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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*).

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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

0 20 40 60 80 100 120 140 160 180 min

15 — 67 HBA
29 — 116 HCA
48 — 81 Flavanols
72 — 157 Anthocyanins
110 — 139 Dihydroflavonols
112 — 138 Flavones
127 — 177 Flavonols

Nucleosil C18 (B)

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	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

0 20 40 60 80 100 120 140 160 180 min

8 — 30 HBA
14 — 67 HCA
22 — 40 Flavanols
55 — 80 Dihydroflavonols
77 — 152 Anthocyanins
83 — 109 Flavones
94 — 161 Flavonols

Nucleodur Spinx (C)

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	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

0 20 40 60 80 100 120 140 160 180 min

7 — 32 HBA
15 — 67 HCA
21 — 37 Flavanols
44 — 133 Anthocyanins
59 — 86 Dihydroflavonols
71 — 95 Flavones
71 — 158 Flavonols

[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.

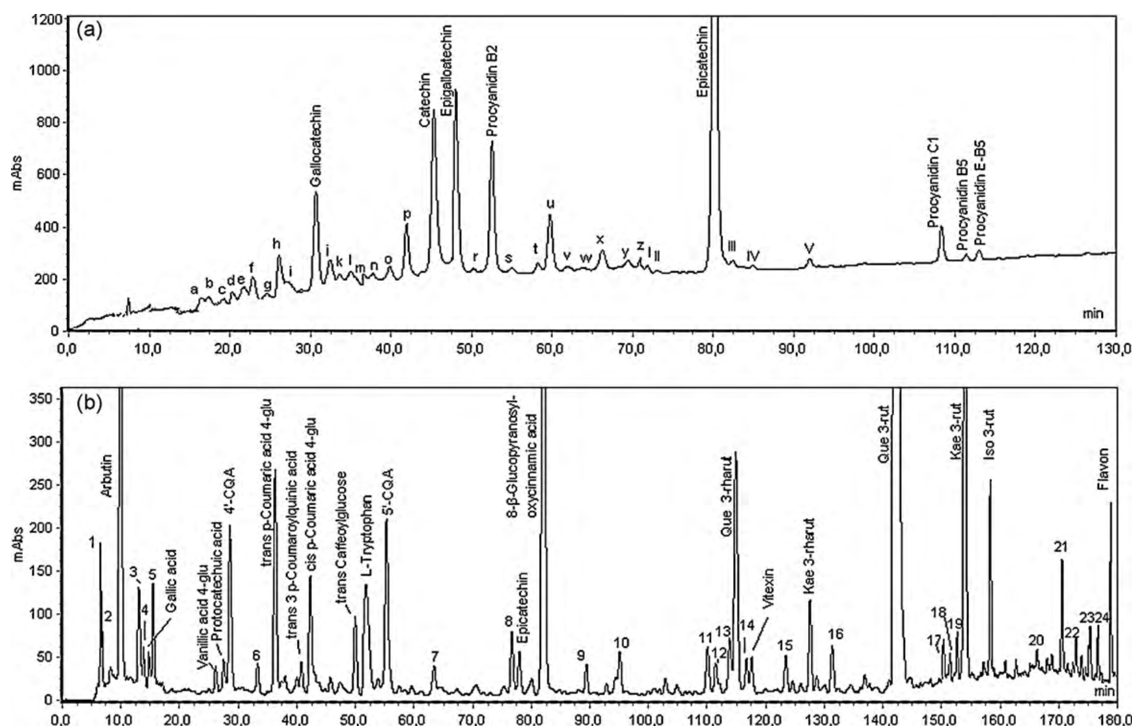

Fig. 1. Chromatograms obtained at 640 nm after post-column derivatization with DMACA (a) and 280 nm (b) from an 80% aq. methanolic extract of whole plants of *Onobrychis viciifolia* variety Cotswold Common using column A (Luna PFP) and system 1 described in Table 2. Abbreviations used: CQA, caffeoylquinic 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,

