, 6.3)	69.6
	6‴	1.14 *d* (6.3)	17.8	1.14 *d* (6.3)	17.6	1.14 *d* (6.3)	17.7	1.13 *d* (6.3)	17.7
2″-*O*-*E*-feruloyl	α			6.39 *d* (15.9)	115.4			6.38 *d* (15.9)	115.4
	β			7.68 *d* (15.9)	147.1			7.69 *d* (16.0)	147.1
	2			7.17 *d* (2.0)	111.7			7.18 *d* (2.0)	111.7
	5			6.81 *d* (8.2)	116.3			6.82 *d* (8.2)	116.3
	6			7.07 *dd* (8.2, 1.9)	123.9			7.08 *dd* (8.3, 2.0)	123.9
	3-OMe			3.91 *s*	56.3			3.91 *s*	56.3
2″-*O*-*E*-sinapoyl	α	6.40 *d* (15.9)	115.7			6.40 *d* (15.9)	115.9		
	β	7.69 *d* (15.8)	147.1			7.70 *d* (15.9)	nd[a]		
	2/6	6.90 *s*	106.8			6.90 *s*	106.8		
	3/5-OMe	3.89 *s*	56.7			3.89 *s*	56.7		

[a] Not detected.

3-*O*-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-*O*-*E*-feruloyl)-β-glucopyranoside] (**10**). Although the 3-*O*-rutinosides of quercetin (rutin) and kaempferol (nicotiflorin) are among the most commonly occurring flavonoid glycosides in flowering plants, these 2″-*O*-feruloyl and 2″-*O*-sinapoyl derivatives have not been reported previously. The corresponding 2″-*O*-coumaroylrutinosides of both quercetin and kaempferol were found by Olea et al. (1997) in leaf extracts of *Alibertia sessilis* Schum. (Rubiaceae), although the kaempferol derivative was first described by Romussi et al. (1981) as a constituent of leaf extracts of *Castanea sativa* Mill. (Fagaceae).

2.5. Quantitative analysis of acylated flavonol glycosides in different aerial parts of sainfoin

The highest concentrations of the acylated flavonol glycosides present in aerial parts of sainfoin were found in mature leaflets (0.89 mg/g dry wt.), mature petioles (0.35 mg/g dry wt.) and stems (0.12 mg/g dry wt.), which collectively represented c. 2.6% of the total flavonol content (33.50, 13.46 and 5.37 mg/g dry wt., respectively). In unfolded young leaflets (0.28 mg/g dry wt.) and young petioles (0.12 mg/g dry wt.) the acylated flavonol glycosides were 0.8% and 0.7%, respectively, of the total flavonol content (Regos et al., 2009). Similarly, flower stalks and buds contained 0.19 and 0.06 mg/g dry wt. of acylated flavonol glycosides, representing 1.7% and 0.6%, respectively, of the total flavonol content in these tissues.

2.6. Conclusions

Extracts of the aerial parts of sainfoin (*O. viciifolia*) yielded ten acylated flavonol glycosides for which structures were elucidated using MS and NMR, including eight new compounds (Fig. 1). Three different patterns of acylation were found; for the triglycosides **1–6**, the terminal Rha residues of the branched trisaccharides *O*-α-Rha*p*(1‴ → 2″)[α-Rha*p*(1⁗ → 6″)]-β-Gal*p* or *O*-α-Rha*p*(1‴ → 2″)[α-Rha*p*(1⁗ → 6″)]-β-Glc*p*, were acylated either at 2⁗- or 3⁗-OH of 6″-*O*-Rha (**1–3**), or 4‴-OH of 2″-*O*-Rha (**4–6**). The diglycosides **7–10** were acylated at 2″-OH of the primary Glc residue of *O*-α-Rha*p*(1‴ → 6″)-β-Glc*p*. Although the acylating groups were identified readily by MS and NMR, a subtle distinction in band I of the UV spectra of feruloylated (λ_{max} 330–334 nm, shoulder at 295 nm; **1–4**, **6**, **8**, **10**) and sinapoylated (λ_{max} 333–336 nm, symmetrical appearance; **5**, **7**, **9**) derivatives was noted, which could be a useful diagnostic feature for the direct annotation of these compounds in hyphenated analyses (Fig. 2). Similarly, for the acylated flavonol 3-*O*-triglycosides (**1–6**), an important distinction noted from MS was that compounds acylated at 2⁗-OH of 6″-*O*-Rha (**2** and **3**) showed a much more abundant ion at [acylRha−H_2O+H]$^+$ following MS2 of [M+H]$^+$ compared to those acylated at 3⁗-OH of 6″-*O*-Rha (**1**) or 4‴-OH of 2″-*O*-Rha (**4–6**).

Although the aerial parts of sainfoin have been found to contain the 3-*O*-α-rhamnopyranosyl(1‴ → 2″)[α-rhamnopyranosyl(1⁗ → 6″)]-β-glucopyranosides of both kaempferol and quercetin (Lu et al., 2000), and the corresponding 3-*O*-rhamnosyl(1‴ → 6″)-glucosides (Marais et al., 2000; Regos et al., 2009), no acylated derivatives were previously reported. As such, the characterisation of **1–10** is a significant contribution to the description of the phenolic metabolome of this important fodder legume, the full elucidation of which is critical to understanding its beneficial nutritional and veterinary properties.

3. Experimental

3.1. General instrumentation

NMR spectra were acquired in MeOH-d_4 at 30 °C on Bruker 600 (Avance) or 700 (Avance II+) MHz instruments equipped with 5 mm 1H/13C/15N triple-resonance PFG cryoprobes. Standard pulse sequences and parameters were used to obtain one-dimensional ^{1}H and two-dimensional COSY, HSQC and HMBC spectra. Chemical shift referencing was carried out using the internal solvent resonances at δ_H 3.31 and δ_C 49.1 (calibrated to TMS at 0.00 ppm).

Accurate mass measurements and MSn analyses were performed with a Thermo Scientific LTQ Orbitrap XL hybrid mass spectrometer. Samples were introduced into the electrospray source via a Thermo Scientific Accela HPLC system fitted with a Phenomenex 150 mm × 3.0 mm × 3 μm Luna C18(2) column using a 30 min linear mobile phase gradient of 0:90:10 to 40:50:10 $CH_3OH/H_2O/CH_3CN$ + 1% HCOOH pumped at 400 μl/min. Positive and negative mode ion-trap MSn spectra were all recorded using an ion isolation window of ±2 *m/z* and a collision energy of 35%. The [M+H]$^+$ ion was selected for MS2 analysis to obtain *m/z* values for the protonated aglycone and ions containing the acyl group (indicating the type of sugar acylated), while the [M+Na]$^+$ ion was fragmented to confirm the single glycosylation site of the flavonol (by observing the neutral loss of the aglycone). The molecular mass of the acyl group was confirmed by selecting the [M−H]$^-$ ion for MS2 analysis and observing the neutral loss of the acyl residue. The resulting product ion was then subjected to further fragmentation experiments by MS3 (diglycosides) or MS4 (triglycosides) analyses to confirm either Glc or Gal as the primary 3-*O*-glycosidic sugar, as described by Kite and Veitch (2009). In Sections 3.5–3.14 below, ions annotated '∗' in LC–ESI-MS/MS

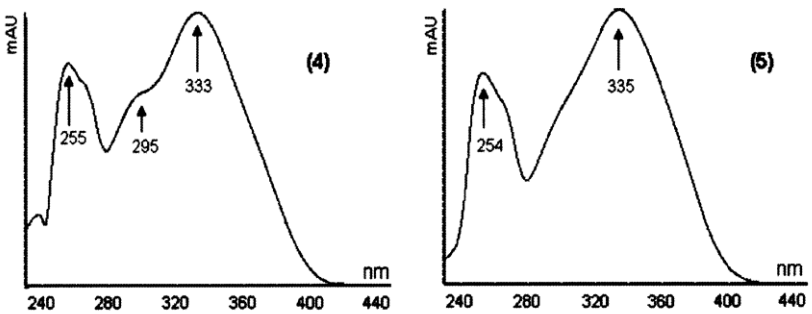

Fig. 2. Diagnostic UV spectra of quercetin 3-*O*-triglycosides acylated by ferulic (**4**) and sinapic acids (**5**).

(ion trap) of $[M+H]^+$ listings invoke the mechanism of internal hexose residue loss described by Ma et al. (2000).

3.2. Plant material

O. viciifolia (var. Cotswold Common) plants, ca. 50 cm high, were harvested at the bud stage by Ian Wilkinson, Cotswold Seeds Ltd., UK. on 31 May 2006, air-dried at room temperature, and ground (<1 mm). For the quantitative analysis of acylated flavonol glycosides from different aerial parts (young and mature 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 leaves were divided into leaflets and petioles. The material was frozen in liquid nitrogen immediately after sampling and lyophilized.

3.3. Extraction and isolation of acylated flavonol glycosides

Ground plant material (50 g) was extracted with 70% ice-cold aq. acetone in an ultrasonic water bath at 7 °C for 30 min and the acetone was removed under reduced pressure (<30 °C). The remaining aq. extract was diluted with H_2O and extracted successively with ice-cold chloroform and EtOAc. The freeze dried EtOAc extract (1.5 g, 3% of total leaf and stem material) was dissolved in 10% aq. MeOH and the solution chromatographed on a Sephadex LH-20 column with aq. MeOH containing increasing proportions of MeOH. The acylated flavonol glycosides were eluted from the column with 50 and 60% aq. MeOH. These fractions were further purified by HPLC using a system consisting of two pumps (Kontron Modell 422), an automatic sample injector (Gilson Abimed Model 231) and a diode array detector (Bio Teck Kontron 540). A Nucleodur Sphinx RP column (250 × 4 mm i.d., Macherey–Nagel) and a mixture of H_2O containing 5% HCO_2H (solvent A) and MeOH (solvent B) was used. The following gradient was applied at a flow rate of 0.5 ml/min: 0–20 min, 20% B; 20–35 min, 20–25% B; 35–40 min, 25–30% B, 40–75 min, 30–40% B; 75–105 min, 40–50% B; 105–125 min, 50–60% B; 125–135 min, 60–90% B; 135–145 min, 90% B. The acylated flavonol triglycosides eluted at t_R = 90.1 (**1**), 102.9 (**2**), 110.6 (**3**), 116.7 (**4**), 120.8 (**5**), and 125.2 min (**6**), and the diglycosides at t_R = 116.6 (**7**), 118.0 (**8**), 122.6 (**9**), and 123.0 min (**10**). After solvent evaporation, the compounds were obtained as pale yellow solids in yields of 50–200 μg. An additional acylated flavonol triglycoside eluting at t_R = 105.8 was a minor component of the extract and isolated only in very low amounts (<50 μg).

3.4. Quantitative analysis of acylated flavonol glycosides from different plant tissues

Freeze-dried material was ground in a mortar or a ball mill, depending on the amount available. Extractions were performed by adding 500 μl of 80% aq. MeOH to 100 mg of powder for 30 min in a cooled ultrasound water bath (7 °C). After centrifugation (10,000 rpm, 10 min and 4 °C), the clear supernatants were transferred to Eppendorf tubes and the residues were washed twice with 250 μl of 80% aq. MeOH. The corresponding supernatants were combined after centrifugation, the solvent was evaporated, and the residues were redissolved in 100 μl of MeOH. For each tissue sampled, 10 μl of the latter extract was subjected to HPLC analysis on the system described above (Section 3.3). The acylated flavonol glycosides were separated on a Nucleosil column (250 × 4 mm i.d., 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. The acylated flavonol glycosides were monitored at 320 nm and calculated as chlorogenic acid (Roth, Karlsruhe).

3.5. Quercetin 3-O-α-rhamnopyranosyl-(1‴ → 2″)[(3⁗-O-E-feruloyl)-α-rhamnopyranosyl-(1⁗ → 6″)]-β-galactopyranoside (**1**)

UV: 254, 266 sh, 295 sh, 333 nm; ^1H and ^{13}C NMR: in agreement with Kang et al. (2000); LC–ESI-MS/MS (ion trap) of $[M+H]^+$, *m/z* (rel. int.): 787 [(M+H)−Rha]$^+$ (65), 611 [(M+H)−Rha−feruloyl]$^+$ (15), 469 [(M+H)−quercetin−Gal]$^+$ (45)*, 449 [(M+H)−feruloyl−Rha−Gal]$^+$ (20)*, 323 [feruloylRha+H]$^+$ (100), 303 [quercetin+H]$^+$ (75); HRESIMS *m/z*: 955.2486 $[M+Na]^+$ (calc. for $C_{43}H_{48}O_{23}Na$, 955.2479).

3.6. Quercetin 3-O-α-rhamnopyranosyl-(1‴ → 2″)[(2⁗-O-E-feruloyl)-α-rhamnopyranosyl-(1⁗ → 6″)]-β-galactopyranoside (**2**)

UV: 254, 266 sh, 295 sh, 332 nm; ^1H and ^{13}C NMR: see Table 1; LC–ESI-MS/MS (ion trap) of $[M+H]^+$, *m/z* (rel. int.): 787 [(M+H)−Rha]$^+$ (35), 613 [(M+H)−quercetin−H_2O]$^+$ (10), 469 [(M+H)−quercetin−Gal]$^+$ (30)*, 449 [(M+H)−feruloyl−Rha−Gal]$^+$ (10)*, 323 [feruloylRha+H]$^+$ (1 0 0), 305 [feruloylRha−H_2O+H]$^+$ (30), 303 [quercetin+H]$^+$ (15); HRESIMS *m/z*: 955.2486 $[M+Na]^+$ (calc. for $C_{43}H_{48}O_{23}Na$, 955.2479).

3.7. Kaempferol 3-O-α-rhamnopyranosyl-(1‴ → 2″)[(2⁗-O-E-feruloyl)-α-rhamnopyranosyl-(1⁗ → 6″)]-β-galactopyranoside (**3**)

UV: 265, 295 sh, 330 nm; ^1H and ^{13}C NMR: see Table 1; LC–ESI-MS/MS (ion trap) of $[M+H]^+$, *m/z* (rel. int.): 771 [(M+H)−Rha]$^+$ (55), 613 [(M+H)−kaempferol−H_2O]$^+$ (10), 469 [(M+H)−kaempferol−Gal]$^+$ (55)*, 433 [(M+H)−feruloyl−Rha−Gal]$^+$ (5)*, 323 [feruloylRha+H]$^+$ (1 0 0), 305 [feruloylRha−H_2O+H]$^+$ (30), 287 [kaempferol+H]$^+$ (15); HRESIMS *m/z*: 939.2543 $[M+Na]^+$ (calc. for $C_{43}H_{48}O_{22}Na$, 939.2529).

3.8. Quercetin 3-O-(4‴-O-E-feruloyl)-α-rhamnopyranosyl-(1‴ → 2″)[α-rhamnopyranosyl-(1⁗ → 6″)]-β-glucopyranoside (**4**)

UV: 255, 266 sh, 295 sh, 333 nm; ^1H and ^{13}C NMR: see Table 2; LC–ESI-MS/MS (ion trap) of $[M+H]^+$, *m/z* (rel. int.): 787 [(M+H)−Rha]$^+$ (10), 625 [(M+H)−Rha−Glc]$^+$ (15)*, 611 [(M+H)−Rha−feruloyl]$^+$ (20), 469 [(M+H)−quercetin−Glc]$^+$ (15)*, 465 [(M+H)−(2 × Rha)−feruloyl]$^+$ (30), 449 [(M+H)−feruloyl−Rha−Glc]$^+$ (10)*, 323 [feruloylRha+H]$^+$ (40), 303 [quercetin+H]$^+$ (100); HRESIMS *m/z*: 955.2478 $[M+Na]^+$ (calc. for $C_{43}H_{48}O_{23}Na$, 955.2479).

3.9. Quercetin 3-O-(4‴-O-E-sinapoyl)-α-rhamnopyranosyl-(1‴ → 2″)[α-rhamnopyranosyl-(1⁗ → 6″)]-β-glucopyranoside (**5**)

UV: 254, 266 sh, 335 nm; ^1H and ^{13}C NMR: see Table 2; LC–ESI-MS/MS (ion trap) of $[M+H]^+$, *m/z* (rel. int.): 817 [(M+H)−Rha]$^+$ (10), 655 [(M+H)−Rha−Glc]$^+$ (10)*, 611 [(M+H)−Rha−sinapoyl]$^+$ (25), 499 [(M+H)−quercetin−Glc]$^+$ (15)*, 465 [(M+H)−(2 × Rha)−sinapoyl]$^+$ (25), 449 [(M+H)−sinapoyl−Rha−Glc]$^+$ (10)*, 353 [sinapoylRha+H]$^+$ (40), 303 [quercetin+H]$^+$ (1 0 0); HRESIMS *m/z*: 985.2585 $[M+Na]^+$ (calc. for $C_{44}H_{50}O_{24}Na$, 985.2584).

3.10. Kaempferol 3-O-(4‴-O-E-feruloyl)-α-rhamnopyranosyl-(1‴ → 2″)[α-rhamnopyranosyl-(1⁗ → 6″)]-β-glucopyranoside (**6**)

UV: 265, 295 sh, 330 nm; ^1H and ^{13}C NMR: see Table 2; LC–ESI-MS/MS (ion trap) of $[M+H]^+$, *m/z* (rel. int.): 771 [(M+H)−Rha]$^+$ (10),

609 [(M+H)−Rha−Glc]$^+$ (10)*, 595 [(M+H)−Rha−feruloyl]$^+$ (25), 469 [(M+H)−kaempferol−Glc]$^+$ (15)*, 449 [(M+H)−(2 × Rha)−feruloyl]$^+$ (25), 433 [(M+H)−feruloyl−Rha−Glc]$^+$ (5)*, 323 [feruloylRha+H]$^+$ (30), 287 [kaempferol+H]$^+$ (1 0 0); HRESIMS *m/z*: 939.2542 [M+Na]$^+$ (calc. for $C_{43}H_{48}O_{22}Na$, 939.2529).

3.11. Quercetin 3-O-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-O-E-sinapoyl)-β-glucopyranoside] (7)

UV: 256, 266 sh, 336 nm; ^{1}H and ^{13}C NMR: see Table 3; LC–ESI-MS/MS (ion trap) of [M+H]$^+$, *m/z* (rel. int.): 671 [(M+H)−Rha]$^+$ (60), 515 [(M+H)−quercetin]$^+$ (75), 369 [sinapoylGlc+H]$^+$ (65), 303 [quercetin+H]$^+$ (1 0 0); HRESIMS *m/z*: 839.2013 [M+Na]$^+$ (calc. for $C_{38}H_{40}O_{20}Na$, 839.2005).

3.12. Quercetin 3-O-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-O-E-feruloyl)-β-glucopyranoside] (8)

UV: 255, 266 sh, 295 sh, 334 nm; ^{1}H and ^{13}C NMR: see Table 3; LC–ESI-MS/MS (ion trap) of [M+H]$^+$, *m/z* (rel. int.): 641 [(M+H)−Rha]$^+$ (55), 485 [(M+H)−quercetin]$^+$ (1 0 0), 339 [feruloylGlc+H]$^+$ (75), 303 [quercetin+H]$^+$ (1 0 0); HRESIMS *m/z*: 809.1907 [M+Na]$^+$ (calc. for $C_{37}H_{38}O_{19}Na$, 809.1899).

3.13. Kaempferol 3-O-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-O-E-sinapoyl)-β-glucopyranoside] (9)

UV: 265, 333 nm; ^{1}H and ^{13}C NMR: see Table 3; LC–ESI-MS/MS (ion trap) of [M+H]$^+$, *m/z* (rel. int.): 655 [(M+H)−Rha]$^+$ (50), 515 [(M+H)−kaempferol]$^+$ (1 0 0), 369 [sinapoylGlc+H]$^+$ (65), 287 [kaempferol+H]$^+$ (1 0 0); HRESIMS *m/z*: 823.2065 [M+Na]$^+$ (calc. for $C_{38}H_{40}O_{19}Na$, 823.2056).

3.14. Kaempferol 3-O-α-rhamnopyranosyl-(1‴ → 6″)-[(2″-O-E-feruloyl)-β-glucopyranoside] (10)

UV: 265, 295 sh, 331 nm; ^{1}H and ^{13}C NMR: see Table 3; LC–ESI-MS/MS (ion trap) of [M+H]$^+$, *m/z* (rel. int.): 625 [(M+H)−Rha]$^+$ (50), 485 [(M+H)−kaempferol]$^+$ (75), 339 [feruloylGlc+H]$^+$ (60), 287 [kaempferol+H]$^+$ (1 0 0); HRESIMS *m/z*: 793.1955 [M+Na]$^+$ (calc. for $C_{37}H_{38}O_{18}Na$, 793.1950).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.01.001.

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Article 3 – Summary

Optimization of a high-performance liquid chromatography method for the analysis of complex polyphenol mixtures and application for sainfoin extracts (*Onobrychis viciifolia*)

REGOS, I. and TREUTTER, D.

2010. Journal of Chromatography A, 1217, 6169-6177.

HPLC using ultraviolet (UV) detection was established as the most convenient method for providing valuable insights into the distribution of phenolics in plants utilizing their feature to absorb UV light. With easily upwards of 5,000 compounds reported, full characterisation of a plant biophenol extract is a challenging analytical exercise. The separation performance of a PFP phase was compared with those of a heterogeneous phase constituted of octadecyl and phenylpropyl bonded silica and three C18 phases by injecting 10 µl methanol extract of the forage legume sainfoin and mixtures of 54 standard substances including: 1 amino acid, 2 simple phenolic acids, 4 hydroxybenzoic and 6 hydroxycinnamic acids, 2 dihydroflavonols, 4 flavones, 3 flavanols, 9 anthocyanins, 1 chalcone, 1 isoflavone and 21 flavonols. The best separation and an excellent peak shape was obtained using the PFP column where except anthocyanins all analytes were considerably more retained compared to the other columns. The positive outcome is particularly the better resolution of early eluting phenolic compounds such as hydroquinones, hydroxybenzoic and hydroxycinnamic acids and flavanols which gave longer elution ranges on the PFP phase compared to conventional columns. An HPLC method with sensitive diode array and chemical reaction detection using the perfluorophase was validated and applied for the analysis of an *Onobrychis viciifolia* sample. Although fluorinated stationary phases were claimed to offer many utilities that could not be accomplished by conventional C8, C18, and phenyl phases and have shown novel selectivity and enhanced retention for several compound classes, no applications for biophenols were previously reported.

Article 3 – Authors' contributions

Optimization of a high-performance liquid chromatography method for the analysis of complex polyphenol mixtures and application for sainfoin extracts (*Onobrychis viciifolia*)

REGOS, I. and TREUTTER, D.

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REGOS, I.: literature search, study design, experimental work, data collection, data analysis, data interpretation, writing, figures.

TREUTTER, D.: supervision and scientific conception, revision of the manuscript

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Optimization of a high-performance liquid chromatography method for the analysis of complex polyphenol mixtures and application for sainfoin extracts (*Onobrychis viciifolia*)

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ABSTRACT

A pentafluorophenylpropyl (PFP) stationary phase was tested for the simultaneous determination of several classes of phenolic compounds. The chromatographic results were compared with those obtained by using a bifunctional phase constituted of octadecyl and phenylpropyl bonded silica and three conventional C18 columns. The elution gradient was optimized with 5% formic acid and sodium acetate in combination with acetic acid as additives and methanol as solvents. For these evaluations, a complex phenolic extract of *Onobrychis viciifolia* (sainfoin) and test mixtures containing 54 standard substances including 2 simple phenolic compounds, 1 amino acid, 4 hydroxybenzoic acids (HBA), 6 hydroxycinnamic acids (HCA), 3 flavan-3-ols, 9 anthocyanins, 2 dihydroflavonols, 1 chalcone, 4 flavones, 1 isoflavone and 21 flavonols have been assayed. The perfluorinated column showed good resolution for the studied phenolic compounds which have the following elution order: HBA, HCA, flavan-3-ols, anthocyanins, dihydroflavonols, flavones, flavonols and isoflavones. Compared with other columns, it provides longer elution ranges for HBA, HCA and flavan-3-ols and increased retention times for all compound classes except anthocyanins which were similarly retained on a C18 column. Its selectivity is different from C18 and bifunctional phases. A high-performance liquid chromatography (HPLC) method with diode array detection (DAD) and post-column derivatization with p-dimethyl-aminocinnamic aldehyde (DMACA) has been validated for the analysis of individual phenolic compounds from a sainfoin plant extract (*O. viciifolia*).

1. Introduction

Phenolic compounds are ubiquitous plant metabolites with a wide variability of structures and chemical characteristics. They exhibit key functions in plant physiology and in the defense against herbivores and pathogens [1]. Moreover, they are considered to be active contributors to the health benefits of foods of plant origin [2,3]. HPLC using ultraviolet (UV) detection was established as the most convenient method for providing valuable insights into the distribution of phenolics in plants utilizing their feature to absorb UV light. By coupling HPLC with diode array and chemical reaction detection, various phenolic compounds from complex mixtures can be separated, quantified and identified in one operation, while the catechins and proanthocyanidins are selectively detected after post-column derivatization with p-dimethylaminocinnamaldehyde [4,5].

However, accurate identification and quantification of the analytes are particularly linked to the optimization of the separation since an HPLC procedure rarely exists that can separate and measure all phenolics from complex samples such as food products, beverages and plant extracts. The separations depend on column characteristics and capacity, and are also influenced by solvent composition, gradient and the flow rates used. A wide range of stationary and mobile phase combinations have been reported in the literature [1,2]. However, C18 reverse-phases combined with binary elution systems containing an aqueous acidified polar solvent and a less polar organic solvent were used almost exclusively. The most common acid modifiers necessary to minimize peak tailing are acetic and formic acid, but phosphate buffers or ammonium acetate is also used. Typical flow rates are in the range of 1–1.5 ml/min and the analyses time depends on how many compounds are analyzed. Despite the large number of investigations, the simultaneous determination of phenolic compounds of different classes remains difficult.

In the recent years, significant research efforts within the synthesis of novel reverse-phase columns including different functionalities offering attractive selectivity have been made. Several

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studies dealing with the comparison of chromatography performance of fluorinated, C18 and phenyl type packing materials have been published [6–10]. Fluorinated stationary phases were claimed to offer many utilities that could not be accomplished by conventional C8, C18, and phenyl phases [11]. In particular, pentafluorophenyl (PFP) phases have shown novel selectivity and enhanced retention for several compound classes and have been proven useful to resolve tocopherol isomers [12], taxanes [13], pentacyclic triterpenoids [14], phenethylamine alkaloids [15], corticosteroids [16] and specific pharmaceutical formulations [17]. Compared to traditional alkyl phases which achieve selectivity mainly through hydrophobic interactions, the pentafluorophenyl (PFP) phase uses multiple retention mechanisms such as ionic interactions, hydrogen bonding, dipole–dipole, aromatic and π–π interactions, and hydrophobic interactions. Moreover, it gave larger capacity factors for aromatics, halogenated aromatics, and polycyclic aromatic hydrocarbons than the phenyl phase, due to donor–acceptor complex formation [18]. Longer retention and greater selectivity were reported for fluorinated and other halogenated aromatic compounds on the PFP column when compared to phenyl column [19].

Another special characteristic of the PFP phase is the dual normal- and reverse-phase retention for polar analytes depending on the composition of the mobile phase. At high concentration of organic modifier in the mobile phase, the free silanol groups available on this packing material can act as a normal phase and provide strong retention of polar and basic compounds. At lower percentages of organic modifier, solute retention resembles that of classical reversed-phase system. The combination of reversed- and normal-phase behavior forms a "U-shape" relationship between retention and organic modifier percentage and can be rationalized by the presence of hydrophobically assisted ion-exchange mechanism or additional independent interactions due to the presence of the pentafluorophenyl ligands [20–22]. Needham et al. found that the pentafluorophenylpropyl modified silica columns gave good retention of several kinds of basic drugs with a mobile phase containing 90% acetonitrile, whereas, to achieve good retention on C18 columns, 40% acetonitrile has to be used [23–25]. In a similar study from Marín and Barbas, both reversed-phase and normal-phase-like characteristics for certain analytes have been observed [17]. The special behavior and the high retention at higher percentage of organic solvent observed for basic compounds make this phase very advantageous in working with LC/MS. The effect of the concentration of the organic solvent was also investigated for phenolic compounds by Blahová et al., who observed that the retention order changes in dependence on the acetonitrile concentration on a pentafluorophenylpropyl column [26].

In this context the purpose of the present study was to test the separation performance of a pentafluorophenylpropyl phase for the analysis of different polyphenolics which includes phenolic acids and flavonoids (both glycosides and aglycones). These results were then compared with those obtained using a bifunctional phase constituted of octadecyl and phenylpropyl bonded silica and three conventional C18 columns. An HPLC method was validated for the analysis of a complex extract from sainfoin plants and consequently could be used for comparative study of different sainfoin varieties.

2. Experimental

2.1. Chemicals

The reference compounds were purchased from Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Extrasynthese (Genay, France), Apin Chemicals Limited (Oxon, UK) and Polyphenols Laboratories AS (Sandnes, Norway). Kaempferol 3-arabinoside, kaempferol 3-glucoside, kaempferol 3-rhamnoside, kaempferol 7-rhamnoside, kaempferol 3-(6-O-acetylglucoside)-7-rhamnoside, kaempferol 3-rutinoside-7-rhamnoside, myricetin 3-galactoside, quercetin 7-glucoside and quercetin 3-rutinoside-7-rhamnoside were kindly provided by Hans Geiger. 3′- and 4′-caffeoylquinic acids were prepared by isomerization of 5′-caffeoylquinic acid in a phosphate buffer solution [27]. All chemicals were dissolved in HPLC-grade methanol (MeOH).

2.2. Extract preparation for selectivity experiments

For column selectivity tests, 6 g fine powder of air-dried *Onobrychis viciifolia* (variety Cotswold Common, harvested at the bud stage by Ian Wilkinson, Cotswold Seeds Ltd., UK, on May 31, 2006) whole plants were extracted with 30 ml of 80% aqueous methanol for 30 min in a cooled ultrasound water bath at 7 °C. After centrifugation at 6000 rcf (relative centrifugal force), 10 min and 4 °C, the clear supernatant was collected and the residue was washed twice with 15 ml of 80% aqueous MeOH. The corresponding supernatants were combined and the solvent was evaporated under reduced pressure at 30 °C. The residue was re-dissolved in 6 ml MeOH, divided into 6 equal portions and stored at −20 °C. A 10 μl sample of these extract was injected into the HPLC.

2.3. Preparation of sainfoin samples

The extraction of phenolic compounds was performed by adding 500 μl aqueous methanol ($MeOH/H_2O$, 80/20, v/v), containing flavone (c = 0.02 mg/ml) as internal standard, to 100 mg dry powder for 30 min in a cooled ultrasound water bath (7 °C). After centrifugation at 10,000 rcf, 10 min and 4 °C, the supernatant was evaporated, the residue was re-dissolved in 100 μl methanol and 10 μl was injected for HPLC analysis.

2.4. Chromatographic instrumentation and conditions

LC experiments were performed on a Kontron HPLC system (Kontron Instruments, Germany) equipped with two pumps, a diode array detector and an automatic sample injector (model 231, Gilson Abimed Systems, Germany). For selective detection of flavan-3-ols using post-column derivatization with DMACA, a further Gynkotek HPLC pump (model 300C, Gynkotek GmbH, Germering, Germany) and a vis-detector (Kontron Detector 432, Kontron Instruments, Germany) was used [4,5]. Five columns were assayed: Luna PFP (A), Nucleosil C18 (B), Nucleodur Sphinx RP (C), Altima HP C18 HiLoad (D) and Reprosil-Pur Basic C18 (E). Except the last one, all other columns have 3 μm particles and the runs were performed at room temperature with a 0.5 ml/min flow rate. Column E with 5 μm particle size was additionally tested at 0.8 and 1.0 ml/min. Chemical and physical features of the columns and their manufacturers are summarized in Table 1. In further attempts for enhancing the selectivity, a gradient elution was optimized and two mobile phases were tested on column A (Table 2). Chromatograms were simultaneously recorded at 280, 320, 350 and 540 nm using DAD and at 640 nm by a vis-detector after post-column derivatization.