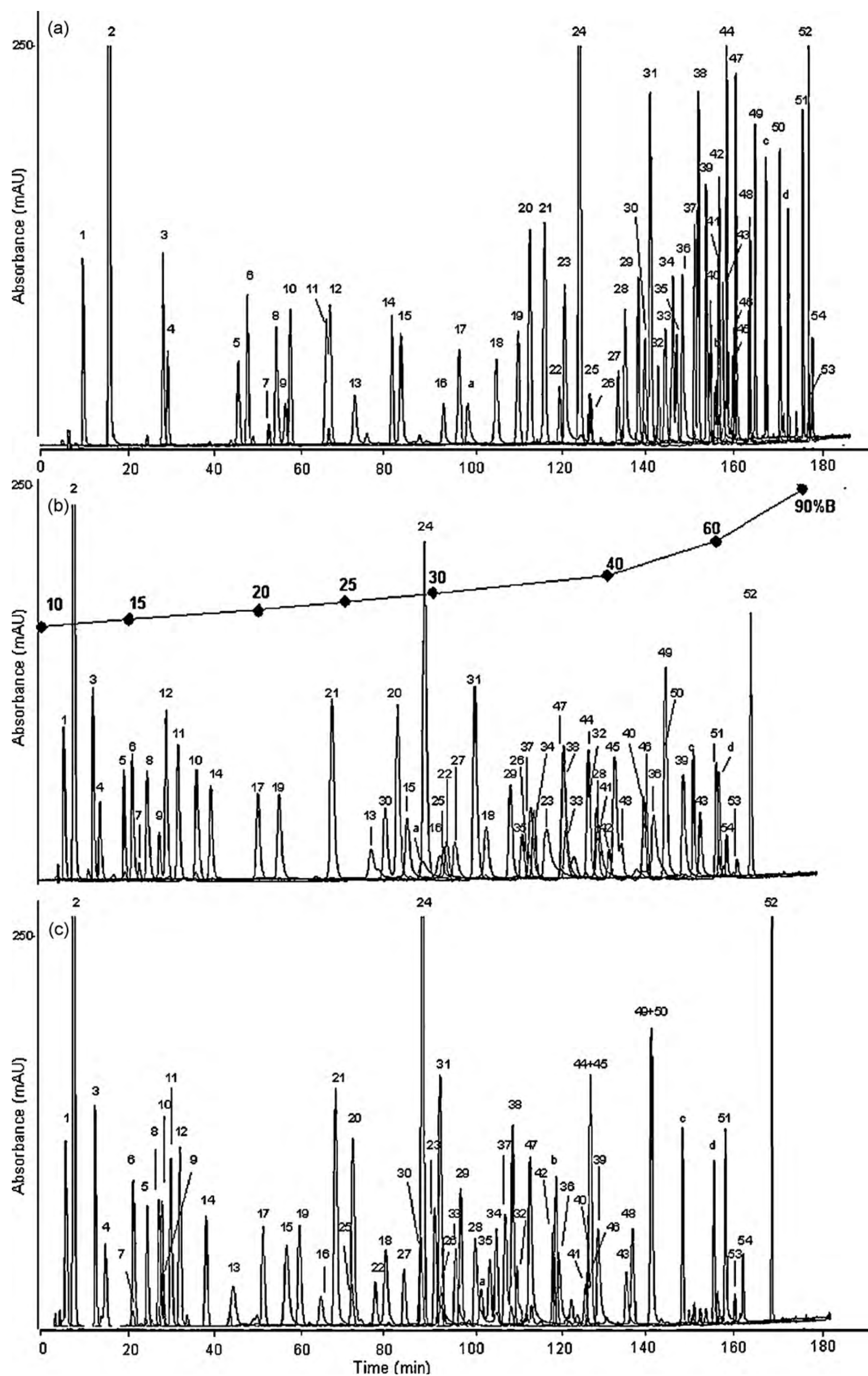

Fig. 2. Chromatogram overlays at 280 nm for the mixtures of standard phenolic compounds employed in the optimization of chromatographic method obtained with: (a) column A (Luna PFP); (b) column B (Nucleosil C18); (c) column C (Nucleodur Spinx) and the gradient steps given in subfigure b. For peak numbers,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

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