2.5. Identification and quantification of phenolic compounds

The peaks were identified by comparing their retention times and UV–vis spectra with those of the standards and of previously isolated compounds.

Quantification was performed using the internal standard method after having calculated response factors for the authentic standards available at each concentration point on the calibration curve within the linear range. The response factor was

Table 1
Stationary phases used in the present study and their features.

Notation	Column	Supplier	Column specifications	Nature of the stationary phase	Endcapping
A	Luna PFP	Phenomenex	4.6 mm × 250 mm, 3 μm, 100 Å, 5.7% C	Pentafluorophenyl propyl ligand bonded silica	Yes
B	Nucleosil C18	Macherey-Nagel	4.0 mm × 250 mm, 3 μm, 120 Å, 11% C	Octadecyl bonded silica	Yes
C	Nucleodur Spinx RP	Macherey-Nagel	4.0 mm × 250 mm, 3 μm, 110 Å, 14% C	Octadecyl and propylphenyl bonded silica	Yes
D	Altima HP C18 HiLoad	Grace	3.0 mm × 150 mm, 3 μm 100 Å, 24% C	Octadecyl bonded silica	No
E	Reprosil-Pur Basic C18	Dr. Maisch	4.6 mm × 250 mm, 5 μm, 120 Å, 17% C	Octadecyl bonded silica	Yes

calculated as the ratio of concentration of the analyte to the area produced by that concentration at the wavelength that gave the most intensive signal for that respective compound. Because its value may change for different concentrations the average of the response factors was used for quantification: flavone (280 nm) 6.57×10^{-6}; arbutin (280 nm) 7.24×10^{-5}; gallic acid (280 nm) 1.05×10^{-5}; protocatechuic acid (280 nm) 1.51×10^{-6}; 3′-caffeoylquinic acid (320 nm) 1.63×10^{-5}; 5′-caffeoylquinic acid (320 nm) 9.69×10^{-6}; catechin (640 nm) 1.78×10^{-6}; epicatechin 1.16×10^{-6}; gallocatechin (640 nm) 1.82×10^{-6}; epigallocatechin (640 nm) 1.95×10^{-6}; procyanidin B2 (640 nm) 1.32×10^{-6}; vitexin (320 nm) 1.35×10^{-5}; rutin (350 nm) 1.78×10^{-5}; kaempferol 3-rutinoside (350 nm) 1.65×10^{-5}; isorhamnetin 3-rutinoside (350 nm) 1.76×10^{-5}. For the unknown compounds and in the case there was no authentic standard available, the response factor of a standard with similar structure was used. Thus, simple phenolic acids and hydroxybenzoic acids were quantified as gallic acid; 8-β-glucopyranosyloxycinnamic acid as cinnamic acid (at 280 nm, response factor 2.87×10^{-6}), hydroxycinnamic acids as 5′-caffeoylquinic acid; flavan-3-ols as procyanidin B2; flavones as vitexin; flavonols as rutin; acylated flavonols as 5′-caffeoylquinic acid and isoflavones as formononetin (at 280 nm, response factor 1.37×10^{-5}).

2.6. Method validation

The selectivity of the method was determined by analysis of an extract of *O. viciifolia* and mixture of 10 standards.

Linearity was measured at five or six concentration levels. Calibration curves were constructed by plotting peak area versus concentration in the range of 0.025–5.0 mg/ml for arbutin and rutin; 0.025–1.0 mg/ml for gallic and protocatechuic acid, 3′-caffeoylquinic acid, epigallocatechin, vitexin, kaempferol and isorhamnetin 3-rutinoside; 0.01–0.5 for the internal standard flavone and 0.01–0.2 for apigenin 8-*C*-glucoside. Linearity was described by a regression equation and by the determination of the correlation coefficient.

The limit of detection (LOD) was defined as the compound concentration that produced a signal-to-noise ratio above three. The limit of quantification (LOQ) was evaluated as the concentration equal to 10 times the signal-to-noise ratio.

Accuracy was determined by analysing the percentage recovery for nine phenolic compounds characterized in the *O. viciifolia* extract. A sainfoin sample was spiked with a known amount of standards before extraction ($n = 6$), at the same concentration level as expected in the extract. The spiked samples were extracted and analysed under the previously established optimal conditions. The repeatability of the analytical run was expresses as the relative standard deviation (% R.S.D.) and evaluated for retention time and for peak area by performing six injections of a solution containing nine phenolic compounds standards. Method precision (% R.S.D.) was investigated using sample preparation procedure for six sainfoin samples.

3. Results and discussion

3.1. Development of gradient elution system and comparison of the columns

The first step in our attempt to find an efficient stationary phase for the LC separation of a high number of different phenolic compounds from complex mixtures, the separation performance of a pentafluorophenyl phase (A) was compared with those of a heterogeneous phase constituted of octadecyl and phenylpropyl bonded silica (C) and three C18 phases (B, D and E) by injecting 10 μl of methanol extract of the sainfoin variety Cotswold Common, using an HPLC protocol previously developed in our laboratory [5,28]. This forage legume contains a broad spectrum of phenolic compounds including: amino compounds, simple phenolic acids, hydroxybenzoic (HBA) and hydroxycinnamic acids (HCA), dihydroflavonols, flavones, flavan-3-ols, flavonols and anthocyanins [28]. A notable performance was observed for the pentafluorophenyl phase (A) which gave a good peak resolution in the first part of the chromatogram. Peaks which eluted too closely to void volume on the other columns (B–D) were sufficiently retained by the first one (A) where, the resolution of other compounds was good too. The phenylpropyl (B) and the Nucleosil C18 (C) columns gave good chromatograms with peaks distributed through the whole run and separated a higher number of peaks when compared to column D (Altima HP C18 HiLoad) and E (Reprosil-Pur Basic C18). Column E, the only one with 5 μm particle size was tested at 0.5 ml/min like the other four phases with 3 μm, and additionally at 0.8 and 1.0 ml/min flow rates.

Secondly, we tried to achieve a better separation of sainfoin compounds on the best three columns A, B and C by optimizing the elution protocol. The gradient steps and the methanol concen-

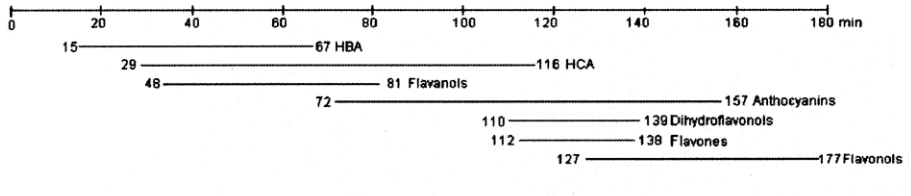
Table 2
Composition of the assayed mobile phases on column A (Luna PFP) and the optimized gradient.

System	Solvent A	Solvent B	Optimized gradient elution	
			Time (min)	%B
1	Water + 5% formic acid	Methanol	0	10
			20	15
			50	20
			70	25
			90	30
2	6% Acetic acid in 2 mM aq. NaOAc (2 mM sodium acetate water solution mixed with acetic acid at the ratio 94:6, v/v)	Methanol	130	40
			155	60
			175	90
			195	90

Table 3
Retention times (t_R) in minutes and the elution ranges of different phenolic classes of pure chemicals and compounds of *Onobrychis viciifolia* methanolic extract on the Luna PFP, Nucleosil C18 and Nucleodur Spinx columns obtained using 5% formic acid in water as solvent A and methanol as solvent B and the optimized gradient steps (Table 2 system 1). Sainfoin compounds are written in italics. Abbreviations used: ara, arabinose; c, cis; gal, galactose; glu, glucose; HBA, hydroxybenzoic acids; HCA, hydroxycinnamic acids; rha, rhamnose; rut; rutinose.

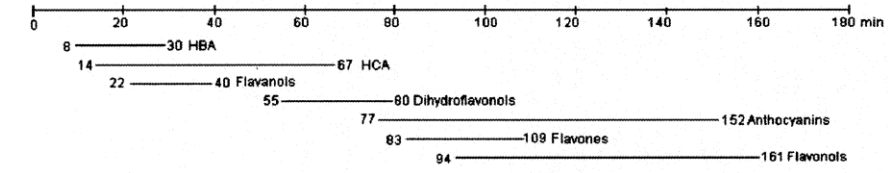
Luna PFP (A)

Other polyphenols	t_R	HBA	t_R	[a]HCA	t_R	Anthocyanins	t_R	[b]Flavanols	t_R	Dihydroflavonols	t_R	Flavons	t_R	Flavonols	t_R
Arbutin	*10*	*Gallic acid*	*15*	*3'-Caffeoylquinic acid*	29	Delphinidin 3-glu	72	*Gallocatechin*	25	Dihydroquercetin	110	Luteolin 8-C-glu	112	*Que 3-rharut*	*118*
L-Tryptophan	*54*	*Vanillic acid 4-glu*	*26*	*p-Coumaric acid 4-glu*	*36*	Cyanidin 3-glu	83	*Catechin*	*48*	Dihydrokaempferol	139	Luteolin 6-C-glu	116	Myricetin 3-gal	127
8-β-Glucopyranosyl-		*Protocatechuic acid*	*28*	*3-p-Coumaroylquinic acid*	*42*	Cyanidin 3-ara	93	*Epigallocatechin*	53			*Apigenin 8-C-glu*	*124*	Quercetin 3-rut-7-rha	127
-oxycinnamic acid	82	4-Hydroxybenzoic acid	45	*c p-Coumaric acid 4-glu*	*43*	Peonidin 3-glu	105	*Procyanidin B2*	*57*			Apigenin 6-C-glu	138	*Kaempferol 3-rharut*	*131*
Ellagic acid	144	Vanillic acid	67	*p-Caffeoylglucose*	*51*	Luteolinidin	120	*Epicatechin*	*81*					Quercetin 7-glu	133
Afrormosin	177			4'-Caffeoylquinic acid	56	Cyanidin	134	*Procyanidin C1*	*103*					Myricetin 3-rha	140
Isoliquiritigenin	177			*5'-Caffeoylquinic acid*	*57*	Pelargonidin	148	*Procyanidin B5*	*111*					Kaempferol 3-rut-7-rha	142
				Caffeic acid	66	Peonidin	151	*Procyanidin E-B5*	*113*					*Quercetin 3-rut*	*146*
				p-Coumaric acid	97	Malvidin	157							Quercetin 3-glu	147
				Ferulic acid	116									Kaempferol 7-rha	150
														Quercetin 3-ara	151
														Kaempferol 7- -(6-O-acetyl-glu)-7-rha	154
														Kaempferol 3-rut	*156*
														Kaempferol 3-glu	156
														Quercetin 3-rha	158
														Isorhamnetin 3-glu	160
														Isorhamnetin 3-rut	*160*
														Myricetin	160
														Kaempferol 3-ara	163
														Kaempferol 3-rha	164
														Quercetin	170
														Kaempferol	175
														Isorhamnetin	177



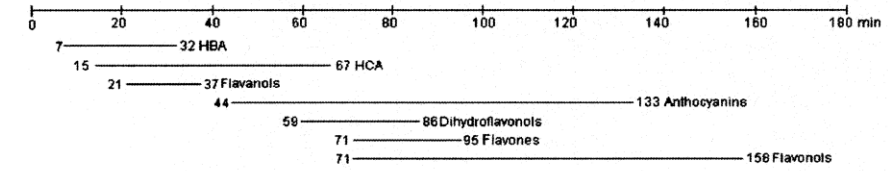
Nucleosil C18 (B)

Arbutin	*6*	*Gallic acid*	*8*	*3'-Caffeoylquinic acid*	*14*	Delphinidin 3-glu	77	*Gallocatechin*	*18*	Dihydroquercetin	55	Luteolin 8-C-glu	83	Myricetin 3-gal	94
L-Tryptophan	*25*	*Vanillic acid 4-glu*	*12*	*3-p-Coumaroylquinic acid*	*20*	Cyanidin 3-glu	85	*Catechin*	*22*	Dihydrokaempferol	80	*Apigenin 8-C-glu*	*89*	Quercetin 7-glu	96
8-β-Glucopyranosyl-		*Protocatechuic acid*	*13*	*c p-Coumaric acid 4-glu*	*21*	Cyanidin 3-ara	92	*Epigallocatechin*	*23*			Luteolin 6-C-glu	94	*Quercetin 3-rharut*	*98*
-oxycinnamic acid	*41*	4-Hydroxybenzoic acid	20	*p-Coumaric acid 4-glu*	*23*	Peonidin 3-glu	103	*Procyanidin B2*	*25*			Apigenin 6-C-glu	109	Myricetin 3-rha	100
Ellagic acid	121	Vanillic acid	30	*4'-Caffeoylquinic acid*	*28*	Luteolinidin	117	*Epicatechin*	*40*					*Kaempferol 3-rharut*	*110*
Isoliquiritigenin	159			*p-Caffeoylglucose*	29	Cyanidin	129							Quercetin 3-glu	111
Afrormosin	164			Caffeic acid	32	Pelargonidin	142							Quercetin 3-rut-7-rha	113
				5'-Caffeoylquinic acid	*36*	Peonidin	148							Kaempferol 7-rha	113
				p-Coumaric acid	50	Malvidin	152							*Quercetin 3-rut*	*114*
				Ferulic acid	67									Myricetin	121
														Quercetin 3-ara	121
														Quercetin 3-rha	127
														Kaempferol 3-rut-7-rha	127
														Kaempferol 3-rut	*131*
														Kaempferol 3-glu	133
														Isorhamnetin 3-glu	134
														Kaempferol 3-ara	139
														Kaempferol 7- -(6-O-acetyl-glu)-7-rha	140
														Isorhamnetin 3-rut	*140*
														Kaempferol 3-rha	144
														Quercetin	144
														Kaempferol	156
														Isorhamnetin	161



Nucleodur Spinx (C)

Arbutin	*6*	*Gallic acid*	*7*	*3'-Caffeoylquinic acid*	*15*	Delphinidin 3-glu	44	*Epigallocatechin*	*21*	Dihydroquercetin	59	Luteolin 8-C-glu	71	Myricetin 3-gal	71
L-Tryptophan	*27*	*Protocatechuic acid*	*12*	*p-Coumaric acid 4-glu*	*21*	Cyanidin 3-glu	56	*Catechin*	*21*	Dihydrokaempferol	86	Luteolin 6-C-glu	76	Quercetin 7-glu	82
8-β-Glucopyranosyl-		*Vanillic acid 4-glu*	*14*	*3-p-Coumaroylquinic acid*	*23*	Cyanidin 3-ara	64	*Epicatechin*	*37*			*Apigenin 8-C-glu*	*86*	*Quercetin 3-rharut*	*88*
-oxycinnamic acid	*69*	4-Hydroxybenzoic acid	24	*5'-Caffeoylquinic acid*	*28*	Peonidin 3-glu	78					Apigenin 6-C-glu	95	Myricetin 3-rha	91
Ellagic acid	94	Vanillic acid	32	4'-Caffeoylquinic acid	28	Luteolinidin	89							Quercetin 3-rha-7-rha	91
Isoliquiritigenin	159			*p-Caffeoylglucose*	*29*	Cyanidin	99							*Kaempferol 3-rharut*	*102*
Afrormosin	166			Caffeic acid	30	Pelargonidin	118							Quercetin 3-glu	102
				c p-Coumaric acid 4-glu	32	Peonidin	127							*Quercetin 3-rut*	*103*
				p-Coumaric acid	51	Malvidin	133							Kaempferol 7-rha	105
				Ferulic acid	67									Quercetin 3-ara	107
														Kaempferol 3-rut-7-rha	108
														Myricetin	111
														Kaempferol 3-glu	116
														Kaempferol 3-rut	*124*
														Kaempferol 7- -(6-O-acetyl-glu)-7-rha	124
														Quercetin 3-rha	125
														Isorhamnetin 3-rut	*125*
														Isorhamnetin 3-glu	125
														Kaempferol 3-ara	135
														Kaempferol 3-rha	138
														Quercetin	138
														Kaempferol	155
														Isorhamnetin	158



[a]Except cis p-coumaric acid 4-glucoside all hydroxycinnamic acids were *trans* isomers.
[b]The flavanol elution range on each of the three columns were defined only for epigallocatechin, catechin and epicatechin at 280 nm. The t_R of the other flavanols is from the 640 nm chromatograms.

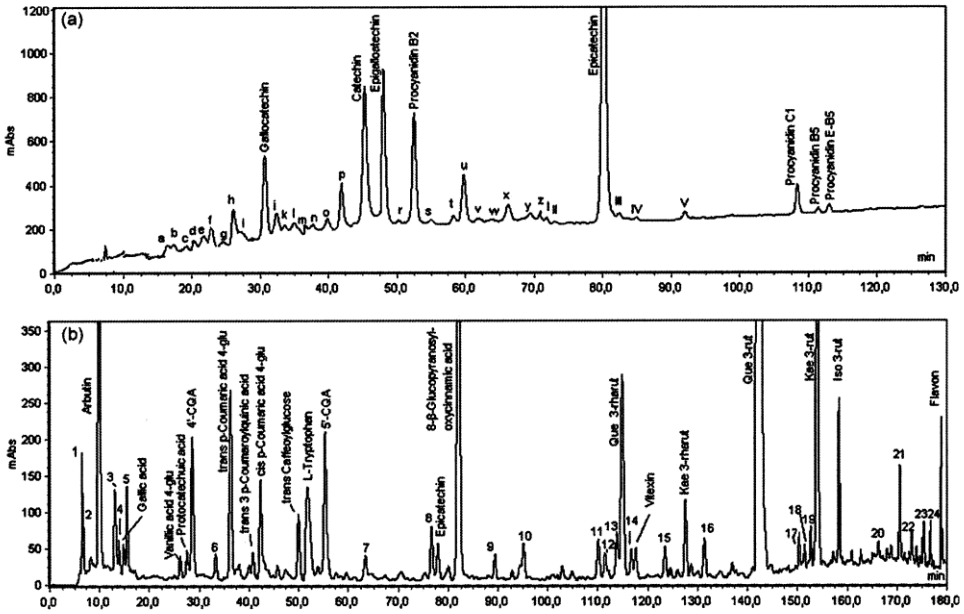


Fig. 1. Chromatograms obtained at 640 nm after post-column derivatization with DMACA (a) and 280 nm (b) from an 80% aq. methanolic extract of whole plants of *Onobrychis viciifolia* variety Cotswold Common using column A (Luna PFP) and system 1 described in Table 2. Abbreviations used: CQA, caffeoylquinic acid; glu, glucose; Iso, isorhamnetin; Kae, kaempferol; rha, rhamnose; rut, rutinose; rharut, rhamnosylrutinoside; Que, quercetin. Unknown peaks were classified as flavan-3-ols (a–z, I–V), hydroxybenzoic acids (1–5, 11), hydroxycinnamic acids (10, 14), flavones (9, 13), flavonol glycosides (8, 12, 15–17, 19–21), acylated flavonols (20, 21) and isoflavones (22, 23).

tration of the gradient we usually used for the analysis of phenolic compounds were suitably modified to yield the best elution program described in Table 2. An analysis time of 195 min was necessary to obtain a good separation of sainfoin broad spectrum of phenolic compounds. Escarpa and González [29,30] optimized short gradient elution methods for the analysis of several groups of the most prominent phenolics in less than 30 min. There is an obvious advantage for those who focus on the major polyphenolics; however, using such short analysis time some minor or unknown compounds may co-elute and lead to a wrong quantification of known compounds also. Among the wide range of investigations, just few methods targeted several classes of phenolic compounds and there longer elution times were necessary for the separation [31–34].

Mobile phase composition in HPLC represents also a critical factor affecting the separation and therefore, the solvent A was changed from 5% formic acid in water (system 1, Table 2) to 6% acetic acid in 2 mM sodium acetate aqueous solution (system 2, Table 2), which was optimized by Tsao and Yang [31] to give a good separation of 25 phenolics commonly found in fruits. In order to evaluate the new solvent, *O. viciifolia* extract was analyzed twice on column A, first using our optimized gradient from Table 2 and then using the 70 min gradient described by these authors [31], whereas the flow was modified at 0.5 ml/min instead 1 ml/min to not exceed the maximum allowable work pressure of the HPLC system. However, when our optimized gradient and sodium acetate in combination with acetic acid as solvent A additive was used the separation was weak, particularly for the early eluting compounds. When using the Tsao gradient at 0.5 ml/min the latest eluting peaks showed a weak separation. On the other hand, Tsao and Yang used acetonitrile exhibiting different solvent strength and selectivity to methanol, which may have affected some of the observations when methanol was used in this study. Consequently, in further experiments we used 5% formic acid in water as solvent A and methanol as solvent B and the gradient steps optimized by us.

The resolution of columns A, B and C was then compared by the LC chromatography of 10 μl sainfoin extract using 5% formic acid in water as solvent A and methanol as solvent B and the optimized gradient steps (Table 2, system 1). The highest number of well separated peaks was obtained by column A (Fig. 1). As can be seen in Table 3, where the retention times and elution ranges of the three columns were compared, sainfoin compounds are longer retained on column A than on B and C. The quantification and identification of poorly resolved or co-eluting compounds are rendered possible on column A. Such critical pairs are *cis* and *trans* p-coumaric acid 4-glucoside on column B or vanillic acid 4-glucoside and 3′-caffeoylquinic acid; caffeoylglucose, 4′- and 5′-caffeoylquinic acid; catechin and epigallocatechin on column C. All three columns showed different elution order for the esters and glucosides of caffeic acid. The coumaric acid derivatives have the same elution order on A and C columns when compared with column B. Besides, compared to the B and C columns, a good separation of the flavonol tri- and di-glycosides kaempferol 3-rhamnosylrutinoside and quercetin 3-rutinoside was observed on the perfluorinated phase (A).

In order to obtain more information about the separation characteristics of the perfluorinated (A), phenylalkyl (B) and C18 (Nucleosil) columns, 54 standard substances including 1 amino acid, 2 simple phenolic acids, 4 HBA, 6 HCA, 2 dihydroflavonols, 4 flavones, 3 flavan-3-ols, 9 anthocyanins, 1 chalcone, 1 isoflavone and 21 flavonols were analyzed. Retention times and elution ranges are given in Table 3 showing many differences between the three columns. The best separation and an excellent peak shape were obtained using column A, which showed only four critical pairs as compared to seven unsolved pairs on columns B and C (Fig. 2). Except for anthocyanins, on column A, the retention times were considerably higher for the phenolic classes tested when compared to the other packing materials. The elution order was the same as for column C: HBA, HCA, flavan-3-ols, anthocyanins,

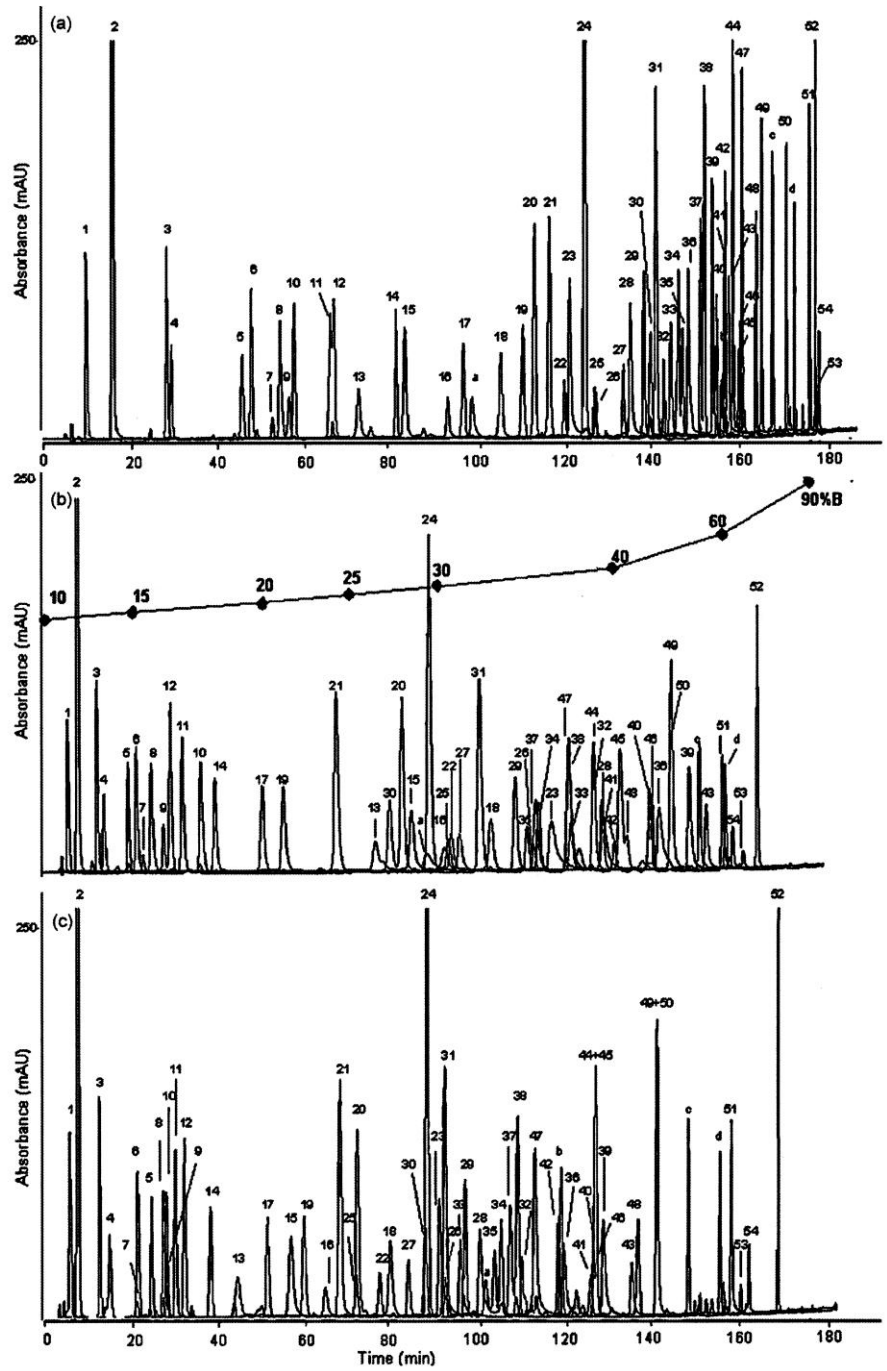


Fig. 2. Chromatogram overlays at 280 nm for the mixtures of standard phenolic compounds employed in the optimization of chromatographic method obtained with: (a) column A (Luna PFP); (b) column B (Nucleosil C18); (c) column C (Nucleodur Spinx) and the gradient steps given in subfigure b. For peak numbers, see Table 4. a, b, c and d are impurity peaks.

Table 4
Retention times (min) and spectroscopic parameters (nm) of the assayed standards of phenolic compounds with their retention factors (k) and selectivity (α) on column A (Luna PFP).

Peak no.	Phenolic compound	Phenolic structure	UV bands (nm)	t_R (min)	k	α
1	Arbutin	Simple phenolic acid	282	10.0	0.51	2.77
2	Gallic acid	Hydroxybenzoic acid	270	15.9	1.42	2.34
3	Protocatechuic acid	Hydroxybenzoic acid	259/293	28.5	3.32	1.05
4	3′-Caffeoylquinic acid	Hydroxycinnamic acid	sh 249/325	29.5	3.49	1.71
5	4-Hydroxybenzoic acid	Hydroxybenzoic acid	255	45.8	5.96	1.05
6	Catechin	Flavan-3-ol	278	47.9	6.28	1.12
7	Epigallocatechin	Flavan-3-ol	269	52.8	7.03	1.04
8	L-Tryptophan	Amino acid	277	54.5	7.28	1.05
9	4′-Caffeoylquinic acid	Hydroxycinnamic acid	sh 252/326	56.6	7.60	1.02
10	5′-Caffeoylquinic acid	Hydroxycinnamic acid	sh 250/326	57.8	7.78	1.16
11	Caffeic acid	Hydroxycinnamic acid	sh 251/323	66.1	9.04	1.01
12	Vanillic acid	Hydroxybenzoic acid	260/292	66.9	9.16	1.10
13	Delphinidin 3-glucoside	Anthocyan	275/525	72.6	10.04	1.13
14	Epicatechin	Flavan-3-ol	277	81.2	11.34	1.03
15	Cyanidin 3-glucoside	Anthocyan	279/514	83.2	11.64	1.13
16	Cyanidin 3-arabinoside	Anthocyan	278/516	93.1	13.14	1.04
17	p-Coumaric acid	Hydroxycinnamic acid	309	96.7	13.69	1.09
18	Peonidin 3-glucoside	Anthocyan	278/515	105.2	14.99	1.05
19	Dihydroquercetin	Dihydroflavonol	288	110.2	15.75	1.03
20	Luteolin 8-C-glucoside	Flavone	sh 258/265/348	112.9	16.16	1.03
21	Ferulic acid	Hydroxycinnamic acid	323	116.3	16.68	1.03
22	Luteolin 6-C-glucosdie	Flavone	268/349	119.8	17.20	1.01
23	Luteolinidin	Anthocyan	278/486	120.9	17.37	1.03
24	Apigenin 8-C-glucoside	Flavone	266/336	124.3	17.90	1.02
25	Myricetin 3-galactoside	Flavonol	260/358	126.7	18.26	1.00
26	Quercetin 3-rutinoside-7-rhamnoside	Flavonol	258/355	127.0	18.30	1.05
27	Quercetin 7-glucoside	Flavonol	255/370	133.3	19.25	1.01
28	Cyanidin	Anthocyan	274/524	134.8	19.49	1.02
29	Apigenin 6-C-glucoside	Flavone	269/336	137.9	19.96	1.01
30	Dihydrokaempferol	Dihydroflavonol	290	139.4	20.19	1.01
31	Myricetin 3-rhamnoside	Flavonol	259/350	140.6	20.37	1.01
32	Kaempferol 3-rutinoside-7-rhamnoside	Flavonol	265/346	142.6	20.66	1.01
33	Ellagic acid	Simple phenolic acid	254/367	144.1	20.90	1.01
34	Quercetin 3-rutinoside	Flavonol	256/355	145.9	21.17	1.01
35	Quercetin 3-glucoside	Flavonol	257/354	146.7	21.30	1.01
36	Pelargonidin	Anthocyan	266/sh 423/513	148.0	21.50	1.02
37	Kaempferol 7-rhamnoside	Flavonol	263/366	150.8	21.92	1.01
38	Quercetin 3-arabinoside	Flavonol	256/355	151.7	22.06	1.01
39	Peonidin	Anthocyan	273/sh439/530	153.5	22.33	1.01
40	Kaempferol 7-(6-O-acetylglucoside)-7-rhamnoside	Flavonol	265/346	154.5	22.49	1.01
41	Kaempferol 3-rutinoside	Flavonol	264/347	156.1	22.73	1.00
42	Kaempferol 3-glucoside	Flavonol	256/349	156.1	22.78	1.00
43	Malvidin	Anthocyan	272/sh 350/541	157.3	22.91	1.01
44	Quercetin 3-rhamnoside	Flavonol	255/347	158.3	23.05	1.01
45	Isorhamnetin 3-glucoside	Flavonol	256/354	160.3	23.25	1.00
46	Isorhamnetin 3-rutinoside	Flavonol	256/355	160.3	23.34	1.00
47	Myricetin	Flavonol	255/375	160.3	23.36	1.00
48	Kaempferol 3-arabinoside	Flavonol	264/346	163.6	23.86	1.01
49	Kaempferol 3-rhamnoside	Flavonol	263/342	164.8	24.05	1.04
50	Quercetin	Flavonol	255/372	170.5	24.92	1.03
51	Kaempferol	Flavonol	264/366	175.8	25.72	1.01
52	Afrormosin	Isoflavone	258/319	177.1	25.91	1.00
53	Isorhamnetin	Flavonol	259/370	177.1	25.97	1.00
54	Isoliquiritigenin	Chalcone	258/372	177.1	26.05	

Table 5
Linearity, limits of detection and quantification, and the wavelength used for data collection for nine determined phenolic compounds in the extract of *Onobrychis viciifolia*.

Compounds	Wavelength (nm)	Linearity range (mg/ml)	Regression equation	LOD (mg/ml)	LOQ (mg/ml)	Correlation coefficient (R^2)
Arbutin	280	0.025–2.5	$y = 139.2x + 0.3733$	0.01	0.05	1
Gallic acid	280	0.025–0.5	$y = 952.71x + 0.2142$	0.002	0.005	0.9999
Protocatechuic acid	280	0.025–1.0	$y = 673.4x + 0.2169$	0.0025	0.008	0.9996
3′-Caffeoylquinic acid	320	0.0125–0.5	$y = 657.8x - 2.1012$	0.008	0.02	0.9997
Epigallocatechin	640	0.025–0.5	$y = 4940.9x + 10.683$	0.0025	0.005	1
Vitexin	320	0.01–0.2	$y = 782.44x - 1.3831$	0.0025	0.01	0.9999
Quercetin 3-rutinoside	350	0.025–2.5	$y = 548.73x + 6.543$	0.08	0.02	0.9998
Kaempferol 3-rutinoside	350	0.025–0. 25	$y = 614x - 0.31$	0.0025	0.008	1
Isorhamnetin 3-rutinoside	350	0.025–0.25	$y = 571.31x - 0.1488$	0.0025	0.008	1
Flavone (IS)	280	0.01–0.1	$y = 1570x - 1.3636$	0.0005	0.002	0.9999

Table 6
Accuracy and precision data for nine determined phenolic compounds in the extract of *Onobrychis viciifolia*.

	Accuracy[a]		Repeatability[b]		Precision R.S.D. (%)[a]	
Compounds	Recovery (%)	R.S.D. (%)	Repeatability t_R	Repeatability area	Intra-day	Inter-day
Arbutin	100.0	1.3	1.63	2.14	1.43	7.09
Gallic acid	89.5	1.1	3.82	2.07	1.86	9.36
Protocatechuic acid	91.6	1.1	4.39	2.63	1.85	10.33
3′-Caffeoylquinic acid	88.8	1.0	4.25	2.25	0.48	8.03
Epigallocatechin	83.8	5.9	6.72	3.70	3.81	9.10
Vitexin	104.1	2.3	1.81	2.11	1.64	10.09
Quercetin 3-rutinoside	87.6	1.5	1.57	2.11	1.87	9.66
Kaempferol 3-rutinoside	89.0	2.2	1.02	2.25	1.72	8.52
Isorhamnetin 3-rutinoside	91.5	3.1	0.18	2.22	3.91	2.68

[a] Made in three replicates.
[b] Made in six replicates.

dihydroflavonols, flavones, flavonol glycosides and, flavonols, isoflavone and chalcone aglycones. Additionally, longer elution ranges were observed on column A for compounds of HBA, HCA and flavan 3-ol classes, whereas column C gave longer range for flavonols. Because the solvent strength is increased rapidly at the end of the gradient elution program the later eluting peaks were very sharp. Table 4 lists peak information of the assayed standards including retention times and λ_{max} of the UV/vis spectra together with the corresponding retention factors (k) and selectivity (α) on column A. Another similarity of column A with C was the elution order of the flavones. Whereas on Nucleosil C18 (column B) the 8-*C* isomers eluted before the 6-*C* isomers, the retention time of these compounds on columns A and C will depend on the number of hydroxyl groups. For the derivatives of quercetin and kaempferol which were available for the tests in many glycosylated forms a very clear elution order was observed on column A: 3-triglycosides, 7-glycosides, 3-rutinosides, 3-glucosides, 3-arabinosides and 3-rhamnosides. On columns B and C the 7-glucoside of quercetin eluted before the 3-triglycosides and the 7-rhamnoside of kaempferol between the two 3-triglucosides of which one was 3-rhamnosylrutinoside and the other 3-rutinoside-7-rhamnoside. The 3-galactoside of myricetin (3-OH groups on the B ring) was the first eluted flavonol on columns B and C. On column A these compound eluted after quercetin 3-rhamnosylrutinoside. The described retention behavior of the PFP phase might be explained by the existence of some π–π interactions between unsaturated solutes and perfluorophenyl ligands as well as greatly different dispersive interactions between solutes and column as a result of large differences in ligand polarizability [8].

3.2. HPLC method validation

The proposed HPLC method for the determination of phenolic compound in sainfoin was evaluated in terms of precision, accuracy, linearity, detection and quantification limit, and selectivity. The results obtained using the standards arbutin, gallic acid, protocatechuic acid, 3′-caffeoylquinic acid, epigallocatechin, vitexin, rutin, kaempferol 3-rutinoside, isorhamnetin 3-rutinoside are summarized in Tables 5 and 6. The repeatability of retention times and peak areas were obtained with R.S.D. values lower than 7% and 4%, respectively. The intra- and inter-day precisions (expressed in terms of % R.S.D.) were found to be in the range of 0.48–3.91% and 2.68–10.33%, respectively, which demonstrated the good precision of the proposed method. The recovery was found to be between 84% and 104%. The linearity ranges, LODs and LOQs (in the range 0.005–0.05 mg/ml) and the regression equations and coefficient of correlations (not less than 0.999) revealed a good sensitivity and linearity response for the developed method.

3.3. Quantification of phenolic compounds in a sainfoin sample

Finally, the developed method using column A (Luna PFP), the binary mobile phase consisting of 5% formic acid in water as solvent A and MeOH as solvent B and the 195 min gradient described in Table 2 was used to analyze the phenolic compounds of a sainfoin extract.

For instance, Fig. 1 shows the chromatograms at 280 nm (Fig. 1b) and at 640 nm (Fig. 1a) after chemical reaction with DMACA. The

Table 7
Quantification of *Onobrychis viciifolia* compounds.

Compound	Concentration (mg/g DW)
Amino compounds	
L-Tryptophan	0.340
Simple phenolic acids	
Arbutin	2.694
8-β-Glucopyranosyloxycinnamic acid	0.179
Hydroxybenzoic acids	
Protocatechuic acid	<LOQ
Gallic acid	0.020
Vanillic acid 4-glucoside	0.018
Unknown simple phenolic acids	0.260
[a]*Hydroxycinnamic acids*	
p-Coumaric acid 4-glucoside	0.084
cis p-Coumaric acid 4-glucoside	0.030
p-Coumaroylquinic acid	0.036
3′-Caffeoylquinic acid	0.328
5′-Caffeoylquinic acid	0.276
p-Caffeoylglucose	0.083
Unknown hydroxycinnamic acids	0.162
Flavones	
Vitexin	0.048
Unknown flavones	0.127
Flavan-3-ols	
Catechin	0.100
Epicatechin	0.261
Gallocatechin	0.058
Epigallocatechin	0.111
Procyanidin B2	0.054
Procyanidin C1	0.013
Procyanidin B5	<LOQ
Procyanidin E-B5	<LOQ
Unknown flavanols	0.201
Flavonols	
Kaempferol 3-rutinoside	1.874
Quercetin 3-rutinoside	6.147
Isorhamnetin 3-rutinoside	0.375
Kaempferol 3-rhamnosylrutinoside	0.285
Quercetin 3-rhamnosylrutinoside	0.997
Unknown flavonol glycosides	0.693
Unknown acylated flavonols	0.110
Isoflavones	
Unknown isoflavones	0.034

[a] Except *cis* p-coumaric acid 4-glucoside all hydroxycinnamic acids were trans isomers.

identification of the detected peaks was carried out by comparison of their retention times and UV spectra with those of available standards and previously isolated compounds. The unknown compounds were classified based on the absorption pattern of the UV spectra into: simple phenolic acids (peaks 1–5, 11), hydroxycinnamic acids (peaks 10, 14), flavones (peaks 9, 13), flavan-3-ols (peaks a–z, I–V), glycosides (peaks 8, 12, 15–17, 19–21) acylated flavonols (peaks 20, 21), and isoflavones (peaks 22, 23). The contents (mg/g dry weight) are shown in Table 7. The described HPLC method using post-column derivatization with DMACA reagent allowed the quantification and the selective detection of catechins and proanthocyanidins in sainfoin. As can be seen in Fig. 1b, only epicatechin could be detected at 280 nm, whereas further flavan-3-ols could be detected by post-column derivatization and measured at 640 nm (Fig. 1a).

4. Conclusions

The suitability of pentafluorophenylpropyl bonded phase for the separation of broad range phenolic compounds was compared with that of a bifunctional phase constituted of octadecyl and phenylpropyl bonded silica and three C18 columns. Except anthocyanins all analytes were considerably more retained on the perfluorophase compared to the other columns. An HPLC method with sensitive diode array and chemical reaction detection using the perfluorophase was validated and applied for the analysis of an *O. viciifolia* sample. The positive outcome is particularly the better resolution of early eluting phenolic compounds such as hydroquinones, hydroxybenzoic acids, hydroxycinnamic acids and flavan-3-ols which gave longer elution ranges on the PFP stationary phase compared to conventional columns.

Acknowledgments

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5. CHARACTERISATION OF PHENOLIC COMPOUNDS IN YOUNG LEAFLETS OF 37 SAINFOIN ACCESSIONS BY HPLC

Chapter 5 offers a brief description of the work regarding the investigation of polyphenol accumulation in 37 sainfoin accessions.

This study is described in great detail in article 4: THILL et al. (2012).

The original article is attached in the Appendix 2.

Article 4 – Summary

Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin)

THILL, J., REGOS, I., FARAG, M.A., AHMAD, A.F., KUSEK, J., HAYOT CARBONERO, C., GADJEV, I.Z., SMITH, L., HALBWIRTH, H., TREUTTER, D., STICH, K.

2012. Phytochemistry 82, 67-80.

During the EU sainfoin research project, Healthy Hay which sought to research fully all the benefits of sainfoin and reinvent its role in modern farming, a collection of 360 sainfoin accessions was evaluated. The existing sainfoin germplasm was found to be highly diverse on its agronomic behaviour, morphology and proanthocyanidin composition. The aim of our study was to gain knowledge about the variability of phenolic profiles of the young leaflets of 37 sainfoin accessions in order to identify interesting candidates for further breeding programs. As determined by HPLC, considerable variations in the quantity and composition of soluble phenolics among the various accessions were observed. Total phenolic contents ranged from 16.16 to 43.13 mg/g freeze-dried sainfoin. Flavanol content varied from 0.50 to 11.07 mg/g DW and included monomers and polymers known also as proanthocyanidins. Flavonols are the most abundant class of flavonoids in sainfoin and their contents varied from 8.86 to 36.52 mg/g DW. Glycosides of kaempferol and quercetin as well as acylated flavonol glycosides were analysed in all 37 samples. The methylated flavonol isorhamnetin was identified only in several samples bound to glucose or rutinose. Except one sample which is drawing a chromatogramm with many peaks giving a spectrum characteristic to flavonol-glycosides, all other 36 samples show the same pattern. Hydroxycinnamic acids, simple phenolics and flavones were also identified and quantified. Principal component analysis revealed that flavonols and flavanols contributed most to variety separation. The heterogeneity between the different varieties was also assessed using hierarchical cluster analysis of sample phenolics profile.

Article 4 – Authors' contributions

Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin)

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This article is the result of collaboration with other two groups.

Group I: HAYOT CARBONERO, C., GADJEV, I.Z. and SMITH, L. selected the plant material.

Group II: THILL, J., AHMAD, A.F., KUSEK J., HALBWIRTH, H. and STICH, K. investigated the 37 sainfoin accessions regarding the antioxidant capacities, peroxidase activity, selected polyphenol enzyme activities designed the manuscript.

REGOS, I: extraction and analysis of phenolic compounds, data analysis and interpretation, providing data for hierarchical cluster analysis and principal component analysis.

FARAG, M.A.: hierarchical cluster analysis and principal component analysis.

TREUTTER, D.: supervision and scientific conception, revision of the manuscript

6. COMPOSITION OF PHENOLIC COMPOUNDS IN VARIOUS ORGANS AT DIFFERENT DEVELOPMENTAL STAGES OF SAINFOIN (*ONOBRYCHIS VICIIFOLIA* var. COTSWOLD COMMON)

6.1. Introduction

Elucidating the phenolic structures in sainfoin is important for understanding potential health beneficial effects of these dietary phytochemicals. From available literature data (BARAU et al., 2005; TERRILL et al., 2009; MANOLARAKI, 2011) it is apparent that the effects are dependent on phenolic structures and concentrations. However, our study (article 1: REGOS et al., 2009) has shown that phenolic compositions of sainfoin plants within one variety (Cotswold Common) differ even when grown at the same place. Qualitative and quantitative differences on phenolics between sainfoin plant organs have also been measured.

The aim of this study was to characterize the metabolite composition in the leaves (leaflets and petioles), stems and flower of sainfoin (*Onobrychis viciifolia*) at three different stages of plant development: vegetative, bud and bloom stage in order to lay a cornerstone for their physiological evaluation.

6.2. Materials and methods

6.2.1. Plant material

Sainfoin seeds (var. Cotswold Common) were planted in a greenhouse in 2007 in Freising (Germany). When the plants reached the vegetative stage (*Figure 6A*), young and mature leaves were collected (*Figure 7A-B, Table 3*) and the plant pots were transferred over winter in a cold chamber. In spring, the plants were pick up and planted in the field. Different tissues (Table 3) were harvested from plants at bud and bloom stage, (*Figure 6B-C*). Plant material was frozen at -20°C and freeze dried. The tested sainfoin tissues are showed in *Figure 7*.

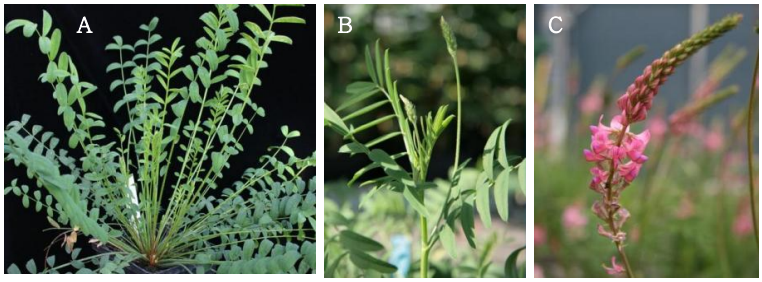

Figure 6. **The three developmental stages use to investigate the phenolic compounds in *Onobrychis viciifolia* tissues.**

(A) Sainfoin plant at vegetative stage from greenhouse.

(B) Sainfoin plant at bud stage from the field.

(C) Sainfoin plant at bloom stage from the field.

Table 3. Plant material.

Stage of plant development	*Sainfoin tissues*	*Number of plants, (n)*
Vegetative (V)	Leaflets (young and mature)	Mixture
	Petioles (young and mature)	Mixture
Bud (B)	Leaflets (young and mature)	3
	Petioles (young and mature)	3
	Stem	6
	Flower buds	6
Bloom (F)	Leaflets (young and mature)	1
	Petioles (young and mature)	1
	Stem	1
	Flowers (bud, open and mature)	1

Figure 7. **Sainfoin plant tissues.**

(A) Young leave with leaflets and petioles.

(B) Mature leave with leaflets and petioles.

(C) Stem sample is a mixture of flower stalks and plant stems.

(D) Flower buds.

(E) Open flowers.

(F) Mature flowers.

6.2.2. General experimental procedures

The phenolic compounds in sainfoin tissues were extracted and analysed by HPLC as described previously by REGOS et al. (2009).

6.3. Results and discussion

Phenolics in stems, leaflets and petioles, buds and flowers of sainfoin collected at vegetative, bud and bloom stage were analysed by reverse phase HPLC with diode array and chemical reaction detection. This yielded information on phenolic content and composition (Table IA - IIIA in Appendix 3 and *Figures 8 – 17*).

Six phenolic classes were detected: simple phenolics, (SP), hydroxycinnamic acids, (HCA), flavanols, flavonols, flavones and anthocyanins. *Figure 8* shows their distribution among sainfoin tissues collected from plants at vegetative (V), bud (B) and bloom (F) stage.

Large quantitative variations related to the total phenolics and phenolic classes were measured in the different plant organs and between the same organs at different plant developmental stages. Total phenolic content showed 7-fold differences, which ranged from 7 to 52 mg total phenolics per g of freeze-dried tissues (open flowers versus young petioles at bud stage). The predominant classes were the flavonols in leaflets (at bloom stage represented 87% on average young/mature leaflets of total phenolics) and flower buds (53% of total phenolics), and the simple phenolic acids in petioles and stems (at vegetative stage represents 57% on average young/mature petioles of total phenolics). The contents of each class and of the identified constituents are shown in *Figure 9 – 17*.

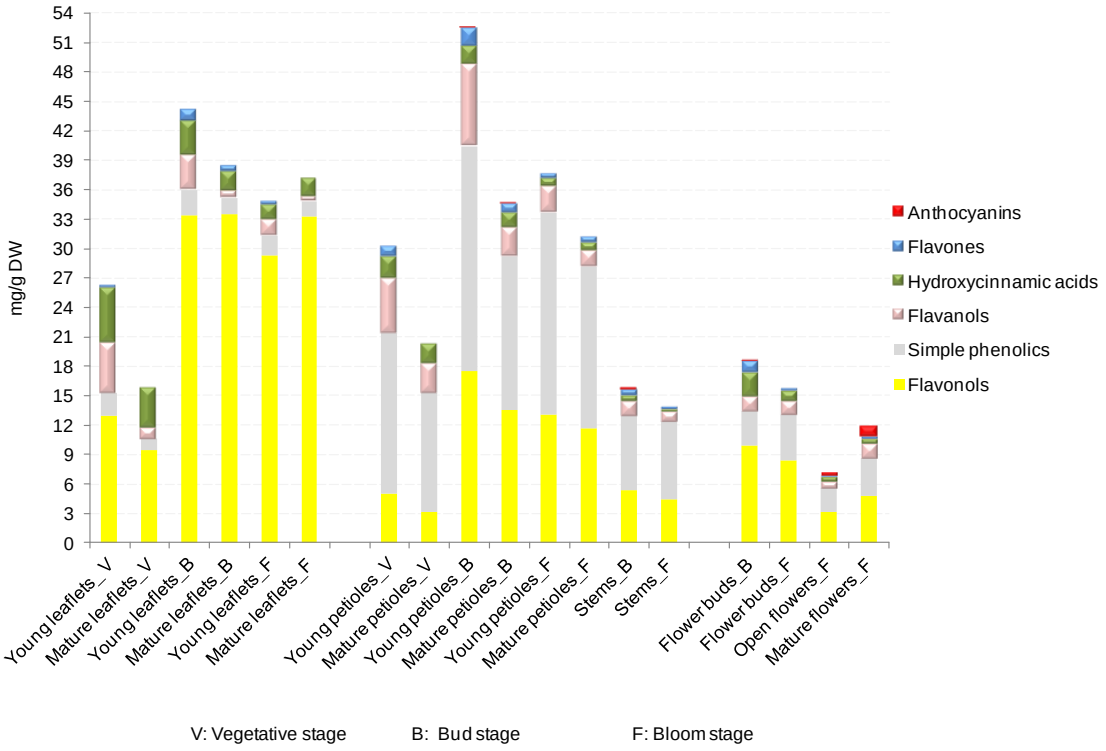

Figure 8. **Phenolic composition in various organs at different developmental stages of sainfoin.** Total phenolic content, expressed as milligram per gram of freeze dried tissue, is achieved by summing up single phenolic pools. Different colors are representing different phenolic groups. The highest phenolic amount was measured in young tissues at bud stage (B) followed by tissues at bloom (F) and vegetative stage (V).

Left: Leaflets contained mostly flavonols. Their quantity increased with 33% on average (young/mature leaflets) from vegetative to bloom stage, whyle the quantity of flavanols and hydroxycinnamic acids declined with 11% and 18%, respectively.

Middle: Petioles and stems contained mostly simple phenolics. Flavonols content increased with 27% on average (young/mature petioles) from vegetative to bloom stage, while the quantity of flavanols and hydroxycinnamic acids declined with 10% and 6%, respectively.

Right: Flower buds contained 53% on average flavonols.Their content declined to 43% in the flowers which accumulated additionally anthocyanins.

6.3.1. Simple Phenolics (SP)

The highest SP levels were measured in petioles, followed by stems, flowers and leaflets (*Figure 9,* upper panel). SP compounds determined in different sainfoin tissues were identified as arbutin, 8-β-glucopyranosyloxycinnamic acid, protocatechuic acid, vanillic acid 4-O-glucoside and p-hydroxybenzoic acid.

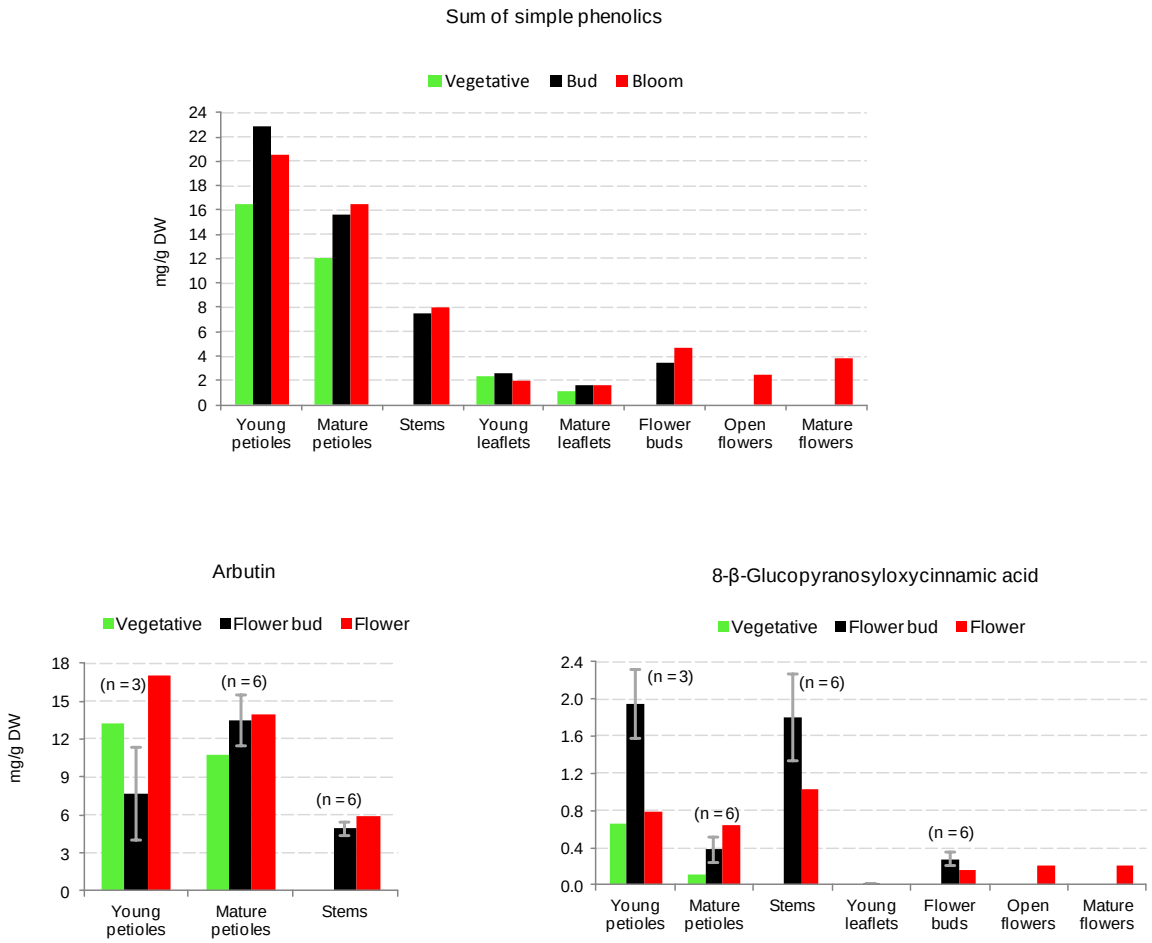

Figure 9. **Quantitative analysis of simple phenolics in various organs at different developmental stages of sainfoin.** Simple phenolic levels measured at vegetative (green), bud (black) and bloom stage (red) in sainfoin tissues were expressed in milligram per gram of freeze dried tissue.

Up: Sum of simple phenolics.

Down: Concentration of the most abundant compounds: arbutin and 8-β-glucopyranosyloxycinnamic acid. Error bars were calculated only for the bud stage and the repetition numbers are shown in each panel in parenthesis.

Low amounts of the last three compounds were measured. The unknown peaks with SP typical spectrum were named other SP derivatives (Table IA - IIIA in Appendix 3).

The hydroquinone arbutin was detectable only in petioles and stems (*Figure 9,* down panel on the left side of the page). It is quantitatively the most abundant compound and represented on average 83% in the SP levels in petioles and 70% in stems. Arbutin had also a high contribution in the total soluble phenolic levels in petioles and stems, on average with approximately 43% and 37%, respectively.

6.3.2. Hydroxycinnamic acids (HCA)

Five hydroxycinnamic acids were identified and quantified in different sainfoin tissues and for different developmental stages of sainfoin plant. These were: cis and trans p-coumaric acid 4-O-glucoside (quantified as one compound), and the trans isomers of 3-p-coumaroylquinic acid, caffeoylglucose, neochlorogenic and chlorogenic acid. The unknown peaks with HCA typical spectrum were named other HCA derivatives.

The upper panel in *Figure 10* shows that the highest HCA amounts were detected in leaflets. During the growth cycle the highest HCA levels were measured at the vegetative stage in young tissues and decreased with increasing maturity of the tissues. This decrease was mainly due to a reduction of neochlorogenic acid.

Inspection of the panels in *Figure 10*, down on the page shows qualitative and quantitative variations of the individual hydroxycinnamic acids in different tissues and different developmental stages of sainfoin:

- 3-p-coumaroylquinic acid was not detectable in young petioles at bloom stage and in open flowers;
- caffeoylglucose was not detectable in tissues at vegetative stage;
- chlorogenic acid could not be detected in petioles at vegetative stage and in tissues at bloom stage and in flowers;
- except in open and mature flowers, neochlorogenic acid was quantitatively the most abundant HCA in all tissues at all developmental stages. Its contents in young petioles and young and mature leaflets at bud stage were considerably lower than in tissues at the vegetative stage (60% reduction in petioles and 71%, in leaflets);
- in open and mature flower the most abundant HCA was p-coumaric acid 4-O-glucoside, followed by neochlorogenic acid, caffeoylglucose and 3-p-coumaroylquinic acid;
- the 3-p-coumaroylquinic acid concentration decreased considerable with the maturity stage;
- chlorogenic acid concentration was higher in tissues at bud stage than in tissues at vegetative stage.

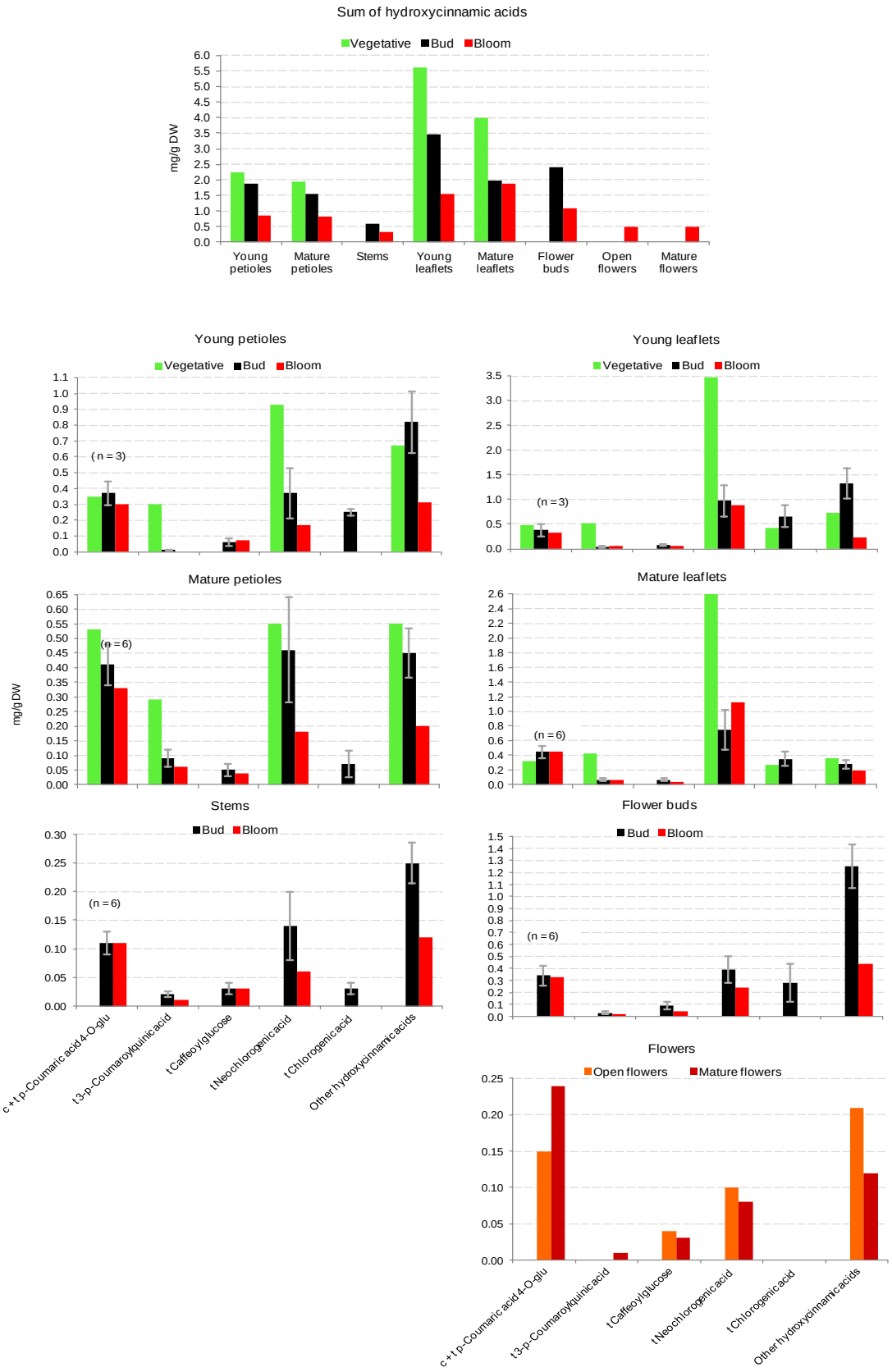

Figure 10. **Quantitative analysis of hydroxycinnamic acids in various organs at different developmental stages of sainfoin.** Hydroxycinnamic acid levels, measured at vegetative (green), bud (black) and bloom stage (red) in sainfoin tissues, were expressed in milligram per gram of freeze dried tissue.

Up: Sum of hydroxycinnamic acid compounds measured for all tissues.

Down: Concentration of individual compounds measured for each tissue. Error bars were calculated only for the bud stage and the repetition numbers are shown in each panel in parenthesis. Abreviations: c: cis; t: trans; glu: glucose.

6.3.3. Flavanols

The following flavanols were identified in the sainfoin tissues: the monomers catechin (C), epicatechin (E), gallocatechin (GC) and epigalocatechin (EGC) and the oligomers procyanidin B2, B5 and E-B5. Their concentration was determined for each developmental stage by RP-HPLC at 640 nm after derivatization with *p*-dimethylaminocinnamicaldehyde (DMACA). The unknown peaks from the same chromatograms were calculated as procyanidin B2 and were named other polymers flavanol derivatives.

The upper panel in *Figure 11* shows that the highest flavanols levels were measured in petioles, followed by leaflets, flowers and stems.

Young petioles accumulated the highest total amount of flavanols at bud stage. Of the four flavanols monomers present, the dihydroxylated B-ring compounds catechin and epicatechin reached together at bud stage 88%. Their concentration decreased with 11% at bloom stage. In contrast, at vegetative stage 73% of the monomeric flavanols were the trihydroxylated B-ring compounds gallocatechin and epigallocatechin. The cis flavanol isomers (-) epigallocatechin and (-) epicatechin were the predominant compounds at vegetative (74%) and bloom stage (61%), whereas at bud stage they represented together only 30% of the flavanols monomers.

Mature petioles showed the same trend except the fact that they accumulated the highest total amount of flavanols at the vegetative stage. Compared to the young tissues the proportion of the cis isomers increased with 6% at vegetative stage and 22% at bud and bloom stage.

Stems exhibited a similar flavanol pattern to the young petioles.

During plant development, young and mature leaflets accumulated the highest total amount of flavanols at vegetative stage. Of the four flavanol monomers present, young leaflets accumulated predominantly trihydroxylated B-ring compounds representing 96, 56 and 78% of the monomers at the vegetative, bud and bloom stage. In the mature leaflets, their proportion declined from 99 to 70% with plant development.

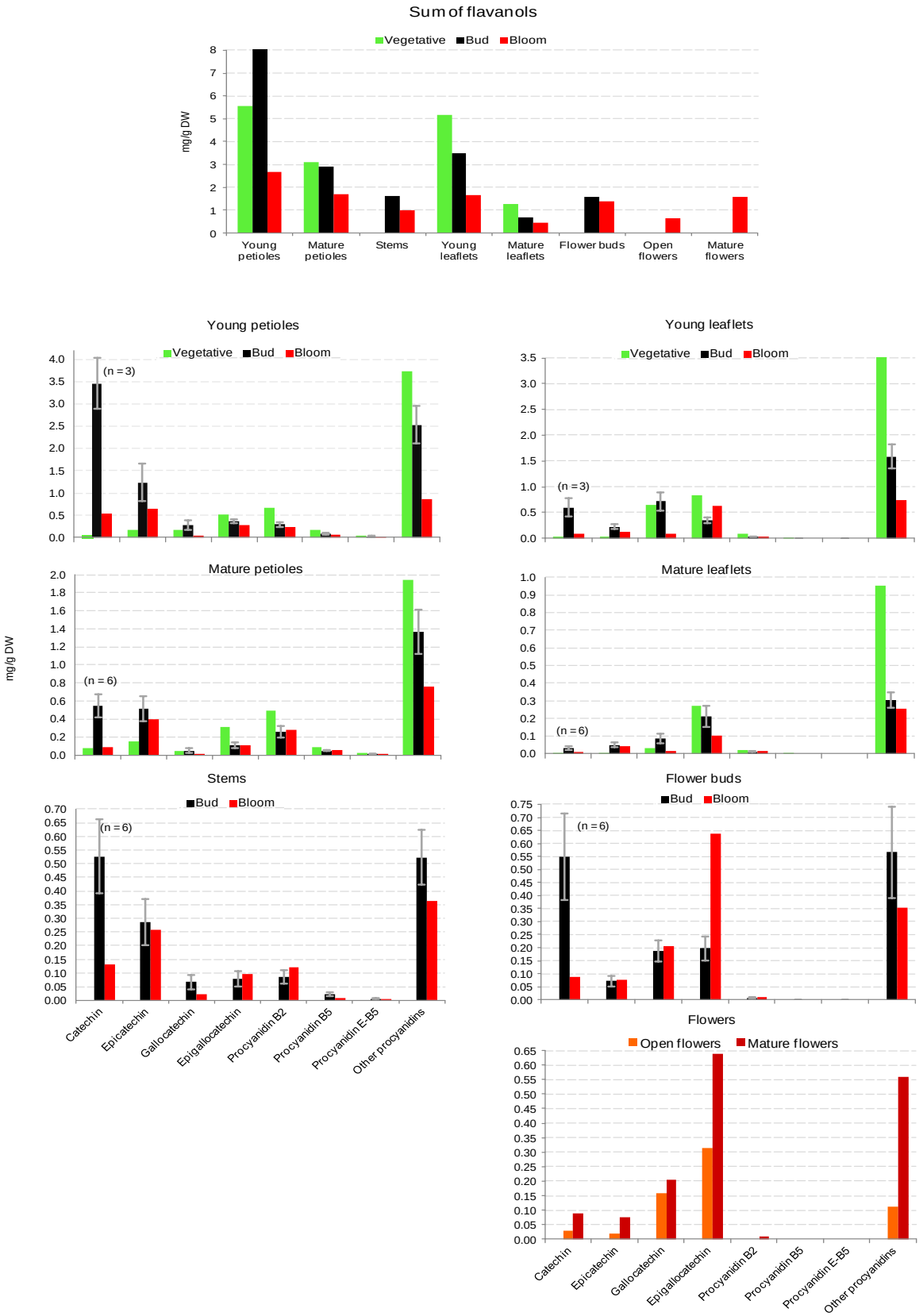

Figure 11. **Quantitative analysis of flavanols in various organs at different developmental stages of sainfoin.** Flavanols levels, measured at vegetative (green), bud (black) and bloom stage (red) in sainfoin tissues, were expressed in milligram per gram of freeze dried tissue.

Up: Sum of flavanols measured for all tissues.

Down: Concentration of flavanol compounds, measured for each tissue. Error bars were calculated only for the bud stage and the repetition numbers are shown in each panel in parenthesis.

The proportion of cis isomers increased with maturity of the leaflets from 56 to 89% at vegetative stage, 30 to 69% at bud stage and from 81 to 85% at bloom stage.

The last two panels drawn in *Figure 11*, on the right side down on the page shows the composition of catechin, epicatechin, gallocatechin and epigallocatechin in the flower tissues (buds, open and mature) of sainfoin plants. In the flower buds collected at the bud and bloom growth stage, the total concentration of the four monomers was similar, while a noticeable variation in their composition was measured with increasing plant maturity. Flower buds at the bloom stage and the open and mature (brown) flowers accumulated approximately 45% more trihydroxylated B-ring flavanols and 37% more cis isomers when compared with the flower buds collected at the bud stage.

Figure 12 shows the changes in the concentration of the polymers and of the two trihydroxylated B-ring monomers, gallocatechin and epigallocatechin measured in the young leaflets (left panel) and in the flower tissues of sainfoin (right panel) at different maturity stages.

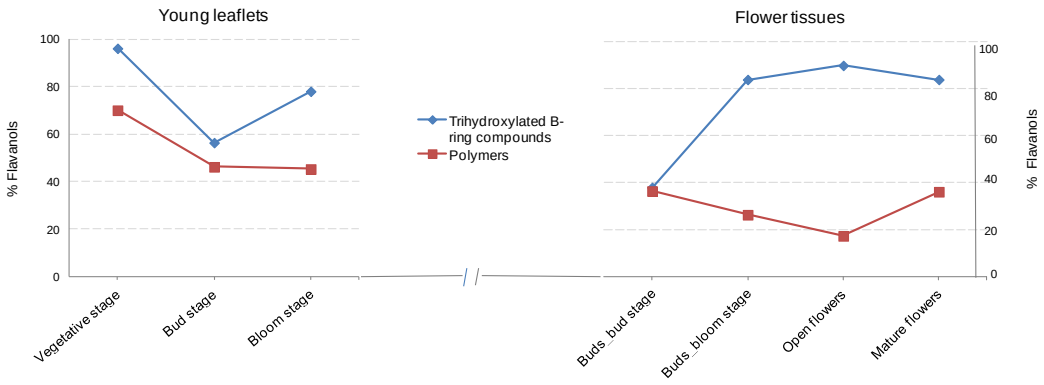

Figure 12. **Changes in the concentration of the two trihydroxylated B-ring monomers, gallocatechin and epigallocatechin and of the polymers measured in the young leaflets (left panel) and in the flower tissues (right panel) of sainfoin at different development stages.**

Left: Of the four monomers determined by HPLC, catechin, epicatechin, gallocatechin and epigallocatechin, the last two with trihydroxy-B rings (blue line) showed similar accumulation effect as the polymers (red line) in young leaflets of sainfoin at vegetative, bud and bloom stages.

Right: In the flower tissues of sainfoin at the bud and bloom stage, of the four monomers, the trihydroxy-B rings monomers proportion increased from 38 to 90% drawing an opposite line (blue) when compared to polymer accumulation (red line).

Of the total flavan-3-ol content measured in the leaflets and the petioles, the greatest polymer content was measured at the vegetative stage. Their accumulation decreased at the

bud stage and then increased slowly at the bloom stage. Of the four flavanol monomers present, a similar effect was seen for the two trihydroxylated B-ring monomers gallocatechin and epigallocatechin. In comparison to leaves and petioles, in the flower tissues, the polymer synthesis runs alinear to gallocatechin and epigallocatechin accumulations and increased as plant and flower maturity increased. The lower accumulation was measured in the open flowers and increased in the mature flowers with 20%.

6.3.4. Flavonols

Flavonols represent one of the predominant class of phenolic compounds identified in sainfoin and their levels and composition varied greatly depending on the plant developmental stage. Highest flavonol levels were found in the tissues collected from plants at the bud stage. For example, leaflets at the vegetative stage contained on average only 33% of the flavonols contained in leaflets at bud stage and 36% at bloom stage (*Figure 13*, left panel). The HPLC analysis showed that the high difference in the total flavonol amount is mainly due to an increasing rutin level which increased with the phenological stage. In the young leaflets at vegetative stage rutin represented 15% in the flavonol levels and its accumulation increased, representing 60% in the flavonol levels at bud stage and 73% at bloom stage (*Figure 13*, right panel). However, rutin accumulation increased also with increasing the maturity of the tissue.

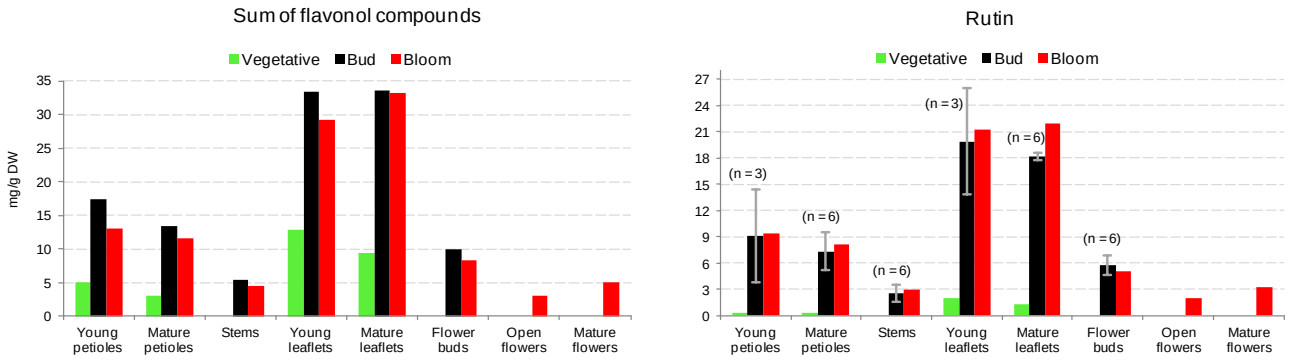

Figure 13. **Quantitative analysis of flavonols in various organs at different developmental stages of sainfoin.** Flavonols levels, measured at vegetative (green), bud (black) and bloom stage (red) in sainfoin tissues, were expressed in milligram per gram of freeze dried tissue.

Left: Sum of flavonols measured for all tissues.

Right: Concentration of the rutin. Error bars were calculated only for the bud stage and the repetition numbers are shown in each panel in parenthesis.

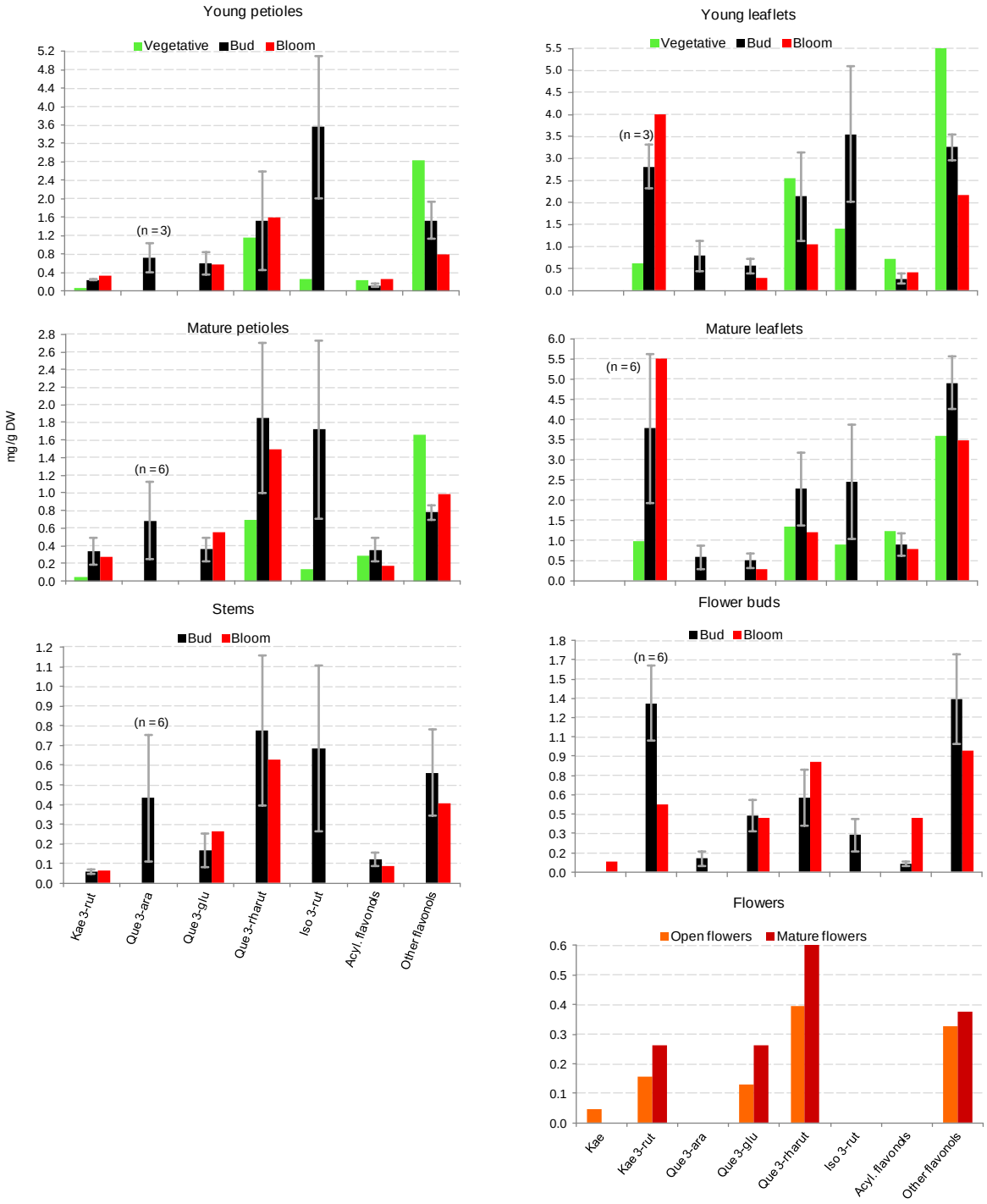

Figure 14. **Quantitative analysis of flavonol compounds in various organs at different developmental stages of sainfoin.** Flavonol compounds levels, measured at vegetative (green), bud (black) and bloom stage (red) in sainfoin tissues, were expressed in milligram per gram of freeze dried tissue.

Error bars were calculated only for the bud stage and the repetition numbers are shown in each panel in parenthesis.

Abbreviations: kae, kaempferol; rut, rutinoside; que, quercetin; ara, arabinoside; glu, glucoside; rharut, rhamnosylrutinoside; iso, isorhamnetin; acyl, acylated.

Kaempferol was accumulated only in flower organs. Quercetin 3-arabinoside was measured only in tissues at bud stage.

The flavonol compounds determined by HPLC in different sainfoin tissues were identified as: kaempferol, kaempferol 3-rutinoside (nicotiflorin), quercetin 3-arabinoside, quercetin 3-

glucoside (isoquercitrin), quercetin 3-rutinoside (rutin), quercetin 3-rhamnosylrutinoside and isorhamnetin 3-rutinoside. The unknown peaks with a flavonol typical spectrum were named other flavonol derivatives. Also peaks showing UV spectra that are typical to acylated flavonols (article 2, VEITCH et. al., 2011) were measured.

Inspection of the panels in *Figure 14*, shows qualitative and quantitative variations of the individual flavonol compounds in different tissues and different developmental stages of sainfoin. Furthermore, the relative high error bars calculated at bud stage underline the variations between individual plant (discussion available in article 1, REGOS et al., 2009).

In particular, among the variations measured at bud stage, it is worth highlighting those between individual plants, which can be deduced from the high standard deviation (Table IIA). *Figure 15* shows the flavonol profiles in the young leaflets of 3 different plants where the concentrations of rutin varied three fold and the presence of quercetin 3-arabinoside and isorhamnetin 3-rutinoside were found only in replicant 1 and 2.

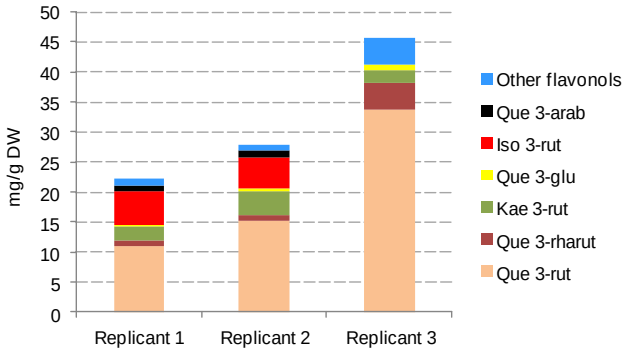

Figure 15. **Quantitative analysis (mg/g DW) of flavonol compounds in the young leaflets of 3 sainfoin plants.**
Abbreviations: kae, kaempferol; rut, rutinoside; que, quercetin; ara, arabinoside; glu, glucoside; rharut, rhamnosylrutinoside; iso, isorhamnetin.

6.3.5. Flavones

Flavone levels in various organs at different developmental stages of sainfoin are shown in *Figure 16*.

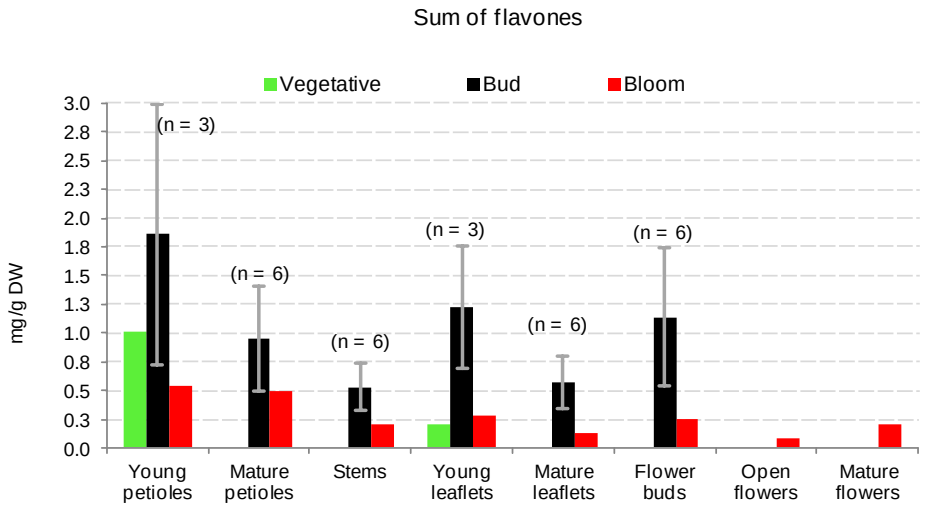

Figure 16. **Quantitative analysis of flavones in various organs at different developmental stages of sainfoin.** Falvones levels, measured at vegetative (green), bud (black) and bloom stage (red) in sainfoin tissues were expressed in milligram per gram of freeze dried tissues.

During the growth cycle the greatest flavone levels were measured at the bud stage.

At vegetative stage the highest flavone levels were measured in young petioles (1 mg/g DW) followed by young leaflets (0.2 mg/g DW). In mature leaflets and petioles no flavones were detectable.

At bud stage the highest flavone levels were measured in young petioles (1.8 mg/g DW) followed by young leaflets (1.2 mg/g DW), flower buds (1.1 mg/g DW) and stems (0.5 mg/g DW). Mature leaflets and petiols had only approximately 50% of the flavones contained in the young tissues. At bud stage the flavones levels for young petioles and leaflets are 46% and 84% higher than in the same tissues at vegetative stage.

At bloom stage the flavone levels were similar in young and mature petioles (0.5 mg/g DW). In contrast, mature leaflets had only 50% of the flavones contained in young leaflets (0.28 mg/g DW). Flower buds had flavone levels similar to those in mature flowers (0.2 mg/g DW) and twice as much as in flowers (0.1 mg/g DW). Flavone levels in tissues at bloom stage were 69% lower than in tissues at bud stage, on average.

6.3.6. Anthocyanins

Anthocyanins were identified in petioles, stems and flowers of sainfoin at bud and bloom stage (*Figure 17*).

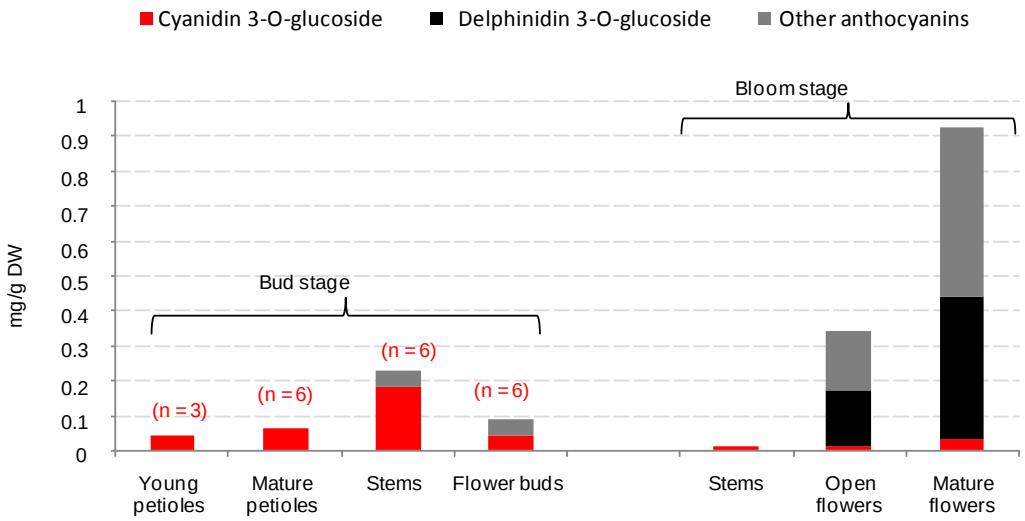

Figure 17. **Quantitative analysis of anthocyanins in various organs at different developmental stages of sainfoin.**

At bud stage (left side of the panel) the cyanidin 3-O-glucoside was detected in stems (0.18 mg/g DW), young and mature petioles (0.04; 0.06 mg/g DW) and flower buds (0.04 mg/g DW). In stems and flower buds additional unknown anthocyanin compounds (named as other anthocyanins) were detected and had similar concentration in both tissues (0.05 mg/g DW).

At bloom stage (right side of the panel) the cyanidin 3-O-glucoside concentration in stems was 25% lower than in stems at bud stage and was not detectable in petioles and flower buds. In open and mature flowers low amounts of cyanidin 3-O glucoside (0.01, 0.03 mg/g DW) were measured. These tissues accumulate higher levels of delphinidin 3-O-glucoside (0.16; 0.4 mg/g DW) and other anthocyanins were also detected (0.17; 0.5 mg/g DW).

Anthocyanin levels in mature flowers were three times higher than in open flowers.

6.4. Conclusions

Screening of the aerial parts of sainfoin plants (*O. viciifolia*) with different ontogenetic stages discovered a large variation in phenolic contents and composition. Furthermore, an organ-specific accumulation tendency was observed. These are important findings because phenolic structures and concentrations have been linked with the anti-parasitic effects of sainfoin.

The exact information about phenolic compound formation lays a cornerstone for their physiological evaluation and is also important for producers to optimize the harvesting period of sainfoin.

7. GENERAL CONCLUSIONS AND FUTURE

There is a considerable body of evidence showing a link between the consumption of sainfoin and reducing nematode (e.g. *Haemonchus contortus*) parasitism in ruminant's guts. Polyphenols including condensed tannins, flavanols and flavonols are the compounds that have been associated with these benefits by affecting several key biological processes of the worm. Although the exact mechanism(s) of action of the compounds involved is still obscure numerous researches in this area have associated the variability in their results with the variability in phenols structure and quantity (HOSTE et al., 2011).

In this study, the soluble low molecular phenolic compounds from sainfoin were investigated and their variation in content and composition of 37 diverse sainfoin accessions was explored. The phenolic profile of the aerial parts of sainfoin plants (*O. viciifolia*) with different ontogenetic stages was also evaluated. Accessions displayed a wide range of differences and the metaboloms of organs from different individuals showed that the phenolic composition of plants within one variety differs not only quantitatively but also qualitatively even when grown at the same place. To maximise the potential benefits to ruminants from sainfoin, plant breeding should focus on developing varieties with predictable polyphenol profiles.

Aqueous acetone extracts of the aerial parts of *O. viciifolia* (var. Cotswold Common) yielded amino acids, alkaloids, phenolic acids, dihydroflavonols, flavone-C-glucosides, flavanols, flavonols and isoflavones. After isolation and purification, their structures were elucidated combining chemical, chromatographic, and spectroscopic methods. Most of these substances have not been described hitherto in this plant material and eight were new compounds.

BRUNET and HOSTE (2006) and BRUNET et al. (2008) showed that not only condensed tannins but also the constitutive monomers have anthelmintic effects. Monomers of prodelphinidins were more active than those of procyanidins. More severe effects were also

found with galloyl derivatives. The results shown suggest that the number of free hydroxy groups of CT monomers is a key factor in interactions with parasite larvae.

KOUPAI-ABYAZANI et al. (1992) identified in sainfoin the monomers catechin (cat), epicatechin (epi), gallocatechin and epigallocatechin and the dimeric procyanidin B2 [epi-(4β→8)-epi]. Recovery of these compounds and the identification of further dimeric [procyanidins B3 (cat-(4α→8)-cat), B4 (cat-(4α→8)-epi) and B5 (epi-(4β→6)-epi)] and trimeric flavanols [procyanidins C1 (epi-(4β→8)-epi-(4β→8)-epi) and E-B5 (epi-(4β→6)-epi-(4β→6)-epi)] was carried out in this work by mass spectrometry and HPLC with post column derivatization with DMACA.

MANOLARAKI (2011) confirmed the anthelmintic activity of the flavonol glycosides kaempferol 3-rutinoside (nicotiflorin), quercetin 3-rutinoside (rutin), and isorhamnetin 3-rutinoside (narcissin) found by Barrau et al. (2005) and demonstrated that the aglycone kaempferol, quercetin and isorhamnetin are more active than the glycoside ones. Of the three glycosides, rutin was more active than narcissin and nicotiflorin.

Of the flavonols previously reported in sainfoin: the aglycones kaempferol and quercetin, the 3-O-rhamnoside of myricetin, the 3-O-rutinosides of kaempferol, quercetin, myricetin and isorhamnetin, and three trisaccharides, the branched 3-O-rhamnosylrutinosides of quercetin and kaempferol and quercetin 3-O-rutinoside-7-O-glucoside (MARAIS et al., 2000; LU et al., 2000), only the last compound and quercetin could not been determined during these work. Further flavonol glycosides reported here for the first time in *O. viciifolia* were the monoglycosides: kaempferol 3-O-glucoside, quercetin 3-O-rhamnoside, quercetin 3-O-glucoside, and myricetin 3-O-rhamnoside; the 3- and 3,7-diglycosides: kaempferol 3-O-rhamnogalactoside and kaempferol 3-O-glucoside-7-rhamnoside; and the isorhamnetin 3-O-rhamnosylrutinoside and quercetin 3-glucuronide.

Furthermore, during the characterisation of the phenolic metabolome of sainfoin, ten acylated flavonol glycosides were investigated. Among these were eight previously unreported examples which comprised either feruloylated or sinapoylated derivatives of 3-O-di- and 3-O-triglycosides of kaempferol or quercetin. Three different patterns of acylation

were found. The diglycosides were acylated at the primary Glc residue of *O*-α-Rha*p*(1→6)-β-Glc*p* (rutinose), whereas the triglycosides were acylated at the terminal Rha residues of the branched trisaccharides, *O*-α-Rha*p*(1→2)[α-Rha*p*(1→6)]-β-Gal*p* or *O*-α-Rha*p*(1→2)[α-Rha*p*(1→6)]-β-Glc*p*. Identification of the primary 3-*O*-linked hexose residues as either Gal or Glc was carried out by negative ion electrospray and serial MS, and cryoprobe NMR spectroscopy. Two of the ten acylated flavonol glycosides had previously been reported only from methanol extracts of the whole plant of the legume, *Vicia amurensis* (KANG et al., 2000). They were: *quercetin 3-O-α-rhamnopyranosyl-(1'''→2'')[(3''''-O-E-feruloyl)-α-rhamnopyranosyl-(1''''→6'')]-β-galactopyranoside* also known as amurenoside (A) ***(1)*** *and quercetin 3-O-α-rhamnopyranosyl-(1'''→2'')[(2''''-O-E-feruloyl)-α-rhamnopyranosyl-(1''''→6'')]-β-galactopyranoside,* known as amurenoside B ***(2)***. The complete structures of the eight new compounds were: *kaempferol 3-O-α-rhamnopyranosyl-(1'''→2'')[(2''''-O-E-feruloyl)-α-rhamnopyranosyl-(1''''→6'')]-β-galactopyranoside (**3**), quercetin 3-O-(4'''-O-E-feruloyl)-α-rhamnopyranosyl-(1'''→2'')[α-rhamnopyranosyl-(1''''→6'')]-β-glucopyranoside (**4**), quercetin 3-O-(4'''-O-E-sinapoyl)-α-rhamnopyranosyl-(1'''→2'')[α-rhamnopyranosyl-(1''''→6'')]-β-glucopyranoside (**5**), kaempferol 3-O-(4'''-O-E-feruloyl)-α-rhamnopyranosyl-(1'''→2'')[α-rhamnopyranosyl-(1''''→6'')]-β-glucopyranoside (**6**), quercetin 3-O-α-rhamnopyranosyl-(1'''→6'')-[(2''-O-E-sinapoyl)-β-glucopyranoside] (**7**), quercetin 3-O-α-rhamnopyranosyl-(1'''→6'')-[(2''-O-E-feruloyl)-β-glucopyranoside] (**8**), kaempferol 3-O-α-rhamnopyranosyl-(1'''→6'')-[(2''-O-E-sinapoyl)-β-glucopyranoside] (**9**) and kaempferol 3-O-α-rhamnopyranosyl-(1'''→6'')-[(2''-O-E-feruloyl)-β-glucopyranoside] (**10**).* Although the aerial parts of sainfoin have been found to contain the parent flavonol glycosides, no acylated derivatives were previously reported. As such, the characterisation of **1–10** is a significant contribution to the description of the phenolic metabolome of this important fodder legume, the full elucidation of which is critical to understanding its beneficial nutritional and veterinary properties. Analysis of HPLC retention time, UV/Vis spectral information, mass fragmentation patterns and the accurate

estimates of mass of the identified phenolics provided additional diagnostic features relevant to direct characterisation of these compounds in hyphenated analyses.

HPLC using ultraviolet (UV) detection was established as the most convenient method for providing valuable insights into the distribution of phenolics in plants utilizing their feature to absorb UV light. With easily upwards of 5,000 compounds reported, full characterisation of a plant biophenol extract is a challenging analytical exercise. Article 3 aimed to improve analyte's separation of the in house existing HPLC method and to optimize it's use for the characterisation of complexe mixtures.

More than 50 standard substances from 10 different classes (hydroxycinnamic acids, flavanols, flavonols, anthocyanins, etc.) and a complex phenolic extract of *O. viciifolia* showed better resolution on a pentafluorophenylpropyl (PFP) column when compared with a bifunctional phase constituted of octadecyl and phenylpropyl bonded silica and three conventional C18 columns. In particularly, it is worth highlighting the better resolution of early eluting phenolic compounds such as hydroquinones, hydroxybenzoic acids, hydroxycinnamic acids and flavanols which gave longer elution ranges on the PFP stationary phase compared to conventional columns.

A HPLC method with sensitive diode array and chemical reaction detection using the perfluorophase was validated and applied for the analysis of an *O. viciifolia* sample. This is the first report of quantitative determination of low molecular phenolic compounds of *O. viciifolia*. Although fluorinated stationary phases were claimed to offer many utilities that could not be accomplished by conventional C8, C18, and phenyl phases and have shown novel selectivity and enhanced retention for several compound classes (ZHANG, 2008), no applications for biophenols were previously reported. As such, the optimization of an HPLC method with sensitive diode array and chemical reaction detection using the perfluoropase for the analysis of biophenols is a significant contribution in the chromatography.

Furthermore this method was successfully used to investigate the phenolic profile of the aerial parts of sainfoin plants (*O. viciifolia*) with different ontogenetic stages. All tested aerial parts contained phenolic compounds and showed organ-specific composition. The exact

information about phenolic compound formation lays a cornerstone for their physiological evaluation and is also important for producers to optimize the harvesting period of sainfoin. The metabolomes of organs from different individuals show that the phenolic composition of plants within one variety differs not only quantitatively but also quantitatively even when grown on the same place. This indicates that the commonly used sainfoin varieties may not yet be homogeneous with respect to their content of bioactive secondary metabolites. Therefore, if the beneficiary 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.

Screening of 37 diverse sainfoin accessions discovered a huge variation in phenolic contents and composition. Principal component analysis revealed that flavonols and flavanols are the most relevant variables for discrimination of the accessions. The influence of genetic factors in the condensed tannins content among the same accessions has been demonstrated by STRIGANO et al. (2012). Anthelmintic data are also available. MANOLARAKI (2011) evaluated the anthelmintic effect of the same accessions and explored how these variations relate to quantitative and /or qualitative changes in phenolic compounds like CTs, flavanols or flavonols. The results obtained confirmed the existence of anthelmintic variability within the accessions. The AH activity of the investigated accessions might relate to the total flavanols, total anthocyanidins, total flavonols, but also to some other small phenolic compounds such as the cinammic and coumaric acids. Such information will help to develop a successful breeding program for sainfoin variety with potential anthelmintic benefits.

8. SUMMARY

Phenolic compounds of sainfoin (*Onobrychis viciifolia* Scop.; Leguminosae subfamily Papilionoidae) are assumed to be responsible for its nutritional and veterinary benefits.

This way, the low molecular weight phenolic compounds from sainfoin have been investigated. By combining chemical, chromatographic and spectroscopic methods, a wide range of diverse phenolic compounds were investigated for the first time in sainfoin. Among these were eight previously unreported compounds which comprised either feruloylated or sinapoylated derivatives of 3-O-di- and 3-O-triglycosides of kaempferol or quercetin.

A high performance liquid chromatography (HPLC) method with diode array detection (DAD) and post column derivatization with p-dimethylaminocinnamicaldehyde (DMACA) using pentafluorophenylpropyl (PFP) phase was optimized to investigate more than 50 standard substances from 10 different classes and was validated for the analysis of individual phenolic compounds from a sainfoin plant extract. Compared to C18 and bifunctional phases, PFP column showed longer elution ranges and better resolution of early eluting phenolic compounds such as hydroquinones, hydroxybenzoic and hydroxycinnamic acids and flavanols.

Furthermore this method was used to investigate the phenolic profile of the aerial parts of sainfoin plants (*O. viciifolia*) with different ontogenetic stages. It was found that all plant parts contained phenolic compounds and showed organ-specific composition.

The metabolomes of organs from different individuals showed that the phenolic composition of plants within one variety differs not only quantitatively but also qualitatively even when grown on the same place.

Accessions were found to be highly variable in their phenolic content and composition. Statistical analysis showed that flavonols and flavan-3-ols are the most relevant variables for discrimination of the accessions.

9. ZUSSAMENFASSUNG

Es wird vielfach angenommen, dass die positive Wirkung von Esparsette (*Onobrychis viciifolia* Scop.; Leguminosae subfamily Papilionoidae) auf die in ihr enthaltenen Polyphenolen zurückzuführen sei.

In dieser Arbeit sollten ihre phenolischen Inhaltsstoffe mit niedrigem Molekulargewicht untersucht werden. Anhand von verschiedenen chemischen, chromatographischen und spektroskopischen Methoden konnten eine Vielzahl an verschiedenen phenolischen Verbindungen in Esparsette erstmals näher charakterisiert und bestimmt werden. Darüber hinaus konnten acht bisher unbekannte Verbindungen identifiziert werden. Es handelt sich um acylierte Flavonole, bei denen entweder Ferulasäure oder Sinapinsäure an 3-O-di- und 3-O-tri-Glukosides des Kämpferols oder Quercetins gebunden sind.

Anhand von mehr als 50 Standardsubstanzen aus 10 unterschiedlichen Stoffklassen wurde eine Methode entwickelt, die Hochdruck-Flüssigkeits-Chromatographie (HPLC) mit Diodenarray-detection (DAD) und Nachsäulen-Derivatisierung mit p-Dimethylaminozimtsäurealdehyd (DMAZA) unter Verwendung von Pentafluorophenylpropyl (PFP) als Festphase kombiniert und anschließend speziell für die Untersuchung von phenolischen Verbindungen eines Esparsette Pflanzenextraktes validiert. Verglichen mit C18- und biofunktionale Säulen weist sich die PFP-Säule durch längere Elutionszeiten und eine bessere Trennung besonders bei früh eluierten phenolischen Verbindungen wie Hydroquinone, Hydroxybenzoesäuren, Hydroxyzimtsäuren und Flavanolen aus.

Mit Hilfe dieser Methode wurden phenolische Profile von oberirdischen Organen der Esparsette (*O. viciifolia*) in unterschiedlichen Entwicklungsstadien analysiert. Dabei zeigte sich, dass alle Pflanzenteile phenolische Verbindungen mit organspezifischen Phenolmustern aufweisen.

Verschiedene Individuen unterscheiden sich innerhalb einer Sorte nicht nur quantitativ sondern auch qualitativ in ihren Phenolmustern, selbst wenn sie am gleichen Standort kultiviert wurden.

Generell zeigte sich eine hohe Variabilität im Phenolgehalt und Phenolmuster, was anhand von statischen Analysen auf starke Schwankungen im Bereich der Flavanole und Flavan-3-ole zurückzuführen ist.

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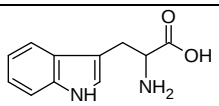
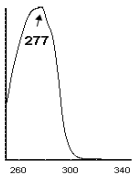
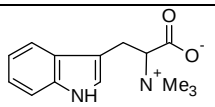
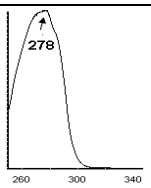
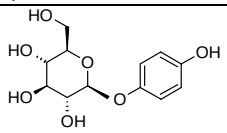
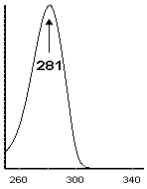
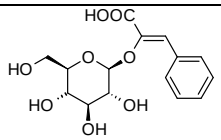
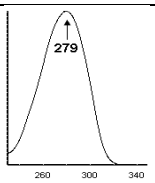
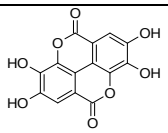
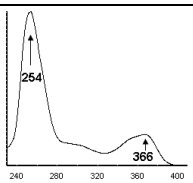
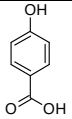
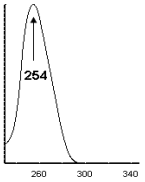
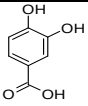
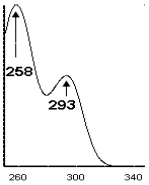
APPENDIX 1

Structure and UV spectra of phenolic and other natural compounds identified in plant material of *Onobrychis viciifolia*

Table 1A. Structure and UV spectra of phenolic and other natural compounds identified in plant material of *Onobrychis viciifolia* during the present work and from past phytochemical studies.

Compound	*Chemical structure*	*UV spectrum*
	amino acids and alkaloids	
[1]L-tryptophan (1)		
[1]hypaphorine = N,N,N-trimethyltryptophan		
	simple phenolic acids	
[1]arbutin = 4-(β-D-glucopyranosyloxy)phenol (1)		
[1]8-β-glucopyranosyloxycinnamic acid (2)		
[1]ellagic acid		
	hydroxybenzoic acids	
[1]p-hydroxybenzoic acid		
[1]protocatechuic acid		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
(1) Compound identified in air-dried leaves and stems of *O. viciifolia* (var. Cotswold Common), (MARAIS et al., 2000).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).

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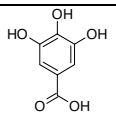
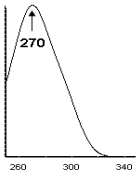
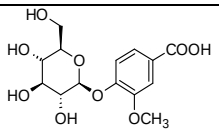
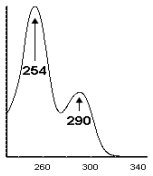
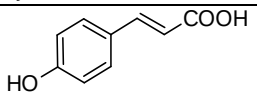
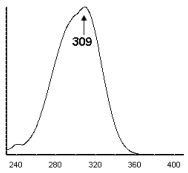
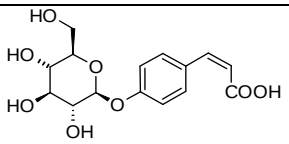
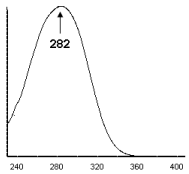
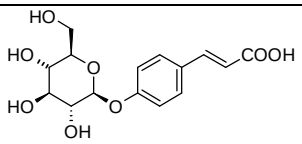
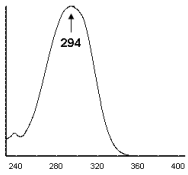
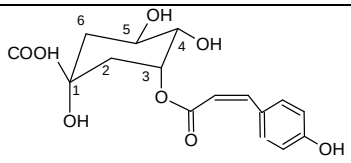
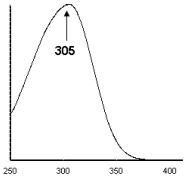
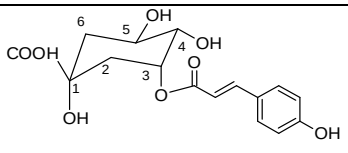
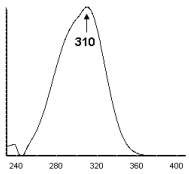
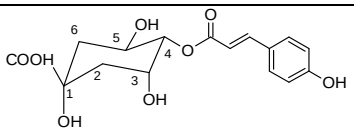
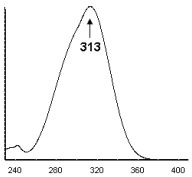
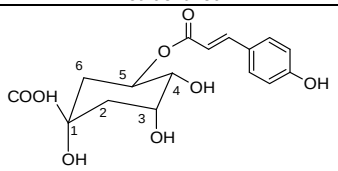
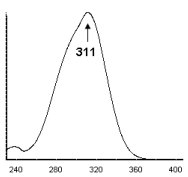
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[1]gallic acid		
[1]vanillic acid 4-O-glucoside		
hydroxycinnamic acids		
[1]*trans* p-coumaric acid		
[1]*cis* p-coumaric acid 4-O-glucoside		
[1]*trans* p-coumaric acid 4-O-glucoside (2)		
[1]*cis* 3-p-coumaroylquinic acid (2)		
[1]*trans* 3-p-coumaroylquinic acid (2)		
[1]*trans* 4-p-coumaroylquinic acid		
cis 5-p-coumaroylquinic acid (2)	Not identified	
[1]*trans* 5-p-coumaroylquinic acid (2)		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).

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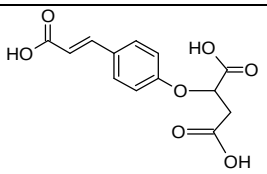
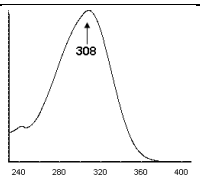
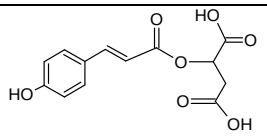
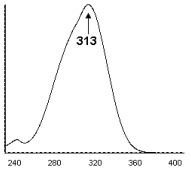
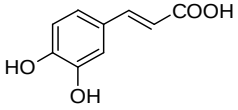
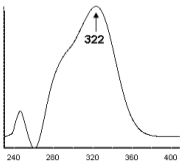
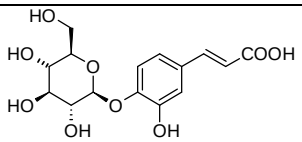
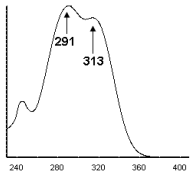
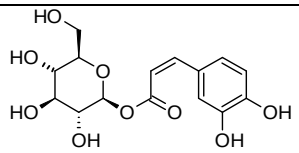
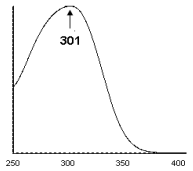
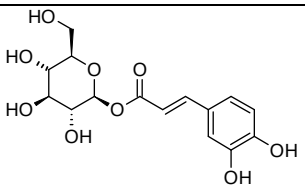
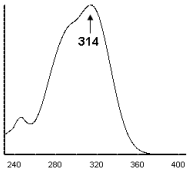
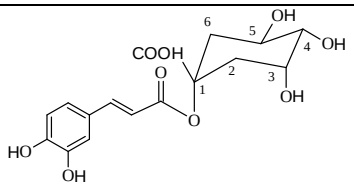
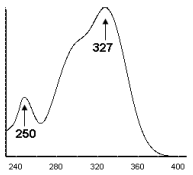
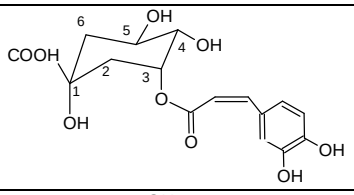
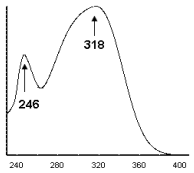
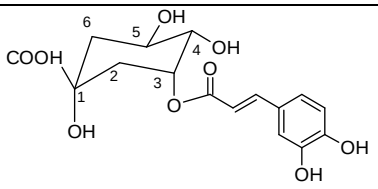
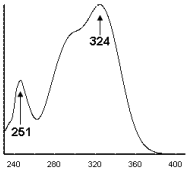
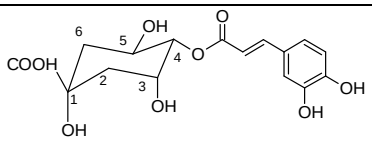
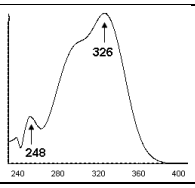
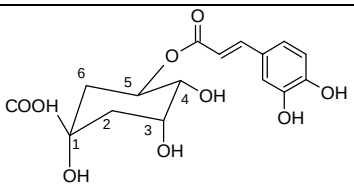
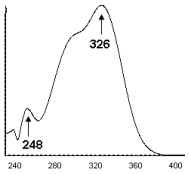
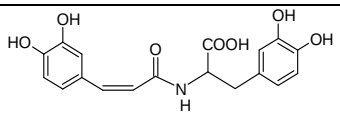
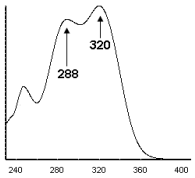
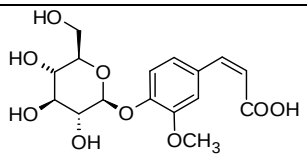
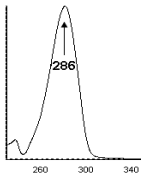
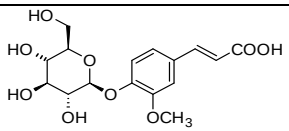
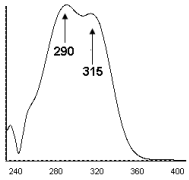
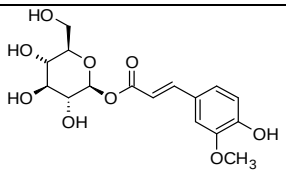
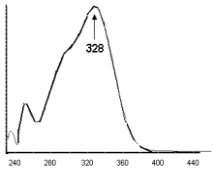
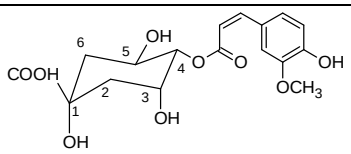
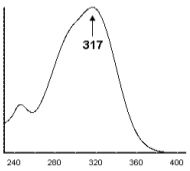
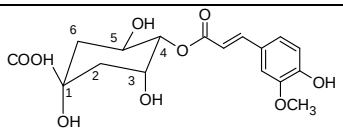
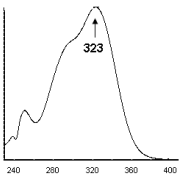
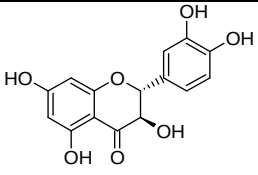
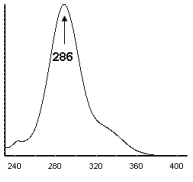
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[1]*trans* p-coumaric acid 4-O-malate		
[1]*trans* p-coumaroylmalic acid		
cis & trans methyl 6-p-coumaroylglucoside (2)	not identified	
[1]*trans* caffeic acid		
[1]*trans* caffeic acid 4-*O*-glucoside		
[1]*cis* caffeoylglucose		
[1]*trans* caffeoylglucose		
[1]*trans* 1-caffeoylquinic acid		
[1]*cis* 3-caffeoylquinic acid		
[1]*trans* 3-caffeoylquinic acid		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).

(continued on next page)

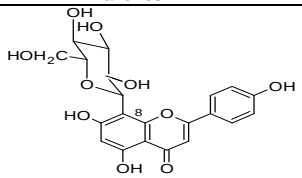
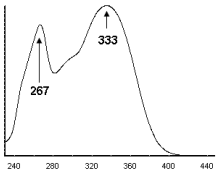
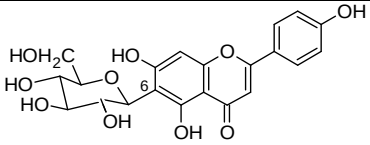
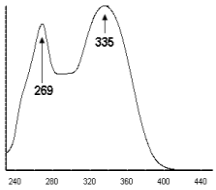
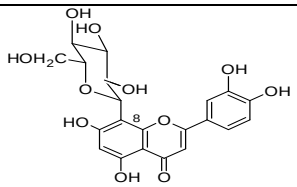
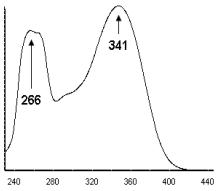
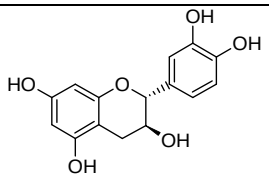
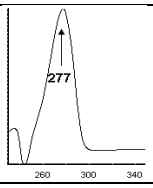
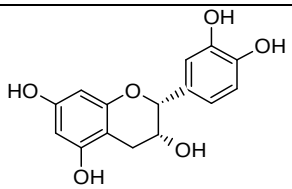
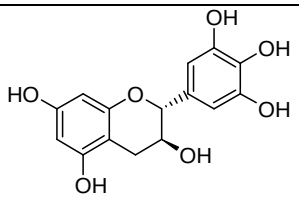
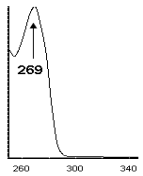
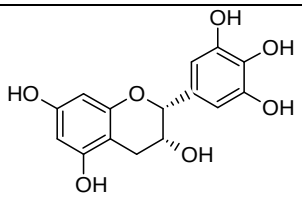
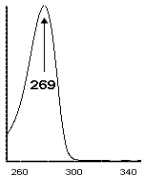
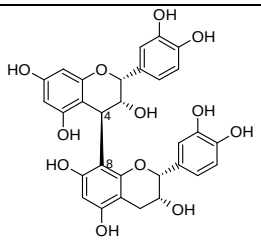
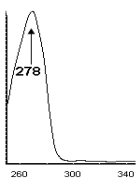
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[1]*trans* 4-caffeoylquinic acid		
[1]*trans* 5-caffeoylquinic acid (2)		
[1]caffeoyl-DOPA		
[1]*cis* ferulic acid 4-O-glucoside		
[1]*trans* ferulic acid 4-O-glucoside		
[1]*trans* feruloylglucose		
[1]*cis* 4-feruloylquinic acid		
[1]*trans* 4- feruloylquinic acid		
dihydroflavonols		
[1]dihydroquercetin		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).

(continued on next page)

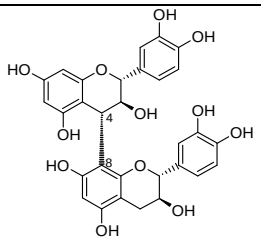
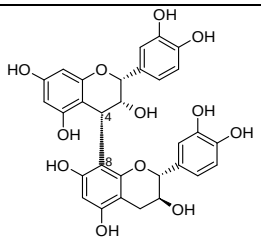
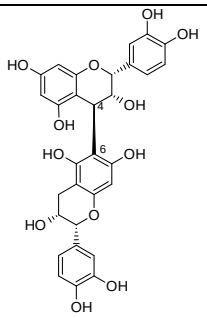
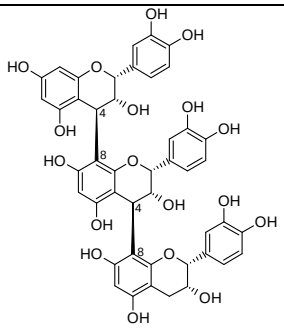
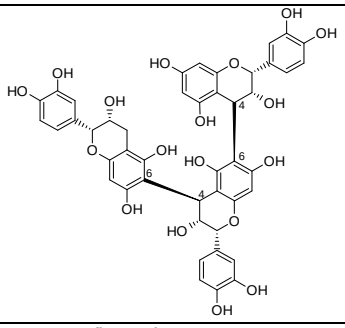
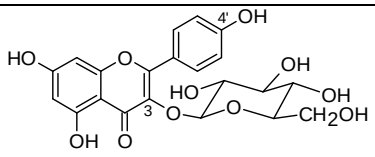
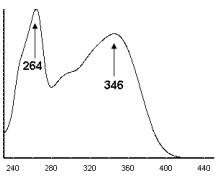
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
	flavones	
[1]vitexin = apigenin 8 *C*-glucoside		
[1]isovitexin = apigenin 6 *C*-glucoside		
[1]orientin = luteolin 8 *C*-glucoside		
chrysoeriol 4'-O-β-D-glucoside (2)	not identified	
chrysoeriol 4'-O-(6"-O-acetyl)-β-D-glucoside (2a)	not identified	
	flavanols	
[1]catechin (cat) (3)		
[1]epicatechin (epi) (3)		
[1]gallocatechin (3)		
[1]epigallocatechin (3)		
[1]procyanidin B2 = epi-(4β→8)-epi (3)		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).
(3) Compound identified in leaves of *O. viciifolia* (var. Melrose), (KOUPAY-ABIAZANI et al., 1992).

(continued on next page)

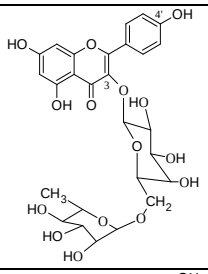
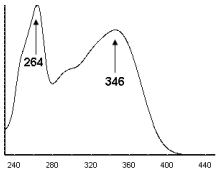
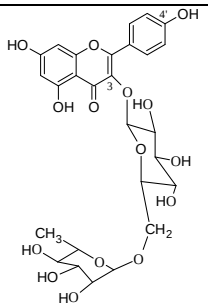
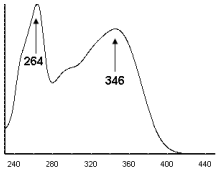
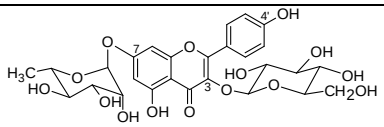
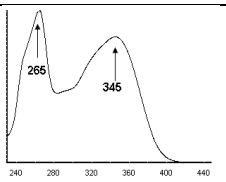
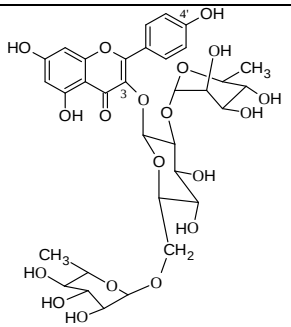
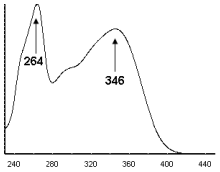
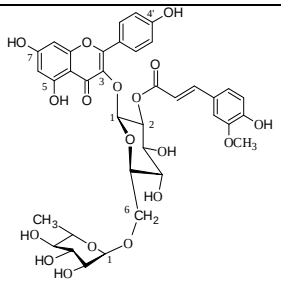
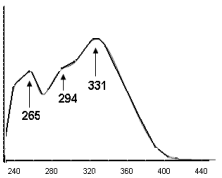
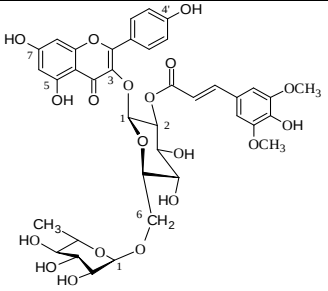
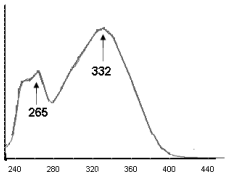
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[1]procyanidin B3 = cat-(4α→8)-cat		
[1]procyanidin B4 = cat-(4α→8)-epi		
[1]procyanidin B5 = epi-(4β→6)-epi		
[1]procyanidin C1 = epi-(4β→8)-epi-(4β→8)-epi		
[1]procyanidin E-B5 = epi-(4β→6)-epi-(4β→6)-epi		
	flavonols	
kaempferol (1)	not identified	
[1]astragalin = kaempferol 3-*O*-glucoside		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
(1) Compound identified in air-dried leaves and stems of *O. viciifolia* (var. Cotswold Common), (MARAIS et al., 2000).

(continued on the next page)

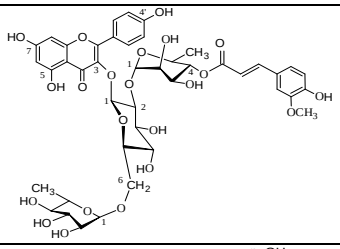
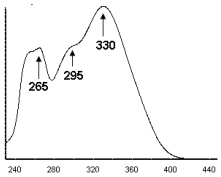
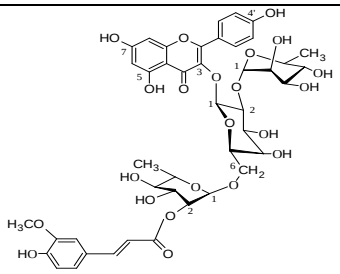
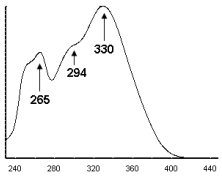
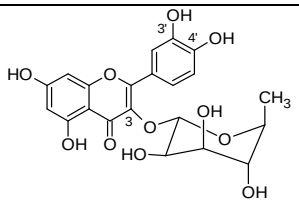
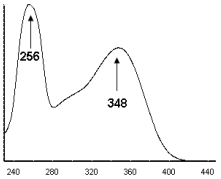
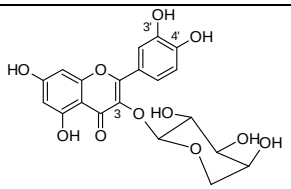
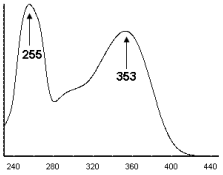
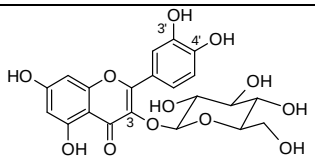
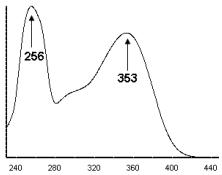
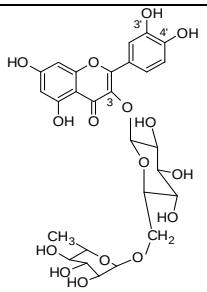
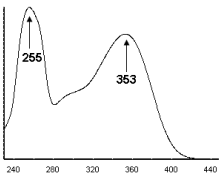
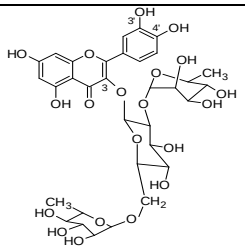
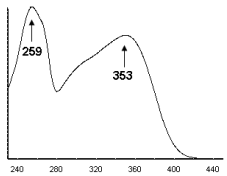
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[1]kaempferol 3-O-rhamnogalactoside		
[1]nicotiflorin = kaempferol 3-*O*-rutinoside (1), (2)		
[1]kaempferol 3-*O*-glucoside-7-O-rhamnoside		
[1]kaempferol 3-*O*-rhamnosylrutinoside (2)		
[2]kaempferol 3-*O*-α-rhamnopyranosyl-(1→6)-[(2-*O*-*E*-feruloyl)-β-glucopyranoside]		
[2]kaempferol 3-*O*-α-rhamnopyranosyl-(1→6)-[(2-*O*-*E*-sinapoyl)-β-glucopyranoside]		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
[2] Chemical, chromatographic and spectroscopic data are shown by VEITCH et al. (2011).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).

(continued on the next page)

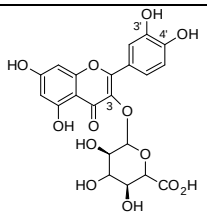
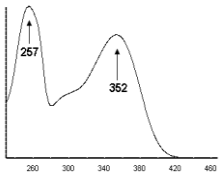
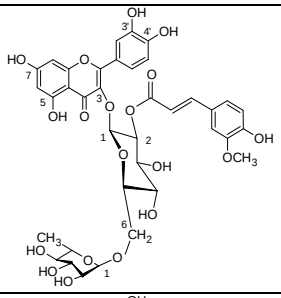
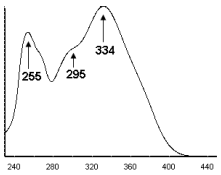
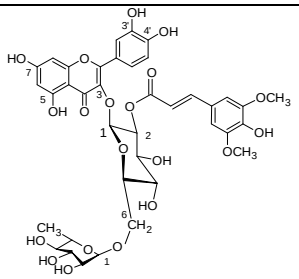
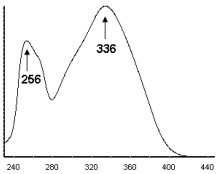
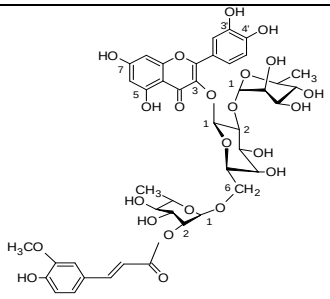
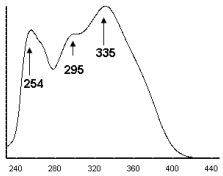
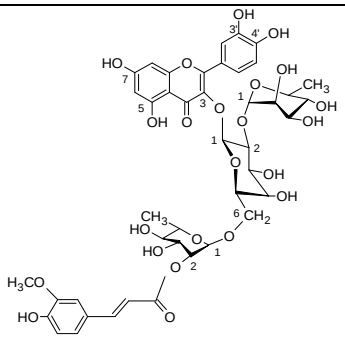
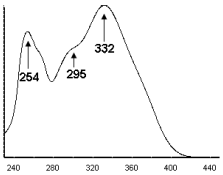
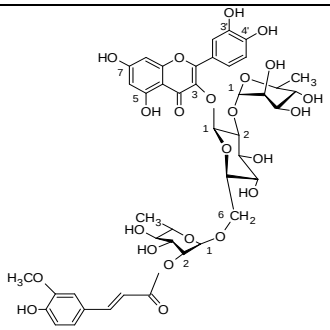
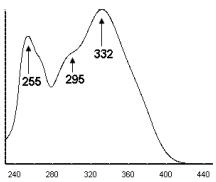
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[2]kaempferol 3-*O*-(4-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1→2)[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside		
[2]kaempferol 3-*O*-α-rhamnopyranosyl-(1→2)[(2-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1→6)]-β-galactopyranoside		
quercetin (1)	Not identified	
[1]quercitrin = quercetin 3-*O*-rhamnoside		
[1]avicularin = quercetin 3-*O*-arabinoside		
[1]isoquercitrin = quercetin 3-*O*-glucoside		
[1]rutin = quercetin 3-*O*-rutinoside (1), (2)		
[1]quercetin 3-*O*-rhamnosylrutinoside (1)		
quercetin 3-O-rutinoside-7-O-glucoside (2)	not identified	

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
[2] Chemical, chromatographic and spectroscopic data are shown by VEITCH et al. (2011).
(1) Compound identified in air-dried leaves and stems of *O. viciifolia* (var. Cotswold Common), (MARAIS et al., 2000).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).

(continued on next page)

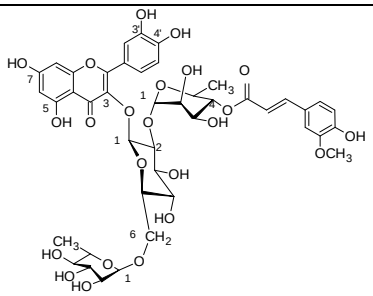
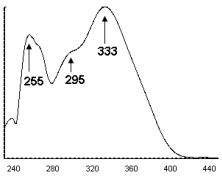
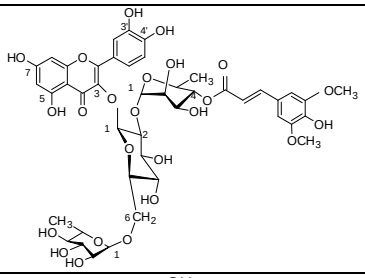
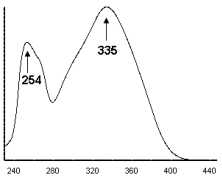
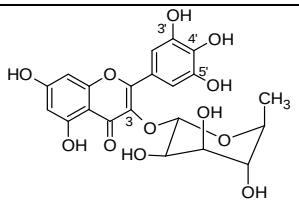
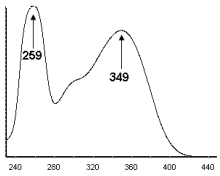
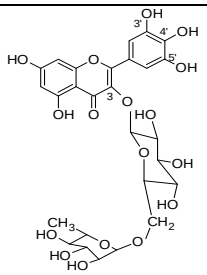
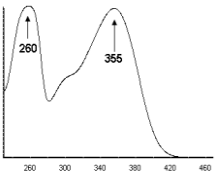
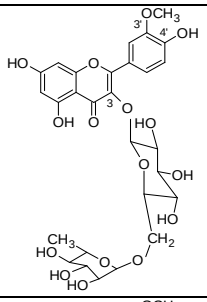
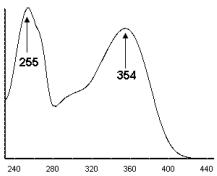
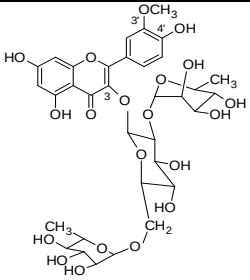
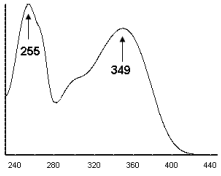
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[1]quercetin 3-glucuronide		
[2]quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-[(2-*O*-*E*-feruloyl)-β-glucopyranoside]		
[2]quercetin 3-*O*-α-rhamnopyranosyl-(1→6)-[(2-*O*-*E*-sinapoyl)-β-glucopyranoside]		
[2]amurenoside A = quercetin 3-*O*-α-rhamnopyranosyl-(1→2)[(3-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1→6)]-β-galactopyranoside		
[2]amurenoside B = quercetin 3-*O*-α-rhamnopyranosyl-(1→2)[(2-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1→6)]-β-galactopyranoside		
[2]quercetin 3-*O*-α-rhamnopyranosyl(1→2)[(2-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1→6)]-β-glucopyranoside		

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
[2] Chemical, chromatographic and spectroscopic data are shown by VEITCH et al. (2011).

(continued on next page)

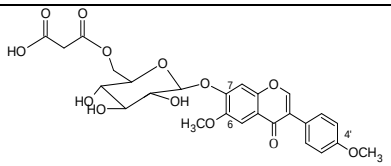
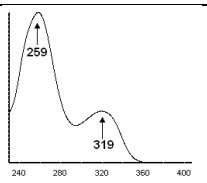
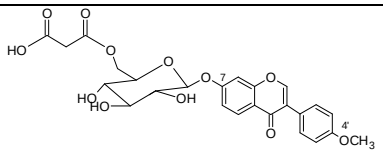
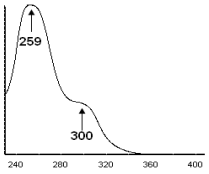
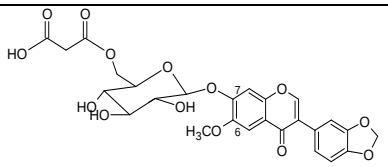
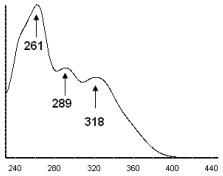
Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[2]quercetin 3-*O*-(4-*O*-*E*-feruloyl)-α-rhamnopyranosyl-(1→2)-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside		
[2]quercetin 3-*O*-(4-*O*-*E*-sinapoy)-α-rhamnopyranosyl-(1→2)-[α-rhamnopyranosyl-(1→6)]-β-glucopyranoside		
[1]myricitrin = myricetin 3-*O*-rhamnoside (2)		
[1]myricetin 3-*O*-rutinoside (2)		
[1]narcissin = isorhamnetin 3-*O*-rutinoside (2)		
[1]isorhamnetin 3-*O*-rhamnosylrutinoside		
isoflavones		
afrormosin (4a)	not identified	

[1] Chemical, chromatographic and spectroscopic data are shown by REGOS et al. (2009).
[2] Chemical, chromatographic and spectroscopic data are shown by VEITCH et al. (2011).
(2) Compound identified in leaves of *O. viciifolia* (unspecified variety), (LU et al., 2000).
(4a) Compound identified in healthy leaves of *O. viciifolia* (unspecified variety), (INGHAM, 1978).

(continued on next page)

Table 1A. (continued)

Compound	*Chemical structure*	*UV spectrum*
[3]afrormosin 7-*O*-(malonylglucoside)		
formononetin (4a)	not identified	
[3]formononetin 7-*O*-(malonylglucoside)		
[3]fujikinetin 7-*O*-(malonylglucoside)		
isoflavans		
vestitol (4b)	not identified	
isoflavanones		
vestitone	not identified	
chalcones		
isoliquiritigenin (4b)	not identified	
flavones		
liquiritigenin (4b)	not identified	
garbanzol (4b)	not identified	
pterocarpans		
medicarpin (4b)	not identified	

[3] Chemical, chromatographic and spectroscopic data are shown in Table 2, chapter 3.
(4a) Compound identified in healthy leaves of *O. viciifolia* (unspecified variety), (INGHAM, 1978).
(4b) Compound identified in leaves of *O. viciifolia* (unspecified variety) inoculated with the fungus *Helminthosporium carbonum* (INGHAM, 1978).

APPENDIX 2

Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin)

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Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin)

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Screening tool

ABSTRACT

Onobrychis viciifolia (sainfoin) is a traditional fodder legume showing multiple benefits for the environment, animal health and productivity but weaker agronomic performance in comparison to other legumes. Benefits can be mainly ascribed to the presence of polyphenols. The polyphenol metabolism in *O. viciifolia* was studied at the level of gene expression, enzyme activity, polyphenol accumulation and antioxidant activity. A screening of 37 accessions regarding each of these characters showed a huge variability between individual samples. Principal component analysis revealed that flavonols and flavan 3-ols are the most relevant variables for discrimination of the accessions. The determination of the activities of dihydroflavonol 4-reductase and flavonol synthase provides a suitable screening tool for the estimation of the ratio of flavonols to flavan 3-ols and can be used for the selection of samples from those varieties that have a specific optimal ratio of these compounds for further breeding.

1. Introduction

Legumes provide a rich source of proteins and polyphenols (Aoki et al., 2000) and can therefore play a major role in the nutrition of humans and animals. Most of the commonly used fodder legumes, such as alfalfa, lupin and a number of clover species cause bloat, which is a cause of considerable economic risk in ruminant farming (Majak et al., 1995). There are, however, a few non-bloating legumes, including sainfoin (*Onobrychis viciifolia*), cicer milkvetch (*Astragalus cicer*) and bird's-foot trefoil (*Lotus corniculatus*). These species are currently not competitive with alfalfa in terms of yield, re-growth and persistence in the stand. Sainfoin is a traditional fodder legume, which was replaced by other legume forages in the middle of the 20th century, due to low productivity, and problems with establishment (Borreani et al., 2003) despite showing multiple benefits on environment and animal health (Hayot Carbonero et al., 2011; Marais et al., 2000). Consumption of sainfoin by many ruminant species leads to increased absorption of amino acids and reduced urinal N-excretion and therefore, to a reduced nitrogen emission (Scharenberg et al., 2007). In addition, methane emission is reduced, one of the major gases that are associated with global warming. Furthermore sainfoin is an excellent food source for bees and other pollinators (Hayot Carbonero et al., 2011).

Abbreviations: 5-DLCy, 5-deoxyleucocyanidin; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; BSA, bovine serum albumin; BUT, butin; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; DHF, dihydrofisetin; DHK, dihydrokaempferol; DHM, dihydromyricetin; DHQ, dihydroquercetin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTE, dithioerythritol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ERI, eriodictyol; F3′H, flavonoid 3′-hydroxylase; FGT, flavonoid glucosyl-transferase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; GAPDH, glycerinaldehyde 3-phosphate dehydrogenase; GAR, garbanzol; HAC, hierarchical cluster analysis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase; LIQ, liquiritigenin; MeOH, methanol; NADPH, nicotinamide adenine dinucleotide phosphate; NAR, naringenin; OMT, *O*-methyl transferase; ORF, open reading frame; PAL, phenylalanine ammonia-lyase; PCA, principal component analysis; PEG, polyethylene glycol; PHF, pentahydroxyflavanone; PKR, polyketide reductase; POX, peroxidase; RACE, rapid amplification of cDNA ends; SAM, *S*-adenosylmethionine; TLC, thin layer chromatography.

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Sainfoin's benefits can mainly be ascribed to its polyphenols (Ingham, 1978; Koupai-Abyazani et al., 1993; Lu et al., 2000; Marais et al., 2000; Mueller-Harvey, 2006; Regos et al., 2009; Theodoridou et al., 2010; Yeap Foo et al., 1982). The effects are dependent on both the concentration and the composition. Polyphenols show antiparasitic properties; in particular anthelminthic effects against nematodes are well established (Barrau et al., 2005; Heckendorn et al., 2006; Hoste et al., 2005). Flavonols are the predominant polyphenols in sainfoin (Regos et al., 2009) and have been shown to inhibit the larval migration of nematodes (Heckendorn et al., 2006; Hoste et al., 2005; Manolaraki et al., 2010). In addition, proanthocyanidins (synonyms: condensed tannins, polymeric flavan 3-ols) are found in sainfoin, which are absent in many other common fodder legumes (Li et al., 1996). The beneficial effect of proanthocyanidins with respect to bloat prevention is ascribed to their ability to form complexes with proteins in the rumen of the animals (Jones and Lyttleton, 1971; Jones and Mangan, 1977). Apart from the very commonly occurring polyphenol classes, a number of isoflavonoids and 5-deoxy(iso)flavonoids could be found, which are typical for legumes (Ingham, 1978) and are known to act as phytoalexins.

Whilst the agronomic performance of other legumes like lucerne and clover has benefitted from extensive breeding programmes, very few breeding programmes have been undertaken for sainfoin (Hayot Carbonero et al., 2011). The existing sainfoin germplasm is highly diverse in its agronomic behaviour (Hayot Carbonero et al., 2011, 2012), morphology and in the polyphenol spectrum (Regos et al., 2009). Therefore, identification of suitable germplasm resources with a known polyphenol spectrum is a promising approach for future breeding of sainfoin varieties with improved agronomic performance whilst maintaining the beneficial effects. A collection of 360 sainfoin accessions at the National Institute of Agricultural Botany (NIAB, Cambridge, UK) was recently evaluated (Hayot Carbonero et al., 2011). A subset of 37 accessions was preselected based on their agronomic performance (Hayot Carbonero, 2011). The accessions were sourced from Europe, Eastern block countries, North America and Asia, and accessions were chosen from this large group to ensure a good cover of countries, with particular emphasis in including environmental conditions. In addition, the three main types were included: the so called single cut or 'common' types, the so called double cut or giant' types and those that were a mixture of the two. Finally attention was paid to good condition in terms of homogeneity or uniformity, within the context of an outbreeding species. Proanthocyanidin composition and anthelmintic data are also available (Manolaraki, 2011; Stringano et al., 2012). This offered the opportunity to study the polyphenol metabolism in terms of gene expression, enzyme activity, antioxidative activity and polyphenol composition, and to identify possible germplasm screening tools. The variety 'Cotswold Common' was chosen as a standard, since already a lot of information about this variety from previous growing trials was available, such as assurance in terms of distinctness, uniformity and stability. Furthermore seeds of this variety are commercially available.

2. Results/discussion

2.1. Determination of polyphenol enzymes in O. viciifolia

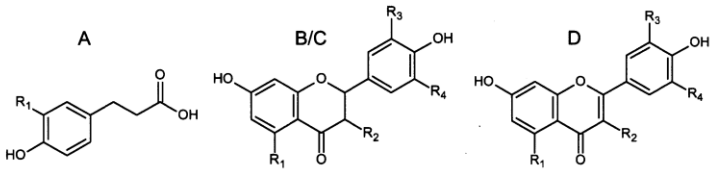
A large variety of polyphenols, namely hydroxycinnamic acids, chalcones, flavanones, dihydroflavonols, flavonols, flavan 3-ols, anthocyanins, and a number of isoflavonoids and 5-deoxyflavonoids were identified in *O. viciifolia* (Ingham, 1978; Koupai-Abyazani et al., 1993; Lu et al., 2000; Marais et al., 2000; Regos et al., 2009; Yeap Foo et al., 1982), which are present in various glycosylated and methylated forms (Lu et al., 2000; Regos et al., 2009). Therefore the presence of the main enzymes of the polyphenol pathway as well as modifying enzymes was expected in tissues of *O. viciifolia*. Preparations from *O. viciifolia*, which were obtained by standard methods (Claudot and Drouet, 1992; Stich et al., 1992) showed no or only low enzyme activities. An optimised extraction method for polyphenol rich material (Dellus et al., 1997) could be successfully adapted for tissues of *O. viciifolia* (refer to Section 3.5) and provided preparations showing high activities for all tested enzymes. The activity of phenylalanine ammonia-lyase (PAL), chalcone synthase/chalcone isomerase (CHS/CHI), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), flavonol synthase (FLS) and flavonoid glucosyl-transferase (FGT) was determined in 11 different tissues of *O. viciifolia* line Cotswold Common: young leaves (still folded), fully developed leaves, young petiols, middle-aged petiols, stems, young flowerstalks, flower buds, open flowers, senescent flowers, unripe seeds, and seedstalks (Table 1). Furthermore the activity of *O*-methyltransferase (OMT) was determined in young leaves. In general, young leaves (still folded) showed the highest enzyme activities and were therefore used as standard material for further investigations. The enzymatic reactions of PAL, CHS/CHI, FHT, DFR, FLS, FGT and OMT were optimised regarding pH optimum, temperature stability and optimum, linearity with time and protein concentration, and resulted in the standard enzyme assays for *O. viciifolia*, described in Section 3.6. IFS activity could not be determined in preparations from leaves, but was demonstrated successfully with microsomes prepared from seedlings of *O. viciifolia*, which had been treated 6 h before harvest with glutathione, an elicitor for the formation of flavonoids (Jung et al., 2000). Flavonoid compounds of three different B-ring hydroxylation patterns were reported to be present in *O. viciifolia* (Regos et al., 2009; Stringano et al., 2012). However, the activity of the microsomal enzymes flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H) activity could not be demonstrated in this study despite many attempts. It is likely that this is due to the high polyphenol content which negatively affects the stability of membrane associated enzymes during preparation.

The enzymes from plant crude extracts were tested for their substrate acceptance (Suppl. Table 1). This included also intermediates for 5-deoxyflavonoid formation, because 5-deoxy(iso)flavonoids such as liquiritigenin, garbanzol, vestiton, vestitol, afrormosin, and formononetin were identified in *O. viciifolia* (Ingham, 1978). Structures of the substrates that were used are shown in Fig. 1.

CHS accepted *p*-coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA and feruloyl-CoA as substrates. Highest conversion rates were observed with *p*-coumaroyl-CoA, which was also preferred, when offered together with a second substrate in equimolar amounts. FHT accepted flavanones of all tested hydroxylation patterns, but converted 5-hydroxyflavanones to a greater extent than 5-deoxyflavanones. DFR was not able to convert DHK to leucopelargonidin by enzyme preparations from *O. viciifolia* indicating the presence of a DFR with narrow substrate specificity as reported for other plants such as tobacco and petunia (Forkmann, 1993; Forkmann and Ruhnau, 1987). This is in agreement with the fact that sainfoin only accumulates anthocyanidins and proanthocyanidins with a 3′,4′- or 3′,4′,5′-hydroxylation pattern, whereas the flavonols carry 1–3 hydroxyl groups in the B-ring. The 5-deoxydihydroflavonols garbanzol and dihydrofisetin were accepted even to a higher extent than DHQ. Since 5-deoxyflavonoids such as garbanzol and liquiritigenin have been identified in *O. viciifolia* (Ingham, 1978), these compounds are natural substrates for the *O. viciifolia* DFR. However, in contrast to DFR, FLS showed a low acceptance of 5-deoxydihydroflavonols reflecting the lack of 5-deoxyflavonols in *O. viciifolia*. DHK was the preferred substrate of FLS. It is obvious that this favours the formation of flavonols at the expense of flavan 3-ol

Table 1
Total protein contents and specific activities of six enzymes of the polyphenol pathway in different tissues of *O. viciifolia*. Plants were grown in the green house, except a few field grown samples marked with * (n.d.: not detectable, FW: fresh weight).

Tissue	Protein content [μg/mg FW]	Specific enzyme activity [nkat/kg protein]					
		PAL	CHS	FHT	DFR	FLS	FGT
Young leaves	9.6; 25.4*	184*	3220*	334*	349	190	59,102
Developed leaves	6.5; 17.4*	20*	307*	59*	0	190	11,715*
Young petiols	9.9	328	614	663	145	505	n.d.
Middle-aged petiols	12.0	241	1745	578	368	n.d.	14,833
Young flower stalks	7.7	555	860	179	561	n.d.	21,267
Stems	9.3	421	605	0	43	n.d.	6818
Seed stalks	11.1	242	626	103	0	n.d.	8483
Unripe seeds	14.6	307	1044	367	56	n.d.	9465
Flower buds	14.9	162	1533	557	76	123	16,263
Open flowers	9.0	34	852	207	0	201	12,130
Senescent flowers	13.5	94	1246	467	172	n.d.	13,224



Compound	Type	R_1	R_2	R_3	R_4
p-coumaric acid	A	H			
ferulic acid	A	CH_3O			
caffeic acid	A	OH			
naringenin	B	OH	H	H	H
eriodictyol	B	OH	H	OH	H
pentahydroxyflavanone	B	OH	H	OH	OH
liquiritigenin	β	H	H	H	H
butin	B	H	H	OH	H
dihydrokaempferol	C	OH	OH	H	H
dihydroquercetin	C	OH	OH	OH	H
dihydromyricetin	C	OH	OH	OH	OH
garbanzol	C	H	OH	H	H
dihydrofisetin	C	H	OH	OH	H
kaempferol	D	OH	OH	H	H
quercetin	D	OH	OH	OH	H
myricetin	D	OH	OH	OH	OH
5-deoxykaempferol	D	H	OH	H	H
fisetin	D	H	OH	OH	H

Fig. 1. Structures of hydroxycinnamic acids (A), flavanones (B), dihydroflavonols (C), and flavonols (D).

and anthocyanidin formation. While DFR and FLS compete for dihydroquercetin and dihydromyricetin as common substrates, DHK is exclusively available for flavonol formation. This provides an explanation for the high flavonol content in *O. viciifolia*, which represents up to 75% of the phenolic compounds in young leaves (Regos et al., 2009). An overview of the pathway created from the obtained results is summarised in Fig. 2.

2.2. Isolation of structural genes involved in the polyphenol biosynthesis

cDNAs containing the full coding regions of CHS, CHI, FHT, DFR, FLS, leucoanthocyanidin reductase (LAR), anthocyanidin synthase (ANS), anthocyanidin reductase (ANR), isoflavone synthase (IFS), isoflavone 7-*O*-methyltransferase (IOMT) and polyketide reductase (PKR) of *O. viciifolia* were isolated. Attempts to isolate the cDNA of IFS from young leaves failed. Therefore, the full open reading frame (ORF) of IFS as well as of PKR and a putative IOMT were isolated using cDNA prepared from seedlings. Furthermore a 422 bp cDNA fragment of putative PAL (GenBank ID HM204487), a 300 bp cDNA fragment of FGT (GenBank ID HQ896307) and a 313 bp cDNA fragment of F3′H (GenBank ID HQ896306) were isolated. Using the sequences from the isolated cDNAs, primers were designed for the quantitative expression studies of 9 structural genes of the polyphenol metabolism in 37 samples.

2.3. Functional activity and characterisation of the recombinant enzymes

The cDNA clones were heterologously expressed to obtain the recombinant enzymes. *Escherichia coli* was chosen as expression system whenever possible (*CHS*, *CHI*, *FHT*, *FLS*, *LAR*, *ANS*, *ANR*). Enzymes, which are typically not or less active when heterologously expressed in *E. coli*, were expressed in yeast (*DFR*, *IFS*, *IOMT*). Functional activity could be demonstrated for all recombinant enzymes with exception of *CHI* and *OMT*. The assays were optimised and

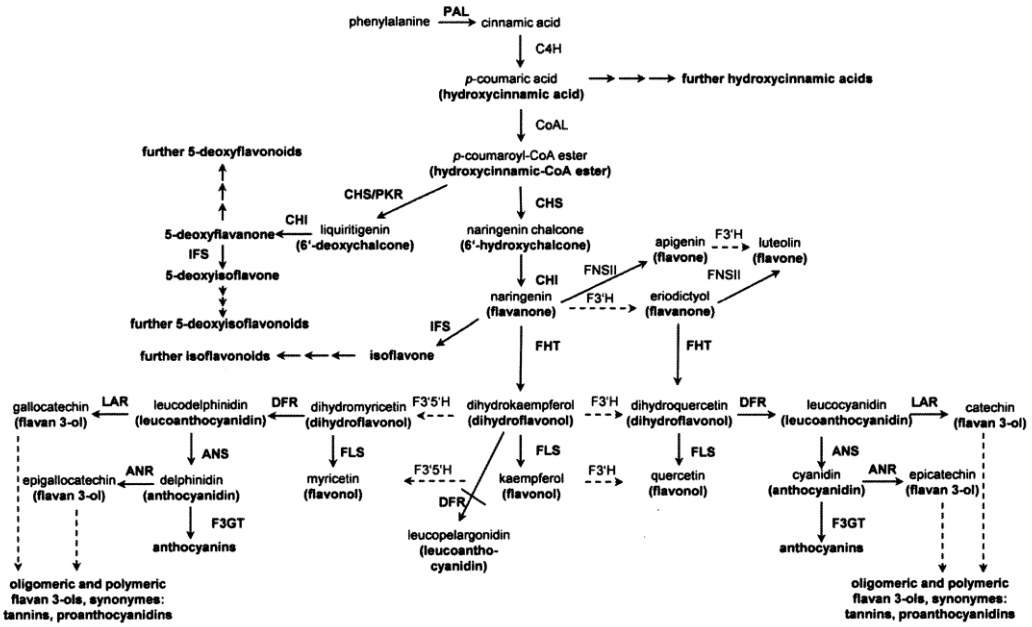


Fig. 2. Summary of the biosynthetic pathway of polyphenols in *O. viciifolia* as established by the current combined studies of gene expression, enzyme activity and metabolites analysis. Bold letters indicate enzymes established and determined in this study either by activity measurement or gene expression studies. Broken arrows indicate not established enzymes. Measurement of the microsomal enzymes F3′H and F3′5′H was not possible, propably due to the high polyphenol content. *Abbreviations:* CoAL, CoA ligase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase; F3GT, flavonoid 3-*O*-glucosyl-transferase; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; FNSII, flavone synthase II; IFS, isoflavone synthase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia-lyase; PKR, polyketide reductase.

standard enzyme assays were adapted accordingly as described in detail in the Section 3.7. The recombinant proteins generally showed similar substrate acceptance as the plant crude extracts, with the exception of recombinant DFR, which showed a low conversion of DHK although it was not accepted by DFR from plant preparations. Structure activity studies suggested that the amino acid in position 134 (*Gerbera* numbering, GenBank ID CAA78930) is a relevant factor for DHK acceptance. DFRs showing an aspartate at position 134 often do not accept DHK as substrate, whereas asparagine or alanine at position 134 seems to result in DHK preference (Fischer et al., 2003; Johnson et al., 1999, 2001; Schlangen et al., 2010; Seitz et al., 2007; Wellmann et al., 2006). The isolated DFR from *O. viciifolia* contains aspartate at position 134, therefore it was expected that it does not convert DHK as a substrate as confirmed by the assays with preparations from leaves. Actually the recombinant protein clearly preferred DHQ over DHK, but a low conversion of DHK could be observed at pH 6.0–7.0. This could be owed to the sometimes unnatural substrate acceptance of recombinant proteins (Fischer et al., 2003; Lukacin et al., 2003; Schlangen et al., 2010; Wellmann et al., 2006) or support indications that the amino acid in position 134 is not the only decisive factors (Petit et al., 2007).

2.4. Screening of O. viciifolia accessions

Samples from 37 accessions (Table 2) were investigated regarding the polyphenol contents, antioxidant capacities, peroxidase activity, selected polyphenol enzyme activities and corresponding gene expressions.

Content and composition of soluble phenolics in sainfoin samples were determined by HPLC and are listed in Table 3. The identified compounds and the unknown peaks from the chromatograms were grouped into five classes based on the maximum UV–VIS: flavan 3-ols, flavonols, hydroxycinnamic acids, simple phenolics and flavones. Considerable variations in the composition and quantity of soluble phenolics among the different samples were observed. Total phenolic contents ranged from 16 to 43 mg/g freeze-dried sainfoin (sample 1163 vs. 1213). Hierarchical cluster analysis (HCA) was performed to examine the relationships between each accession according to their production of phenolic compounds. The samples were distributed among two clear major clusters referred to as groups I and II (Fig. 3). Group I contained 27 samples with high phenolic amount (>27 mg/g DW) and had three subgroups: Ia (from 27 to 33 mg/g DW), Ib (from 33 to 38 mg/g DW) and Ic (from 38 to 43 mg/g DW), respectively. Group II comprised the remaining 10 accessions with low phenolic amounts (from 16 to 27 mg/g DW).

Flavan 3-ol content varied from 0.5 to 11 mg/g DW (sample 1017 vs. 1199) and included monomers (catechin and epicatechin, simple and galloylated derivatives) and soluble polymers known also as proanthocyanidins. Flavonols are the most abundant class of flavonoids in sainfoin (e.g. about 85% in sample 1213) and their contents varied from 8 to 36 mg/g DW (sample 1163 vs. 1213). Glycosides of mono- and dihydroxylated flavonols (kaempferol and quercetin) as well as acylated flavonol glycosides were analysed in all samples (Table 3). The methylated flavonol isorhamnetin was identified only in several samples bound to glucose or rutinose. Except sample 1017 which is drawing a chromatogram with many peaks giving a spectrum characteristic to flavonol

Table 2
Sainfoin samples. All accessions were *O. viciifolia* with the exception of 1264, which was *O. antasiatica*. (na: not available).

Accession number	Variety	Country of origin
1001	Cotswold Common	UK
1007	na	China
1012	Ambra	Italy
1013	Somborne	UK
1017	Teruel	Spain
1019	Taja	Poland
1026	Buciansky	Slovakia
1028	Simpro	France
1041	Camaras	Romania
1043	Bivolari	Romania
1071	Hampshire Common	UK
1077	Nova	Canada
1103	Korunga	Turkey
1104	na	Turkey
1113	CPI 63753	Spain
1118	CPI 63758	Iran
1123	CPI 63763	Turkey
1127	CPI 63767	USA, Washington
1156	Dukorastushchii	Former Soviet Union
1157	Miatiletka	Former Soviet Union
1163	Giant	England
1165	Rees "A"	England
1169	CPI 63810	Lithuania
1179	CPI 63820	Spain
1197	CPI 63838	Norway
1199	CPI 63840	Former Soviet Union
1200	CPI 63841	Germany
1210	Premier	Switzerland
1211	Perly	Switzerland
1213	CPI 63854	Switzerland
1220	247	Morocco
1230	Visnovsky	Czech Republic
1253	Tu86-43-03	Turkey, Hakkari
1260	X93234	China, Xinjiang
1261	Line 107	Armenia
1262	Cotswold Common	UK
1264	Sisiani Local	Armenia

glycosides (calculated as other glycosylated flavonols), all other 36 samples show the same pattern.

Hydroxycinnamic acids, simple phenolics and flavone contents are shown in Table 3. Hierarchical cluster analysis (HCA) of the different sainfoin samples based on 5 variables as analytical data (flavan 3-ols, flavonols, hydroxycinnamic acids, simple phenolics, flavones) was used to assess the heterogeneity between different samples. The dendrogramme in Fig. 4 showed two clear major clusters of 16 and 21 samples referred to as groups 1A and 1B, respectively. Loading plot (Fig. 5B) derived from principal component analysis (PCA) characterisation of sainfoin samples using the same polyphenol profile (5 variables) suggested that the variables referring to flavonols contributed the most for discrimination of samples. PC1 explained 82% of the variation captured between samples and was related to flavonols, contributing negatively to PC1. The PC1/PC2 scores plot (Fig. 5A) shows that on the left side of the plot, the samples 1213_Switzerland, 1169_Lithuania, 1220_Morocco, 1210_Switzerland, 1001_UK and 1071_UK are positioned (negative PC1 values), whereas on the far right side, most other samples are located (positive PC1 values). Samples from same origin were not always clustered close together i.e. 1210 & 1213 from Switzerland (Fig. 4). In a second step, HCA was performed using the same variables as data, albeit data was scaled. This provides similar weights for all the variables, as described elsewhere (van den Berg et al., 2006). HCA was then performed on the scaled data to visualise general clustering, trends, and outliers among samples. A closer clustering of samples from same origin i.e. Former Soviet Union, Romania and Turkey was observed (Fig. 6) and this suggests that phenolic composition is affected by the country of origin. The same sainfoin accessions were recently screened for proanthocyanidin content and composition and a broad agreement between geographic origin and tannin clusters was suggested (Stringano et al., 2012). In contrast, no relationship between phenolic compounds clusters and the geographic origin could be found in our study.

The specific enzyme activities (Table 4) for enzymes from the flavonoid pathway were calculated using the total protein content of the crude extracts. In all samples all investigated enzymes were present. Only in sample 1163 no DFR activity was observed. In general the samples showed a huge diversity regarding their enzyme activities. As observed for the enzyme activities, the expression levels of the single genes in the different samples were highly diverse. Nevertheless, all samples with the exception of samples 1019, 1210, and 1230 (Suppl. Fig. 1) showed a similar expression profile considering the genes' relation to each other. The prevalent expression pattern is shown in Fig. 7. It is striking, that especially FHT, ANS and ANR are expressed several times over the other determined structural genes. Surprisingly the expression level of PAL was very low (<1% of GAPDH expression), which was not expected, since the enzyme activity of PAL was determined in all samples. Enzymes from the polyphenol pathway are often encoded by multigene families (Fukasawa-Akada et al., 1996; Koes et al., 1994; Shimada et al., 2003). We assume that the low PAL expression is due to the existence of isoforms which were not amplified with the used primers.

The expression of IFS and PKR was observed in all the tested samples. These are structural genes responsible for the biosynthesis of isoflavonoids and 5′-deoxy(iso)flavonoids, which play a role as phytoalexins. Some of the samples showed remarkably higher expression of these genes. Since the formation of these compounds is inducible (Dixon, 2001; He and Dixon, 2000), it can be assumed, that those samples may have been infected by pathogens, fungi or other pests. However, no isoflavonoids or 5-deoxyflavonoids were found in the samples. This demonstrates that increased gene expression does not necessarily result in increased metabolite contents.

The majority of the samples showed conforming profiles of gene expression and specific activity of the enzymes (Fig. 8). Gene expression and activity of FHT was considerably higher than those of the other steps in the pathway, indicating a strong drift downstream at the expense of flavone and isoflavone formation. Despite the high correlation of gene expression profiles and enzyme activity profiles the variability between the samples was very high. When comparing the expression of single genes with the corresponding enzyme activities, a high gene expression in individual samples in comparison to other samples was not necessarily reflected as higher enzyme activity and vice versa. Various reasons such as posttranslational modifications and the existence of isoenzymes, which have not been determined by qPCR can be considered.

2.5. Possible screening tools

A screening tool for the selection of sainfoin germplasm with respect to a beneficial polyphenol composition would facilitate future breeding programmes. However, so far, the biochemical and molecular background is not sufficiently understood. Our study showed that flavonols are the predominant polyphenols in sainfoin and that the flavonols and flavan 3-ols are the most relevant variables for discrimination of the accessions. However, there was no correlation between the flavonol or flavan 3-ol content and the anthelmintic activities (Manolaraki, 2011). Peroxidase activity, which converts phenol groups into quinoid structures and can have an impact on the physiological activity, was included in the studies as potential indicator for biological activity. However, no

Table 3
Soluble phenolic composition (mg/g dry weight) in young leaves of 37 samples; 1001–1156: Breeding codes (A) and soluble phenolic composition (mg/g dry weight) in young leaves of 38 samples of the 40 selected most promising accessions; 1157–1264: Breeding codes (B).

Compound	Phenolics quantity (mg/g dry weight)																		
(A)	1001	1007	1012	1013	1017	1019	1026	1028	1041	1043	1071	1077	1103	1104	1113	1118	1123	1127	1156
Gallocatechin	0.17	0.22	0.46	0.14	0.02	0.08	0.96	0.32	1.58	3.00	0.28	0.48	1.28	0.78	0.38	0.68	0.73	0.75	1.62
Epigallocatechin	0.52	0.38	0.34	0.60	0.10	0.18	0.46	0.73	1.12	0.35	0.28	0.37	0.86	0.49	0.57	1.11	0.39	1.32	0.69
Catechin	0.13	0.05	0.23	0.13	0.03	0.02	0.18	0.13	0.37	0.59	0.10	0.12	0.12	0.05	0.16	0.30	0.11	0.15	0.44
Epicatechin	0.18	0.08	0.15	0.22	0.07	0.03	0.06	0.17	0.18	0.05	0.07	0.07	0.08	0.03	0.15	0.30	0.05	0.18	0.13
Procyanidin B2	0.05	0.02	0.03	0.04	0.03	0.01	0.01	0.03	0.02	–	0.02	0.01	0.01	–	0.03	0.04	–	0.01	0.02
Other flavan 3-ols	1.05	1.03	1.08	0.78	0.26	0.56	1.56	1.07	1.94	3.74	0.77	0.58	2.02	1.33	0.87	1.59	0.98	1.60	2.31
Sum flavan 3-ols	2.09	1.77	2.28	1.90	0.50	0.87	3.24	2.46	5.21	7.73	1.53	1.63	4.37	2.68	2.15	4.02	2.26	4.00	5.21
Galic acid	0.02	–	0.01	0.01	0.01	–	0.01	0.02	0.01	0.04	0.01	0.02	0.02	0.05	0.03	0.04	0.02	0.01	0.02
Other simple phenolic acids	1.79	1.49	1.46	1.57	0.84	0.81	1.32	0.86	1.85	4.17	1.84	1.79	1.12	2.80	1.50	1.32	1.00	1.85	2.11
Sum simple phenolic acids	1.81	1.49	1.46	1.58	0.85	0.82	1.34	0.89	1.86	4.22	1.86	1.81	1.15	2.85	1.53	1.35	1.02	1.87	2.13
Cis + trans *p*-coumaric acid 4-glucoside	0.71	0.38	0.83	1.19	1.00	0.81	0.32	0.22	0.61	0.18	1.00	0.41	1.31	1.15	0.97	0.67	0.56	0.86	0.77
Trans 3 *p*-coumaroylquinic acid	0.07	–	0.12	0.05	0.07	0.05	–	0.04	0.05	0.11	0.29	0.03	–	0.15	0.13	0.30	–	0.11	0.07
3′-Caffeoylquinic acid	0.52	–	0.65	1.12	0.57	0.13	0.19	0.36	0.36	0.85	1.11	0.16	–	0.60	0.75	0.29	–	0.64	–
5′-Caffeoylquinic acid	0.90	0.44	1.56	–	0.55	0.54	0.43	1.10	2.16	0.52	0.40	0.43	1.50	–	2.08	2.55	1.24	0.72	–
Other hydroxicinnamic acids	1.27	0.94	1.46	1.82	0.44	0.65	1.58	1.66	2.34	4.89	0.65	1.28	1.92	1.94	1.69	3.67	1.90	1.48	4.74
Sum hydroxicinnamic acids	3.47	1.76	4.63	4.17	2.63	2.19	2.52	3.39	5.52	6.55	3.44	2.30	4.73	3.84	5.62	7.48	3.71	3.81	5.58
Flavones	0.44	0.30	0.54	–	0.23	0.12	0.25	–	0.69	0.69	1.01	0.28	0.72	0.89	0.33	0.36	0.25	0.43	0.61
Kaempferol 3-rutinoside	2.02	2.09	0.43	1.80	0.80	2.07	0.52	1.96	2.83	4.81	4.99	3.05	1.63	0.36	3.95	1.01	0.82	0.55	1.30
Kaempferol 3-rhamnosidrutinoside	0.69	–	–	0.32	–	–	–	–	–	–	0.72	1.40	–	–	0.24	–	–	–	–
Quercetin 3-glucoside	–	–	–	–	1.09	0.61	–	0.33	–	–	–	–	0.20	3.12	–	–	–	0.31	–
Quercetin 3-rutinoside	18.66	18.87	25.09	13.86	10.15	13.09	18.76	11.97	15.10	14.91	17.46	8.03	9.29	13.51	17.92	19.98	18.70	13.00	16.99
Quercetin 3-rhamnosidrutinoside	2.64	–	1.12	1.48	0.15	0.11	–	–	–	–	1.34	3.89	–	–	0.23	–	–	–	–
Isorhamnetin 3-glucoside	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Isorhamnetin 3-rutinoside	–	1.53	–	–	0.14	1.44	0.06	0.65	0.06	–	0.14	–	0.64	0.16	0.39	0.77	0.74	–	0.38
Other glycosylated flavonols	3.00	1.95	1.65	3.04	10.14	2.30	1.21	1.36	1.08	0.95	2.12	2.42	1.48	1.14	1.33	1.17	0.80	1.46	1.57
Acylated flavonols	0.83	0.17	0.89	0.15	1.26	0.92	0.19	0.07	0.19	0.16	0.10	0.55	0.03	0.10	0.62	0.16	0.15	0.09	0.11
Sum flavonols	27.83	24.61	29.18	20.65	23.73	20.54	20.75	16.33	19.26	20.82	26.88	19.35	13.27	18.39	24.68	23.10	21.22	15.40	20.34
(B)	1157	1163	1165	1169	1179	1197	1199	1200	1210	1211	1213	1220	1230	1253	1260	1261	1262	1264	
Gallocatechin	1.78	0.48	0.11	0.15	1.35	0.64	3.50	0.13	0.13	0.53	0.32	0.57	0.59	0.75	1.91	1.67	0.28	0.95	
Epigallocatechin	1.10	0.50	0.29	0.08	0.03	0.57	0.06	0.18	0.50	1.24	0.09	0.76	0.55	1.50	0.18	0.91	0.63	0.49	
Catechin	0.45	0.08	0.20	0.12	1.48	0.15	0.58	0.08	0.08	0.10	0.09	–	0.04	0.09	0.16	0.19	0.16	0.09	
Epicatechin	0.15	0.07	0.22	0.05	0.06	0.11	0.02	0.07	0.12	0.16	0.03	0.05	0.03	0.16	0.01	0.08	0.21	0.04	
Procyanidin B2	0.01	0.01	0.11	0.02	–	0.02	–	0.02	0.03	0.03	–	0.06	–	0.01	–	0.01	0.05	–	
Other flavan 3-ols	3.22	0.77	1.34	0.82	3.79	0.79	6.90	0.61	1.19	1.85	0.83	1.85	0.95	1.86	2.37	2.34	1.31	1.02	
Sum flavan 3-ols	6.71	1.89	2.27	1.22	6.71	2.28	11.07	1.09	2.04	3.90	1.37	3.29	2.17	4.37	4.64	5.19	2.63	2.59	
Galic acid	0.03	0.02	0.02	0.01	0.03	0.02	0.07	0.01	0.01	0.03	–	0.03	0.02	0.02	0.04	0.04	0.01	0.01	
Other simple phenolic acids	2.85	1.94	1.51	1.92	6.74	0.80	1.59	0.87	1.78	1.23	1.47	1.11	0.71	1.58	2.57	1.77	1.34	0.91	
Sum simple phenolic acids	2.88	1.96	1.53	1.93	6.76	0.82	1.66	0.88	1.79	1.26	1.47	1.14	0.73	1.60	2.61	1.81	1.35	0.92	

Cis + trans *p*-coumaric acid 4-glucoside	2.32	0.77	2.00	4.59	0.72	0.45	0.27	0.84	2.11	0.57	0.29	0.76	0.81	0.90	0.91	0.29	0.67	1.07		
Trans 3 *p*-coumaroylquinic acid	0.09	–	0.65	0.06	–	–	–	–	–	0.36	0.11	–	0.04	0.05	0.33	–	–	0.10		
3′-Caffeoylquinic acid	0.43	0.13	0.57	0.68	0.26	–	–	0.05	0.30	0.07	0.59	0.56	0.24	0.14	–	0.24	0.40	0.11		
5′-Caffeoylquinic acid	0.37	1.17	0.52	0.08	0.98	0.92	1.39	1.89	2.22	0.13	0.93	0.75	1.22	0.74	–	0.90	0.72	1.73		
Other hydroxicinnamic acids	2.53	1.37	2.09	0.83	0.91	1.61	1.32	1.85	1.61	3.58	1.85	1.51	1.52	2.64	4.10	2.37	1.00	1.87		
Sum hydroxicinnamic acids	5.73	3.44	5.83	6.26	2.87	2.99	2.98	4.62	6.24	4.72	3.76	3.58	3.82	4.47	5.33	3.81	2.80	4.88		
Flavones	0.78	–	0.37	0.45	0.81	0.16	0.10	0.17	0.66	0.04	–	0.54	0.44	0.96	0.22	0.14	0.48	–		
Kaempferol 3-rutinoside	1.47	0.60	1.15	1.38	1.72	2.78	3.45	3.13	0.88	1.46	5.61	1.57	0.36	0.81	2.32	1.24	2.32	1.46		
Kaempferol 3-rhamnosidrutinoside	–	–	0.13	0.18	0.05	0.22	–	0.25	0.16	0.14	–	–	–	1.07	0.15	–	1.17	0.18		
Quercetin 3-glucoside	0.58	0.19	–	–	–	–	–	–	–	–	–	–	–	0.29	–	–	–	–		
Quercetin 3-rutinoside	19.22	7.65	16.67	24.82	7.21	16.68	10.78	17.69	16.86	17.11	25.11	26.81	17.55	7.91	16.28	16.88	10.09	9.77		
Quercetin 3-rhamnosidrutinoside	–	–	0.96	1.21	0.08	1.32	0.69	0.64	1.93	0.57	0.82	–	–	2.16	0.38	–	3.40	0.26		
Isorhamnetin 3-glucoside	–	–	–	–	0.51	–	–	–	0.14	–	–	–	–	–	–	–	–	–		
Isorhamnetin 3-rutinoside	1.20	–	–	–	5.40	0.19	–	–	4.15	–	0.85	0.12	–	0.06	1.06	0.04	–	–		
Other glycosylated flavonols	1.68	0.38	3.08	4.30	3.19	1.24	1.68	2.70	3.64	1.26	3.44	2.22	1.10	0.68	1.25	1.25	1.73	0.79		
Acylated flavonols	1.18	0.05	0.15	0.43	0.73	0.31	0.41	0.21	0.02	0.25	0.70	0.70	0.11	0.33	1.02	0.31	0.18	0.53		
Sum flavonols	25.34	8.86	22.14	32.32	18.88	22.75	17.01	24.62	27.79	20.80	36.52	31.42	19.13	13.32	22.47	19.71	18.89	12.99		

correlation could be found either. In the same way there was no correlation between antioxidant capacities and the total polyphenol contents and only a very weak correlation between antioxidant capacity and anthelmintic effects. However, a significant positive correlation between the ratios of FLS:DFR and flavonols:flavan 3-ols (Figs. 6 and 9) was determined, $r(35) = 0.86$, $p = 0.01$. Channelling of the metabolism towards either flavonols or leucoanthocyanidins as the intermediates for anthocyanidin and proanthocyanidin formation occurs at the branching point of dihydroflavonols which are used as substrates for both DFR and FLS. Thus decreasing FLS activity and increasing DFR activity results in increased flavan 3-ol formation at the expense of flavonol formation and vice versa. This could allow a systematic selection of plants showing favoured flavan 3-ol formation.

2.6. Conclusion

Flavan 3-ols are active ingredients in sainfoin and have an influence particularly on nitrogen absorption and bloat occurrence in ruminant feedings. Thus, an introduction of flavan 3-ol formation in forage crops by a transgenic approach, was suggested as a promising disease prevention by dietary means (Xie et al., 2006). As a molecular breeding approach for sainfoin, introduction of a DHK-accepting DFR from carnations or apple could increase flavan 3-ol formation at the expense of flavonol formation. For classical breeding approaches, screening of sainfoin accessions or other species for DFRs with improved DHK acceptance could be a possible strategy. Testing germplasm for the ratio FLS:DFR would be additionally useful to ensure that the selected accessions possess an improved flavan 3-ol content.

3. Experimentals

3.1. General

TLC was performed on pre-coated cellulose plates (Merck, Darmstadt, Germany); radiolabeled substances were detected with a Berthold LB 2842 TLC Linear Analyzer (Wildbad, Germany) and a Winspectral 1414 scintillation counter (Perkin-Elmer, Vienna, Austria).

3.2. Plant material

Onobrychis viciifolia, line "Cotswold Common" (accession 1001), was grown at the Technical University Munich and young folded leaves were used for the establishment of standard enzyme assays and for the isolation of the cDNA of polyphenol genes. Morphological criteria were used for defining different tissues collected for the enzyme demonstration. The following material was collected: young leaves (still folded), developed leaves (completely unfolded), petiols separated from young leaves, petiols separated from developed leaves, flower stalks separated from buds, stems, stalks separated from unripe seeds, unripe seeds, buds (still closed flowers), open flowers (fully developed), senescent flowers (wilting). 36 preselected accessions of *O. viciifolia* and one of *O. antasiatica*, which were grown at the National Institute of Agricultural Botany (NIAB), UK, were used for the screening (Table 2). All plant material was shock frozen in liquid nitrogen immediately after harvesting and stored at −80 °C.

3.3. Chemicals

[^{14}C]phenylalanine and [^{14}C]malonyl-CoA were purchased from Amersham International (Freiburg, Germany), *p*-coumaroyl-CoA, caffeoyl-CoA, cinnamoyl-CoA and feruloyl-CoA from TransMIT

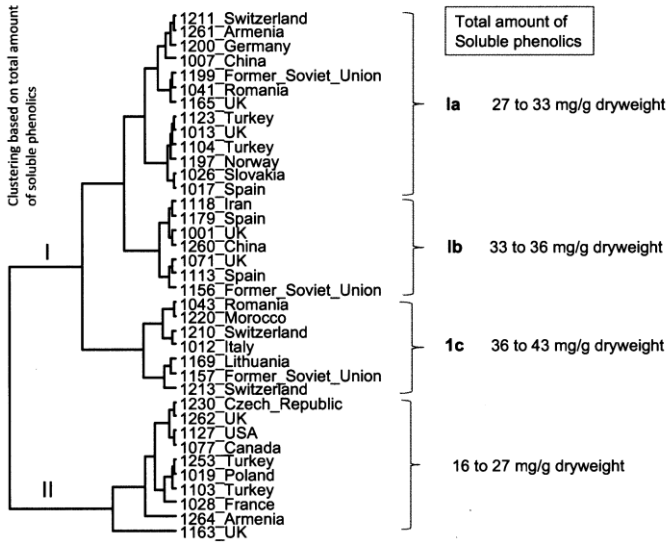


Fig. 3. Hierarchical cluster analysis (HCA) of sainfoin samples based on group average cluster analysis of total amount of soluble phenolics as the analytical data.

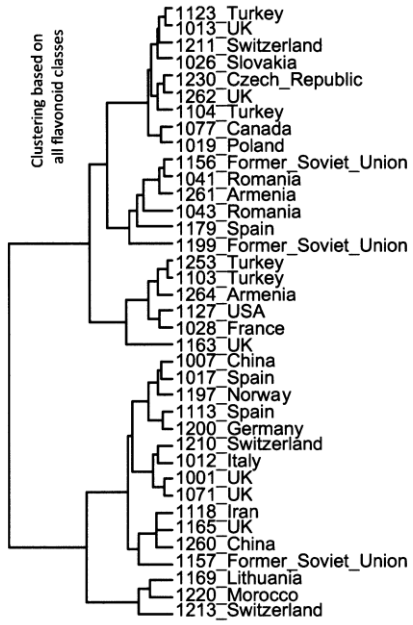


Fig. 4. Hierarchical cluster analysis (HCA) of sainfoin samples based on group average cluster analysis of 5 the variables flavan 3-ols, flavonols, hydroxycinnamic acids, simple phenolics, and flavones as the analytical data.

(Marburg, Germany), *S*-adenosylmethionine (SAM) from Sigma (Vienna, Austria) and cyanidinchlordide from Roth (Karlsruhe, Germany). [^{14}C]naringenin (NAR), [^{14}C]eriodictyol (ERI), [^{14}C]pentahydroxyflavone (PHF), [^{14}C]liquiritigenin (LIQ), [^{14}C]butin (BUT), [^{14}C]dihydrokaempferol (DHK), [^{14}C]dihydroquercetin (DHQ), [^{14}C]dihydromyricetin (DHM), [^{14}C]garbanzol (GAR), [^{14}C]dihydrofisetin (DHF) and [^{14}C]quercetin were synthesised as described before (Halbwirth et al., 2004, 2006). [^{14}C]5-deoxyleucocyanidin (5-DLCY) was synthesised enzymatically from DHF using recombinant DFR of *O. viciifolia*.

Sephadex G-25 (medium) was purchased from GE Healthcare (Munich, Germany), HEPES, NADPH, DTT and *O*-dianisidin from Sigma–Aldrich (Vienna, Austria), PEG 20000 from VWR International (Vienna, Austria) and $CaCl_2$ was purchased by Merck (Darmstadt, Germany).

3.4. Buffers

Extraction buffer: 0.1 M HEPES, 40 mM sucrose, 0,75 mM PEG 20000, 0.1 M Na-ascorbate, 1 mM DTE, and 0.025 mM $CaCl_2$, pH 7.3 (Dellus et al., 1997). To remove oxygen, the HEPES and sucrose containing buffer was initially filled up to 150 ml and cooked for approx. 15 min until a final volume of 100 ml was reached. Thereafter, it was cooled on ice under nitrogen flow, until a temperature of 30 °C was reached, and PEG 20000, Na-ascorbate and DTE were dissolved in the buffer under gentle stirring.

Buffer 1: 0.1 M H_3BO_3 + 0.4% Na-ascorbate; buffer 2: 0.1 M KH_2PO_4 + 0.4% Na-ascorbate; buffer 3: 0.1 M Tris/HCl + 0.4% Na-ascorbate; buffer 4: 0.1 M KH_2PO_4; buffer 5: 0.1 M KH_2PO_4 + 0.003% H_2O_2.

3.5. Enzyme preparation from plant material

The method described by (Dellus et al., 1997) was modified as follows: 0.2 g plant material were ground in a mortar with 0.2 g Polyclar AT (Serva, Heidelberg, Germany), 0.2 g quartz sand and 3 ml of the extraction buffer. The homogenate was centrifuged for 10 min at 4 °C and 10,000g. Low molecular weight substances were removed via gel chromatography by passing the supernatant through Sephadex G-25 columns as described (Stich and Forkmann, 1988).

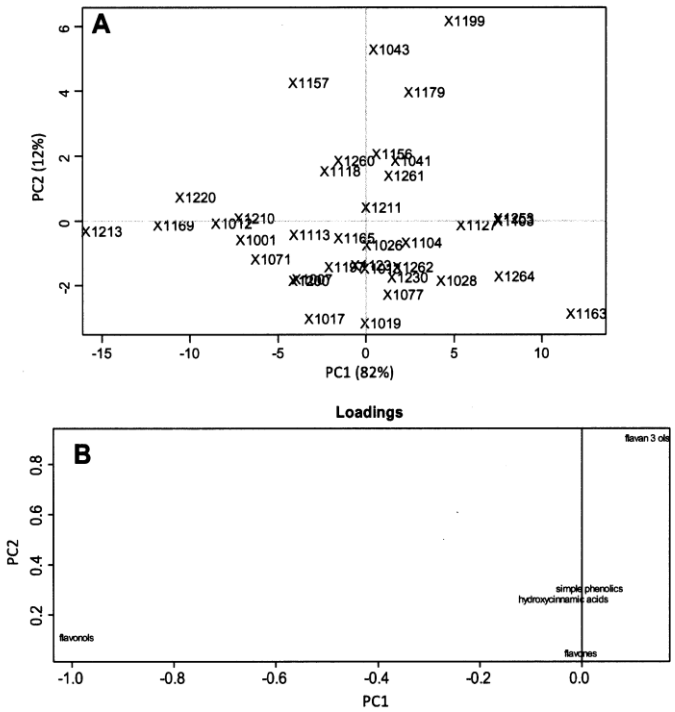


Fig. 5. Principal component analysis characterisation (PC1 vs. PC2) of sainfoin samples using (A) the polyphenol profile (5 variables flavan 3-ols, flavonols, hydroxycinnamic acids, simple phenolics, and flavones) as the analytical data and (B) Loading plot for PC1 & PC2 components: Group discrimination in samples is related to quantitative differences in flavonols and flavan-3-ols.

3.6. Standard enzyme assays with preparations from plant material

In a final volume of 100 µl the PAL assay contained 40 µl enzyme preparation, 55 µl buffer 1 (pH 9.25) and [^{14}C]phenylalanine (0.027 nmol); the CHS/CHI assay contained 40 µl enzyme preparation, 50 µl buffer 2 (pH 7.5), *p*-coumaroyl-CoA (1.0 nmol) and [^{14}C]malonyl-CoA (1.5 nmol, 1300 Bq); the FHT contained 20 µl of a 1:10 diluted enzyme preparation, 70 µl buffer 2 (pH 7.0), [^{14}C]naringenin (0.036 nmol, 100 Bq), 5 µl 1 mM 2-oxoglutarate and 5 µl 2 mM $FeSO_4{\cdot}7H_2O$; the FLS assay contained 30 µl enzyme preparation, 60 µl buffer 3 (pH 7.5), 0.036 nmol [^{14}C]dihydrokaempferol (100 Bq), 5 µl 1 mM 2-oxoglutarate and 5 µl 2 mM $FeSO_4{\cdot}7H_2O$; the OMT assay contained 40 µl buffer 3 (pH 7.75), 50 µl enzyme preparation, [^{14}C]quercetin (0.036 nmol, 100 Bq), 5 µl 10 mM $MgCl_2$, 30 nmol SAM; the IFS assay contained 65 µl buffer 2 (pH 7.5), 30 µl enzyme preparation, 0.036 nmol [^{14}C]naringenin and 5 µl 50 mM NADPH. In a final volume of 50 µl the DFR assay contained 15 µl buffer 2 (pH 6.5), 30 µl enzyme preparation, [^{14}C]dihydroquercetin (0.036 nmol, 100 Bq) and 5 µl 5 mM NADPH; the FGT assay contained 25 µl buffer 2 (pH 6.25), 20 µl enzyme preparation, 30 nmol quercetin, [^{14}C]uridindiphosphatglucose (1600 Bq); in a final volume of 1110 µl the POX assay contained 1095 µl buffer 5 (pH 6.0), 5 µl enzyme preparation, and 10 µl *O*-dianisidin (1% in MeOH).

The reactions were incubated at 40 °C (PAL, CHS/CHI, FGT, OMT) or 30 °C (FHT, DFR, FLS) for 10 min (FHT), 20 min (PAL, CHS/CHI) and 30 min (DFR, FLS, FGT, OMT). PAL and CHS/CHI reaction were stopped by addition of 10 µl acetic acid and 200 µl ethylacetate. Product formation was quantified at a Winspectral 1414 scintillation counter (Perkin-Elmer, Vienna, Austria). FHT, DFR, FLS and OMT reactions were stopped by addition of 10 µl acetic acid and 70 µl ethyl acetate. In case of FLS, 10 µl 0.1 M EDTA was added. The organic phase was transferred to thin-layer cellulose plates (Merck, Darmstadt, Germany) and developed in chloroform/acetic acid/water (10:9:1, v:v:v) in case of FHT and DFR and in acetic acid/water (3:7, v:v) in case of FLS and OMT. The evaluation was carried out on a Berthold LB 2842 TLC Linear Analyzer (Wilbad, Germany) by integration of the peak areas. The POX activity was determined by the measurement of the extinction at 460 nm on a DU-65 spectrophotometer (Beckman instruments) in intervals of 20 s for a period of 12 min. The activity was calculated for the linear range of ΔE/min, using the extinction coefficient of oxidised *O*-dianisidine according to the data sheets of Sigma–Aldrich.

The protein content of the crude extract was determined by a modified Lowry procedure (Sandermann and Strominger, 1972) using crystalline BSA as a standard.

3.7. Standard assays with recombinant enzymes

The assays with recombinant proteins were carried out as described for plant crude extract with the following modifications according to the optimisation. The CHS/CHI assay was incubated at 30 °C for 15 min, using 30 µl of the protein preparation and pH 8.0; the FHT assay contained 2 µl protein preparation and was incubated for 5 min at 20 °C using pH 6.75; The DFR assay was incubated at 30 °C for 15 min, using pH 6.75 and 20 µl protein

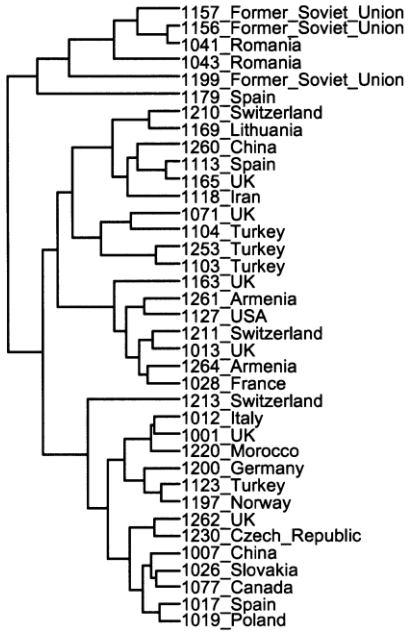


Fig. 6. Hierarchical cluster analysis (HCA) of sainfoin samples based on group average cluster analysis of 5 variables flavan 3-ols, flavonols, hydroxycinnamic acids, simple phenolics, and flavones as the analytical data with scaling of data.

preparation. The FLS assay was incubated at 30 °C for 5 min, using pH 6.75 and 10 µl protein preparation.

In a volume of 500 µl the ANR assay contained 350 µl buffer 4 (pH 5.75), 50 µl enzyme preparation, 50 µl of 3 mM cyanidin, and 50 µl of 20 mM NADPH. The reaction was incubated at 37 °C for 40 min and stopped by addition of 200 µl ethyl acetate. After vacuum evaporation of the organic extract, the residue was dissolved in 80 ml MeOH, transferred to TLC plates (Merck, Darmstadt, Germany), developed in butanol/acetic acid/water (6:1:2, v:v:v) and stained with solution containing 0.1% dimethylaminocinnamic acid in 6 N HCl:butanol (1:1, v:v). In a final volume of 120 µl the PKR assay contained 25 µl buffer (pH 6.0), 40 µl enzyme preparation, 40 µl CHS, *p*-coumaroyl-CoA (1.0 nmol), [^{14}C]malonyl-CoA (1.5 nmol, 1300 Bq) and 5 µl 5 mM NADPH. The reaction was incubated at 30 °C for 30 min and stopped by addition of 100 µl ethyl acetate. The organic phase was transferred to thin-layer cellulose plates and developed in chloroform/acetic acid/water (10:9:1, v:v:v). In a final volume of 50 µl the LAR assay contained 70 µl buffer 2 (pH 7.0), 50 µl protein preparation, [^{14}C]5-DLCY (0.5 nmol, 133 Bq) and 10 µl 5 mM NADPH. The reaction was incubated at 30 °C for 30 min and stopped by the addition of 5 µl acetic acid and 80 µl ethyl acetate. After vacuum evaporation of combined organic extracts, the residue was dissolved in 25 µl MeOH. Product formation was determined by HPLC using a Perkin-Elmer system equipped with a series 200 LC pump, series 200 autosampler and Radiomatic 610TR Flow Scintillation Analyzer. Separation was done on a BDS Hypersil C 18 column (Thermo, 250 mm × 4.6 mm ID, 5 µm) and eluted with a mixture of H_2O (solvent A) and acetonitrile (solvent B). The following gradient was applied using a flow rate of 1.0 ml/min: 0–1 min, 5% B; 1–8 min, 13% B; 8–19 min, 25% B; 19–20 min, 5% B.

Table 4
Specific activities of the polyphenol enzymes [*n*kat/kg total protein], peroxidase [kat/kg total protein], and antioxidant activity [µmol trolox/g DW] in 37 *O. viciifolia samples.* Substrates in the standard assays were used as described in Section 3.6.

No	Protein content (µg/µl)	PAL	CHS/CHI	FHT	DFR	FLS	FGT	POX	Antioxidant activity
1	0.73	710 ± 20	4501 ± 549	13,411 ± 1140	331 ± 1	43 ± 12	46,459 ± 2806	63 ± 1	309 ± 1
2	0.71	146 ± 24	988 ± 1	1947 ± 23	38 ± 2	16 ± 0	24,041 ± 4504	51 ± 2	163 ± 0
3	0.93	429 ± 58	478 ± 2	6303 ± 1107	118 ± 16	19 ± 6	n.d.	36 ± 2	279 ± 2
4	1.03	485 ± 22	2659 ± 249	16,795 ± 2469	344 ± 26	51 ± 3	56,275 ± 4359	25 ± 0	262 ± 1
5	1.05	265 ± 4	11,011 ± 763	1696 ± 10	28 ± 8	40 ± 6	64,187 ± 5925	49 ± 2	79 ± 1
6	0.84	361 ± 7	1265 ± 121	1183 ± 289	12 ± 0	41 ± 16	12,117 ± 3940	45 ± 2	n.d.
7	0.85	601 ± 27	3994 ± 92	20,915 ± 918	532 ± 12	25 ± 4	1,28,363 ± 8062	46 ± 0	277 ± 0
8	0.95	531 ± 11	384 ± 5	15,644 ± 1358	289 ± 17	38 ± 7	n.d.	41 ± 0	285 ± 2
9	1.15	452 ± 40	4594 ± 213	18,071 ± 1004	422 ± 32	65 ± 8	57,577 ± 791	31 ± 1	299 ± 1
10	0.70	572 ± 78	5326 ± 201	21,137 ± 4524	825 ± 33	85 ± 5	68,864 ± 34,432	41 ± 3	138 ± 8
11	0.93	448 ± 38	3862 ± 16	14,288 ± 1167	140 ± 11	66 ± 2	94,958 ± 1062	46 ± 3	234 ± 2
12	1.06	426 ± 8	4286 ± 136	6335 ± 821	504 ± 0	20 ± 1	77,379 ± 13,274	24 ± 2	182 ± 2
13	1.36	303 ± 1	2607 ± 89	13,546 ± 149	126 ± 2	17 ± 4	n.d.	32 ± 3	252 ± 2
14	1.17	464 ± 13	5822 ± 296	15,377 ± 20	214 ± 20	17 ± 6	74,262 ± 891	20 ± 0	293 ± 0
15	1.11	433 ± 15	5455 ± 367	13,430 ± 263	282 ± 10	26 ± 1	n.d.	26 ± 0	145 ± 0
16	1.49	270 ± 12	3899 ± 118	12,387 ± 948	250 ± 21	13 ± 11	n.d.	42 ± 1	214 ± 2
17	1.19	457 ± 15	3813 ± 126	13,605 ± 4236	163 ± 26	18 ± 1	51,959 ± 7943	43 ± 0	174 ± 0
18	1.17	320 ± 4	4621 ± 189	18,534 ± 3028	435 ± 11	15 ± 6	n.d.	48 ± 5	289 ± 1
19	1.04	444 ± 8	7791 ± 256	14,441 ± 3453	269 ± 15	23 ± 13	46,249 ± 1122	40 ± 1	354 ± 0
20	1.05	534 ± 39	5381 ± 250	18,873 ± 816	290 ± 14	41 ± 15	62,909 ± 4135	28 ± 1	444 ± 0
21	1.07	421 ± 14	1122 ± 97	2340 ± 661	–	25 ± 0	71,579 ± 812	30 ± 1	269 ± 0
22	0.87	456 ± 27	3871 ± 34	4128 ± 468	172 ± 16	40 ± 8	78,005 ± 12,158	51 ± 3	417 ± 1
23	0.94	350 ± 24	5708 ± 129	15,140 ± 405	281 ± 36	61 ± 4	n.d.	40 ± 2	386 ± 0
24	0.86	530 ± 13	4200 ± 239	4219 ± 781	590 ± 17	58 ± 3	n.d.	10 ± 2	373 ± 1
25	1.15	429 ± 36	8113 ± 364	13,719 ± 608	323 ± 12	45 ± 1	n.d.	34 ± 0	549 ± 0
26	0.99	570 ± 7	8942 ± 330	13,968 ± 939	581 ± 9	36 ± 1	61,732 ± 7938	63 ± 0	290 ± 0
27	1.06	395 ± 57	2769 ± 167	6789 ± 208	143 ± 0	29 ± 2	n.d.	48 ± 1	249 ± 0
28	0.91	555 ± 48	4788 ± 158	6832 ± 1883	146 ± 7	51 ± 1	38,328 ± 7751	23 ± 3	328 ± 0
29	1.18	337 ± 23	3514 ± 90	6521 ± 611	300 ± 17	36 ± 0	n.d.	31 ± 2	384 ± 0
30	0.86	180 ± 4	995 ± 9	1822 ± 1008	26 ± 4	22 ± 1	n.d.	25 ± 1	144 ± 4
31	1.13	403 ± 43	3190 ± 148	3681 ± 937	86 ± 8	20 ± 1	n.d.	47 ± 1	345 ± 0
32	1.18	317 ± 23	3569 ± 0	3781 ± 444	37 ± 3	40 ± 3	33,793 ± 8597	61 ± 2	261 ± 0

Table 4 (*continued*)

No	Protein content (μg/μl)	PAL	CHS/CHI	FHT	DFR	FLS	FGT	POX	Antioxidant activity
33	1.31	402 ± 48	6870 ± 629	11,918 ± 451	332 ± 2	76 ± 4	n.d.	45 ± 0	366 ± 0
34	1.07	438 ± 48	7945 ± 270	17,065 ± 138	402 ± 22	23 ± 3	n.d.	47 ± 1	100 ± 6
35	1.07	476 ± 43	6190 ± 137	15,795 ± 694	430 ± 26	27 ± 0	59,021 ± 3745	43 ± 4	567 ± 0
36	1.15	406 ± 13	4985 ± 176	4427 ± 1017	414 ± 2	20 ± 0	76,088 ± 11,615	54 ± 2	261 ± 1
37	1.13	324 ± 27	6030 ± 300	7139 ± 254	329 ± 10	12 ± 1	69,057 ± 5239	55 ± 2	200 ± 2

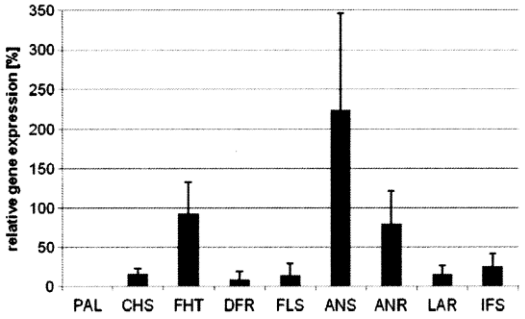


Fig. 7. Prevalent gene expression profile. expression in relation to glycerinaldehyde 3-phosphate dehydrogenase.

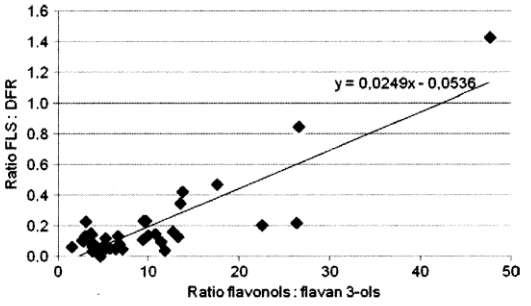


Fig. 9. Correlation between the ratio of specific activities of FLS and DFR and the ratio of flavonols and flavan 3-ols.

3.8. Determination of the antioxidant activity

The antioxidant activity of methanolic plant extracts was determined according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method as described by Brand-Williams et al. (1995) with slight modifications. 100 μl of methanolic plant extracts were briefly mixed with 2 ml of DPPH solution. The absorbance of the reaction was measured at 517 nm for 30 min using methanol as a blank. A negative control was taken by addition of 2 ml of DPPH solution in 100 μl of methanol. The antioxidant activity was calculated with the help of linear regression equation of trolox.

3.9. Cloning of the polyphenol genes

Total RNA was prepared from young leaves or seedlings using a method described for pine trees (Chang et al., 1993) and used for the synthesis of cDNA. cDNA was prepared using the RevertAid H Minus MuLV reverse transcriptase (Fermentas Life Science, St. Leon-Rot, Germany) with the oligo(-dT) anchor Primer GACCACGCGTATCGATGTCGAC$(T)_{16}$V.

Based on information available in the NCBI-GenBank, nucleotide or amino acid sequences of the respective genes derived from other plants, mostly leguminosae, were aligned (GenBank ID: D10001, AB283040, DQ073811, X58180, EF192469 (PAL); M91079, NM126020, DQ835284, AB054801, X16470, PSU03433 (CHI); AB185899, AF198451, AF308855S2, X72593, U86837 (FHT); P51109, EF187612, AF117263 (DFR); AB038247, AB246668, AB185903, DQ087252 (FLS); AY382828, AB185902, EF544389 (ANS); BN000164, AY184243, EF197823 (ANR); BN000698, AY730617, AJ550154 (LAR); AB091686, AB091684, U97125 (IOMT); U13924, EU921437, AJ223291 (PKR). DQ835285, DQ205408, AY939826, AF532999, AF462633, AF195811, AF195813, AF195803, AF195804, AF195807 (IFS). Conserved regions were used for the design of degenerated primers. The degenerated primer for CHS was provided by L. Janvary (TUM, Germany). The obtained cDNA-fragments were isolated, ligated into the vector pCR®2.1-TOPO (Invitrogen, Paisley, UK) and transformed in *E. coli* (TOP10, Invitrogen Paisley, UK). The fragments were then sequenced (Eurofins MWG Operon, Ebersberg, Germany; StarSEQ, Mainz, Germany). The achieved specific sequence information was then used for the design of specific 5′- and 3′-primers for the amplification of the ends of the respective genes by RACE-tech-

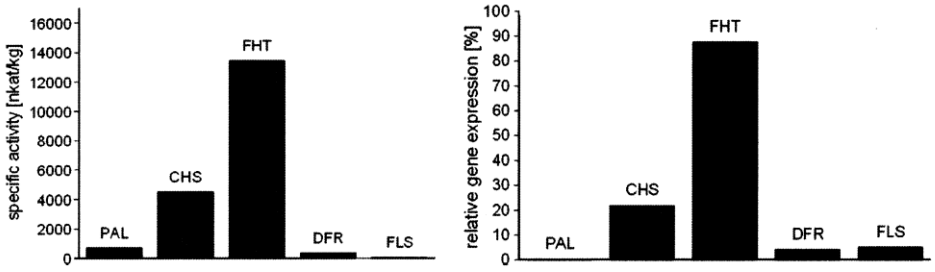


Fig. 8. Profile of the specific activities of the polyphenol enzymes (left) and the expression of the respective genes in relation to the glycerine aldehyde 3-phosphate dehydrogenase (right). shown is accession 1001.

Table 5
Primers used for amplification of the full ORF.

Gene		Sequence	Size (bp)	GenBank ID
CHI	f	5′-GAGCATTGAATTGAATTGAATGGC-3′	696	HM204480
	r	5′-CCTTCCTTCGGTTCTGCAATAA-3′		
CHS	f	5′-CACTGCAAACCAGAAAAGAA-3′	1170	HM204482
	r	5′-AGCAAGTGAGATTACATCTT-3′		
FHT	f	5′-CAAAGAAACTAATAATCAAATAAACAATG-3′	1098	HM204483
	r	5′-CAGGCACATGCTAGCTAG-3′		
DFR	f	5′-GAAAAGAGAAGAGAAAACATGGG-3′	1026	HM152979
	r	5′-GACACAAGCTTAGGCATAAGG-3′		
FLS	f	5′-ATGGAGGTGCTAAGAGTGC-3′	1005	HM204484
	r	5′-GTTATTTCACAGTAGAGGCCA-3′		
ANS	f	5′-AAGATGGGAACTTTGGCTCAAAG-3′	1080	HM204479
	r	5′-GCAAAGCAAAGCAATAAGTGATCATATA-3′		
ANR	f	5′-CAGAGAGTGGGTGTGCATAT-3′	1020	HM152980
	r	5′-GATGAACCTATTGTTCCCTCC-3′		
LAR	f	5′-AACTCATCATCCATCCGTTTCA-3′	1086	HQ896305
	r	5′-CTGCTAGCTTAGCTAGGTG-3′	1095	HM152981
IFS	f	5′-CGTACTAACATCTTCTTCCTCT-3′	1587	HM204485
	r	5′-ACATGATTCATGGAAACACTGG-3′		
IOMT	f	5′-CAGCAACTATGGCTTCTTCTTC-3′	1086	HM204486
	r	5′-GAGATTTATCCTTAAAAGTTATTCTGC-3′		
PKR	f	5′-TCAACATCAAAATGGGTAGTGTTG-3′	945	HM204481
	r	5′-TCTGAATTAGCTCTGGCTTTCTC-3′		

Table 6
Primers used for qPCR.

Gene		Sequence	Fragment length
PAL	f	5′- CTGGTGATCTAATTCCTCTATCATAC-3′	164
	r	5′- CACAGCTGTTGATCTGGTGCA -3′	
CHS	f	5′-GCGCATGTGTGACAAGTCAATG-3′	242
	r	5′-CACAAGTGGTGTGGACATGC-3′	
FHT	f	5′-CCATCTCCAAGGAGAAGCTGT-3′	288
	r	5′-GAGCCTGATCTCACACTTGG-3′	
DFR	f	5′-CAAAGATCCTGAGAACGAAGTGAT-3′	249
	r	5′-CATTAGCGGAACAAGAAGCGTG-3′	
FLS	f	5′-GGAGATGACATGATTCACTTACT-3′	228
	r	5′-CGACCAAATGGAGATACTAAGCAAT-3′	
ANS	f	5′-GACACCACCTTATTATACTGAGGTA-3′	224
	r	5′-GAGTTGAAGCCCACACGGATA-3′	
ANR	f	5′-TCTCACCTGGTGGCACTTCA-3′	243
	r	5′-GTGAAACGAGTCGTCTTAACATCT-3′	
LAR	f	5′-GTGCAAGAGTTATTTACGGTGTGG-3′	323
	r	5′-TCCATTGCTTCTTGGCCCTATTAT-3′	
IFS	f	5′-GATCTTCTCAACGCCACTACTG-3′	208
	r	5′-GAGATATGGCTCGTGATGTGCT-3′	
G3PDH	f	5′-GCTCCCATGTTTGTTGTTGGTG-3′	225
	r	5′-GAGAGGTGGAAGAGCTGCTT-3′	

niques, using the SMART™ Race cDNA amplification kit (Clontech, Takara Bio Company) according to the manufacturer's instructions. Proofreading amplification of the complete open reading frame was carried out using specific forward and reverse primers (Table 5) and the Expand High Fidelity PCR System (Roche, Mannheim, Germany).

3.10. Heterologous expression in E. coli

Proofreading cDNA amplicons were ligated into the *E. coli* expression vector TrcHis2-TOPO® (Invitrogen, Paisley, UK). Sense constructs were isolated, and confirmed by sequencing. Single colonies containing the sense construct were used for heterologous expression in *E. coli* as described (Lukacin and Britsch, 1997).

3.11. Heterologous expression in Saccharomyces cerevisiae

Proofreading cDNA amplicons were ligated into the pYES2.1/V5-His-TOPO® vector (Invitrogen, Paisley, UK). Sense constructs were isolated and confirmed by sequencing. Transformation of the plasmid into the *S. cerevisiae* strain INVSc1 followed. The heterologous expression was carried out according to (Pompon et al., 1996).

3.12. Quantitative RT-PCR

mRNA was prepared from total RNA using the μMACS mRNA isolation kit (Miltenyi Biotech, Auburn, CA). cDNA was prepared as described above. Gene expression was quantified using the StepOnePlus™ Real-Time PCR System with the SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA). Used primers are listed in Table 6. The efficiency of the PCR-reaction was determined on the basis of standard curves which were obtained by applying different DNA concentrations. Results were calculated in relation to the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The product specificity was confirmed by melt curve analysis and gel electrophoresis.

3.13. Extraction of phenolic compounds

Freeze-dried leaves were ground in a ball mill. The extraction was performed by adding 500 μl of 80%aqueous methanol containing flavone (0.02 mg/ml) as internal standard to 100 mg of powder for a period of 30 min in a cooled ultrasound water bath (7 °C). After centrifugation (10,000 rcf, 10 min, 4 °C), the clear supernatant was transferred to an Eppendorf tube. A 10 μl sample of the extract was injected for HPLC analysis.

3.14. Quantitative HPLC analysis of phenolic compounds

The phenolic compounds from *O. viciifolia* were analysed 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 post-column 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. The phenolic 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.

The identification is described by Regos et al. (2009) and Veitch et al. (2011). Quantification was performed as follows (Regos et al., 2009): 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 flavan 3-ols, gallocatechin and epigallocatechin were calculated as epicatechin and the oligomers as procyanidin B2. The flavonols were calculated as rutin.

3.15. Cluster analysis (HCA) and principal component analysis (PCA)

PCA and HCA analyses were performed using three R packages, PCA methods, Heatplus, and gplots, which can be downloaded as an R package from the Metlin Metabolite Database (http://137.131.20.83/download/) under R 2.9.2 environment from phenolic classes (5 variables) or total soluble phenolics. For scaling of data, metabolites levels were autoscaled (the mean area value of each feature throughout all samples was subtracted from each individual feature area and the result divided by the standard deviation) prior to clustering analysis. This provides similar weights for all the variables, as described elsewhere (van den Berg et al., 2006).

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.05.030.

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APPENDIX 3

Composition of phenolic compounds in organs of sainfoin plants (variety Cotswold Common)

Table IA. Composition of phenolic compounds (milligrams per gram of dry weight) in organs of sainfoin plants (variety Cotswold Common) at vegetative stage.

Compound	Young leflets	Mature leaflets	Young petioles	Mature petioles
	simple phenolic acids			
arbutin	-	-	13.22	10.71
8-β-glucopyranosyloxycinnamic acid	-	-	0.66	0.11
other simple phenolic acids	2.31	1.14	2.45	1.28
total simple phenolic acids	*2.31*	*1.14*	*16.33*	*12.09*
	hydroxybenzoic acids			
protocatechuic acid	-	-	0.09	-
	hydroxycinnamic acids			
cis+trans p-coumaric acid 4-O-glucoside	0.48	0.32	0.35	0.53
trans 3-p-coumaroylquinic acid	0.51	0.43	0.30	0.29
trans neochlorogenic acid = trans 3-caffeoylquinic acid	3.47	2.61	0.93	0.55
trans chlorogenic acid = trans 5-caffeoylquinic acid	0.43	0.27	-	-
other hydroxycinnamic acids	0.72	0.36	0.67	0.55
total hydroxycinnamic acids	*5.61*	*3.99*	*2.24*	*1.93*
	flavanols			
catechin	0.03	0.002	0.07	0.07
epicatechin	0.03	0.001	0.18	0.15
gallocatechin	0.65	0.03	0.17	0.04
epigallocatechin	0.84	0.27	0.51	0.31
procyanidin B2 = epi-(4β→8)-epi*	0.08	0.02	0.67	0.50
procyanidin B5 = epi-(4β→6)-epi	0.01	0.001	0.18	0.09
procyanidin E-B5 = epi-(4β→6)-epi-(4β→6)-epi	-	-	0.04	0.02
other flavanols	3.54	0.95	3.74	1.94
total flavanols	*5.18*	*1.27*	*5.56*	*3.12*
	flavones			
flavone derivatives	*0.20*	-	*1.01*	-
	flavonols			
nicotiflorin = kaempferol 3-O-rhamnoglucoside	0.62	0.98	0.06	0.05
rutin = quercetin 3-O-rhamnoglucoside	1.99	1.32	0.38	0.28
quercetin 3-O-rhamnosylrutinoside	2.56	1.34	1.15	0.69
isorhamnetin 3-O-rhamnoglucoside	1.42	0.89	0.25	0.13
acylated flavonols	0.72	1.24	0.24	0.29
other flavonols	5.56	3.60	2.82	1.66
total flavonols	*12.87*	*9.37*	*4.92*	*3.09*

*epi is the abbreviation for epicatechin;
-, not detectable

Table IIA. Composition of phenolic compounds (milligrams per gram of dry weight) in organs of sainfoin plants of the variety Cotswold Common at the bud stage.

Compound	Young leaflets (n = 3)	Mature leaflets (n = 6)	Young petioles (n = 3)	Mature petioles (n = 6)	Stems (n = 6)	Flower buds (n = 6)
			simple phenolic acids			
arbutin	-	-	17.67 ± 7.33	13.51 ± 4.04	4.90 ± 1.03	-
8-β-glucopyranosyloxycinnamic acid	0.01 ± 0.01	-	1.94 ± 0.74	0.38 ± 0.27	1.80 ± 0.93	0.28 ± 0.15
other simple phenolic acids	2.59 ± 1.12	1.49 ± 0.65	3.31 ± 1.34	1.68 ± 0.62	0.72 ± 0.13	3.08 ± 0.92
total simple phenolic acids	*2.61*	*1.49*	*22.92*	*15.57*	*7.42*	*3.36*
			hydroxybenzoic acids			
vanillic acid 4-O-glucoside	-	0.08 ± 0.06	-	0.10 ± 0.06	0.07 ± 0.05	0.07 ± 0.03
			hydroxycinnamic acids			
cis+trans p-coumaric acid 4-O-glucoside	0.38 ± 0.25	0.45 ± 0.17	0.37 ± 0.15	0.41 ± 0.14	0.11 ± 0.04	0.34 ± 0.16
trans 3-p-coumaroylquinic acid	0.04 ± 0.04	0.07 ± 0.05	0.01 ± 0.01	0.09 ± 0.06	0.02 ± 0.01	0.03 ± 0.02
trans caffeoylglucose	0.08 ± 0.05	0.07 ± 0.05	0.06 ± 0.05	0.05 ± 0.04	0.03 ± 0.02	0.09 ± 0.06
trans neochlorogenic acid = trans 3-caffeoylquinic acid	0.97 ± 0.64	0.75 ± 0.55	0.37 ± 0.32	0.46 ± 0.36	0.14 ± 0.12	0.39 ± 0.22
trans chlorogenic acid = trans 5-caffeoylquinic acid	0.66 ± 0.43	0.35 ± 0.19	0.25 ± 0.04	0.07 ± 0.09	0.03 ± 0.02	0.28 ± 0.32
other hydroxycinnamic acids	1.32 ± 0.62	0.28 ± 0.11	0.82 ± 0.39	0.45 ± 0.17	0.25 ± 0.07	1.25 ± 0.36
total hydroxycinnamic acids	*3.45*	*1.97*	*1.87*	*1.53*	*0.58*	*2.39*
			flavanols			
catechin	0.59 ± 0.34	0.03 ± 0.22	3.46 ± 1.15	0.54 ± 0.26	0.53 ± 0.27	0.55 ± 0.33
epicatechin	0.22 ± 0.1	0.05 ± 0.03	1.23 ± 0.83	0.52 ± 0.27	0.29 ± 0.17	0.07 ± 0.04
gallocatechin	0.72 ± 0.36	0.09 ±0.05	0.28 ± 0.20	0.05 ± 0.05	0.07 ± 0.05	0.19 ± 0.08
epigallocatechin	0.35 ± 0.13	0.21 ± 0.12	0.36 ± 0.09	0.11 ± 0.05	0.08 ± 0.06	0.20 ± 0.09
procyanidin B2 = epi-(4β→8)-epi‡	0.03 ± 0.002	0.01 ± 0.01	0.30 ± 0.11	0.25 ± 0.13	0.09 ± 0.05	0.01 ± 0.004
procyanidin B5 = epi-(4β→6)-epi	-	-	0.09 ± 0.05	0.01 ± 0.04	0.02 ± 0.01	-
procyanidin E-B5 = epi-(4β→6)-epi-(4β→6)-epi	-	-	0.03 ± 0.01	0.01 ± 0.04	0.01 ± 0.004	-
other flavanols	1.59 ± 0.47	0.31 ± 0.09	2.53 ± 0.84	1.40 ± 0.50	0.54 ± 0.2	0.57 ± 0.35
total flavanols	*3.50*	*0.70*	*8.26*	*2.89*	*1.60*	*1.58*
			flavones			
flavone derivatives	1.22 ± 1.06	0.57 ± 0.45	1.86 ± 2.27	0.95 ± 0.90	0.53 ± 0.42	1.14 ± 1.21
			flavonols			
nicotiflorin = kaempferol 3-O-rhamnoglucoside	2.82 ± 0.98	3.77 ± 2.05	0.24 ± 0.04	0.34 ± 0.29	0.06 ± 0.02	1.31 ± 0.58
quercetin 3-arabinoside	0.79 ± 0.69	0.58 ± 0.76	0.72 ± 0.62	0.69 ± 0.88	0.43 ± 0.64	0.10 ± 0.11
isoquercitrin = quercetin 3-O-glucoside	0.57 ± 0.33	0.50 ± 0.36	0.60 ± 0.49	0.36 ± 0.27	0.17 ± 0.17	0.44 ± 0.24
rutin = quercetin 3-O-rhamnoglucoside	19.94 ±12.07	18.18 ± 7.13	9.14 ± 10.46	7.37 ± 4.42	2.57 ± 1.92	5.78 ± 2.30
quercetin 3-O-rhamnosylrutinoside	2.14 ± 2.00	2.28 ± 1.81	1.52 ± 2.12	1.85 ± 1.71	0.78 ± 0.76	0.58 ± 0.44
isorhamnetin 3-O-rhamnoglucoside	3.56 ± 3.08	2.45 ± 2.83	3.56 ± 3.08	1.72 ± 2.03	0.69 ± 0.84	0.29 ± 0.25
acylated flavonols	0.28 ± 0.21	0.89 ± 0.56	0.12 ± 0.07	0.35 ± 0.16	0.12 ± 0.07	0.06 ± 0.03
other flavonols	3.26 ± 1.08	4.91 ±1.28	1.53 ± 0.81	1.78 ± 0.27	0.56 ± 0.44	1.35 ± 0.70
total flavonols	*33.34*	*33.50*	*17.43*	*13.46*	*5.37*	*9.91*
			anthocyanins			
cyanidin 3-O-glucoside	-	-	0.04 ± 0.04	0.06 ± 0.04	0.18 ± 0.14	0.04 ± 0.04
other anthocyanins	-	-	-	-	0.05 ± 0.02	0.05 ± 0.03
total anthocyanins	-	-	*0.04*	*0.06*	*0.23*	*0.09*

*epi is the abbreviation for epicatechin
-, not detectable

Table IIIA. Composition of phenolic compounds (milligrams per gram of dry weight) in organs of sainfoin plants (variety Cotswold Common) at bloom stage.

Compound	Young leflets	Mature leaflets	Young petioles	Mature petioles	Stems	Flower buds	Open flowers	Mature flowers
			simple phenolic acids					
arbutin	-	-	17.07	13.97	5.94	-	-	-
8-β-glucopyranosyloxycinnamic acid	-	-	0.79	0.64	1.02	0.16	0.21	0.20
other simple phenolic acids	1.91	1.55	2.61	1.66	0.95	4.33	2.19	3.29
total simple phenolic acids	*1.91*	*1.55*	*20.47*	*16.27*	*7.91*	*4.49*	*2.40*	*3.49*
			hydroxybenzoic acids					
p-hydroxybenzoic acid	-	-	-	-	-	0.13	-	0.19
protocatechuic acid	-	-	-	-	-	-	0.03	-
vanillic acid 4-O-glucoside	0.08	-	0.10	0.20	0.05	0.07	0.06	0.09
total hydroxybenzoic acids	*0.08*	-	*0.10*	*0.20*	*0.05*	*0.20*	*0.09*	*0.28*
			hydroxycinnamic acids					
cis+trans p-coumaric acid 4-O-glucoside	0.33	0.45	0.30	0.33	0.11	0.33	0.15	0.24
trans 3-p-coumaroylquinic acid	0.05	0.07	-	0.06	0.01	0.02	-	0.01
trans caffeoylglucose	0.05	0.04	0.07	0.04	0.03	0.04	0.04	0.03
trans neochlorogenic acid = trans 3-caffeoylquinic acid	0.88	1.12	0.17	0.18	0.06	0.24	0.10	0.08
trans chlorogenic acid = trans 5-caffeoylquinic acid	-	-	-	-	-	-	-	-
other hydroxycinnamic acids	0.23	0.19	0.31	0.20	0.12	0.44	0.21	0.12
total hydroxycinnamic acids	*1.54*	*1.87*	*0.85*	*0.81*	*0.33*	*1.07*	*0.50*	*0.48*
			flavanols					
catechin	0.08	0.01	0.53	0.09	0.13	0.09	0.03	0.09
epicatechin	0.12	0.04	0.65	0.40	0.26	0.08	0.02	0.08
gallocatechin	0.09	0.02	0.05	0.01	0.02	0.21	0.16	0.20
epigallocatechin	0.62	0.11	0.27	0.11	0.1	0.64	0.31	0.64
procyanidin B2 = epi-(4β→8)-epi*	0.02	0.01	0.23	0.27	0.12	0.01-	-	0.01
procyanidin B5 = epi-(4β→6)-epi	-	-	0.06	0.05	0.01	-	-	-
procyanidin E-B5 = epi-(4β→6)-epi-(4β→6)-epi	-	-	0.02	0.01	0.01	-	-	-
other flavanols	0.74	0.26	0.86	0.75	0.36	0.35	0.11	0.56
total flavanols	*1.67*	*0.45*	*2.67*	*1.69*	*1.01*	*1.37*	*0.63*	*1.58*
			flavones					
total flavone derivatives	*0.28*	*0.13*	*0.54*	*0.49*	*0.20*	*0.25*	*0.09*	*0.21*
			flavonols					
nicotiflorin = kaempferol 3-O-rhamnoglucoside	4.00	5.50	0.33	0.27	0.07	0.52	0.16	0.26
isoquercitrin = quercetin 3-O-glucoside	0.29	0.29	0.58	0.56	0.26	0.42	0.13	0.26
rutin = quercetin 3-O-rhamnoglucoside	21.29	21.96	9.40	8.11	2.97	5.08	2.02	3.25
quercetin 3-O-rhamnosylrutinoside	1.05	1.20	1.59	1.50	0.63	0.86	0.40	0.61
kaempferol	-	-	-	-	-	0.08	0.05	-
acylated flavonols	0.43	0.79	0.26	0.17	0.01	0.42	-	-
other flavonols	2.18	3.48	0.80	0.99	0.41	0.94	0.33	0.38
total flavonols	*29.24*	*33.22*	*12.96*	*11.60*	*4.35*	*8.32*	*3.09*	*4.76*
			anthocyanins					
cyanidin 3-O-glucoside	-	-	-	-	0.01	-	0.01	0.03
delphinidin 3-O-glucoside	-	-	-	-	-	-	0.16	0.41
other anthocyanins	-	-	-	-	-	-	0.17	0.49
total anthocyanins	-	-	-	-	-	-	*0.34*	*0.93*

*epi is the abbreviation for epicatechin
-, not detectable

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LIST OF PUBLICATIONS

Publications emerged from this work

THILL, J., REGOS, I., AHMAD, A.F., KUSEK, J., HAYOT CARBONERO, C.H., GADJEV, I.Z., SMITH, L., HALBWIRTH, H., TREUTTER, D., STICH, K., 2012: Polyphenol metabolism provides a screening tool for beneficial effects of *Onobrychis viciifolia* (sainfoin). Phytochemistry 82, 67-80

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