Seasonal variations of the phenolic constituents in bilberry (Vaccinium myrtillus L.) leaves, stems and fruits, and their antioxidant activity

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Seasonal variations of the phenolic constituents in bilberry (*Vaccinium myrtillus* L.) leaves, stems and fruits, and their antioxidant activity

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A B S T R A C T

The seasonal variations of the content and diversity of phenolic compounds, as well as the antioxidant activity of leaves, stems and fruits of bilberry collected in May, July and September, were evaluated for two consecutive years. UPLC/MS^n analyses showed the predominance of anthocyanins in fruits, caffeic acid derivatives in leaves whereas flavanol oligomers represented more than half of the phenolic compounds in stems. Thioacidolysis revealed degrees of polymerization between 2 and 4 and (−)-epicatechin as the main flavanol unit. The sum of the phenolic compounds by UPLC was highly correlated with the total polyphenol content and the antioxidant activity in the DPPH test for all the extracts except for May leaves. The latter were relatively rich in *p*-coumaric acid derivatives. Seasonal effects were more marked for leaves, which exhibited higher antioxidant activities and phenolic contents in July and September when these parameters were at their highest in July for stems.

Keywords:
Polyphenols
Antiradical capacity
Vegetation periods
UPLC/MS^n
Folin–Ciocalteu
DPPH, Thioacidolysis

1. Introduction

Bilberry (*Vaccinium myrtillus L.*), also known as European blueberry, whortleberry, and huckleberry, is a wild shrub, which can be found in the mountains and forests of Europe, and the north of America. Fruits and aerial parts of bilberry are known as a natural source of food and beverage, due to its richness in nutritional and bioactive compounds and are also consumed as dietary supplements and pharmaceutical products for health benefits. Bilberry fruit extracts have been studied for the prevention and treatment of chronic pathologies, such as diabetes, cardiovascular disease and obesity (Erlund et al. 2008; Mauray et al., 2010; Mykkänen et al., 2014; Rouanet et al., 2010). Anti-inflammatory properties of bilberry fruits are central to this health protection.

Leaves and stems of bilberry are used as herbal tea, the most consumed form, or hydro-alcoholic extract in traditional herbal medicine and have also been shown to exhibit antibacterial and antioxidant activities (Vučić et al., 2013). These benefits are attributed to the high content in phenolic compounds (flavonoids, phenolic acids and proanthocyanidins) in bilberry leaves (Martz, Jaakola, Julkunen-Tiitto, & Stark, 2010). The *in vitro* and *in vivo* biological activities of phenolic compounds from natural sources involve application as antioxidants, antibacterial and anticarcinogenic agents, allelochemicals, and plant growth regulators (Bujor, Talmaciu, Volf, & Popa, 2015).

The quality and quantity of phenolic compounds in *V. myrtillus* L. are generally influenced by the parts of the plant to be used, the stage of growth, the environmental conditions and genetic factors (Akerström, Jaakola, Bång, & Jäderlund, 2010; Jovančević et al., 2011; Martz et al., 2010; Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2015; Uleberg et al., 2012). It may appear that higher phenolic contents are favoured by northern latitudes, altitude and a sunny environment. In bilberry fruits, high amounts of anthocyanins and hydroxycinnamic acid derivatives as well as low amounts of flavonols, proanthocyanidins and coumaroyl iridoids were identified (Mikulic-Petkovsek et al., 2015). In contrast, leaves are known to contain, in decreasing levels, hydroxycinnamic acids, flavonol glycosides and proanthocyanidins but also cinchonains and iridoids in unknown amounts (Liu et al., 2014; Martz et al., 2010). A comparative study conducted by Teleszko and Wojdyło (2015) showed that phenolic compounds were found in a markedly higher content in leaves than in fruits, in agreement with the strongest antioxidant capacity displayed by leaves compared to fruits.

To date, most works have focused on the study of a single morphological part of the bilberry plant, fruits most commonly, leaves and stems sometimes. In this context, the primary aim of this study is to simultaneously assess the seasonal variations of phenolic

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compounds and antioxidant activity in leaves, stems, and fruits of bilberry, with the purpose of determining which harvest period or part of the *Vaccinium* plant can be selected as the best source of phenolic antioxidants. Indeed, a good knowledge of the phenolic compound distribution in the various plant tissues of bilberry can play a key role in guiding their fields of use, either as nutraceuticals or food additives. Moreover, this knowledge is of the upmost importance to investigate the mechanisms involved in the health effect of these different polyphenol extracts. Thus, contents in total polyphenols were assessed globally by the Folin–Ciocalteu method or specifically by UPLC/MS and were tentatively correlated with the antioxidant capacity in the DPPH test. Finally, an original analysis of the oligomeric proanthocyanidins is proposed, addressing both the degree of polymerisation and flavanol unit constitution.

2. Methodology

2.1. Materials

2.1.1. Bilberry samples

Leaves and stems of wild bilberry (*V. myrtillus* L.) were collected in May, July and September, while the fruits were collected in July at the ripening stage during the years 2013 and 2014, from mountains near Borca (Neamt, Romania, coordinates: 47° 11′34″ N and 25° 47′ 8″ E).

Fresh bilberry fruits were frozen at −24 °C, then lyophilised in a Christ Alpha 1-4 LSC (Osterode, Germany) freeze dryer for 3 days and finally ground for 25 s at 2000 rpm in a knife mill (Retsch Grindomix GM 200) to a fine powder. Leaves and stems of bilberry were dried at room temperature, in the shade, for 7 days. After drying, leaves were manually separated from stems, ground as above and sieved to a final particle size <0.315 mm. Before extraction, the residual moisture of grinded samples was determined using a RADWAG MAX 50/1 moisture analyzer (RADWAG Balances & Scales, Radom, Poland). Residual moistures between 7% and 9.5% were found for all plant materials.

2.1.2. Chemicals and solvents

Chemicals: Anhydrous sodium carbonate, 37% hydrochloric acid, Folin and Ciocalteu's phenol reagent, DPPH (1,1-diphenyl-2-picrylhydrazyl), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and toluene-α-thiol were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Polyphenol standards, gallic acid, chlorogenic acid, *p*-coumaric acid, (+)-catechin, (−)-epicatechin were from Sigma-Aldrich; quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-galactoside, procyanidin B2, procyanidin A2, (−)-epigallocatechin and cyanidin-3-*O*-galactoside were purchased from Extrasynthese (Genay, France); procyanidin C1 was extracted from apple fruits (*Malus domestica* Borkh.) of the Kermerrien variety as described previously (Watrelot, Renard, & Le Bourvellec, 2015).

Solvents: 96% ethanol was purchased from S.C. Chemical Company (Iași, Romania), HPLC–MS grade methanol and acetonitrile from Fisher Scientific (Illkirch, France), formic acid from Merck (Darmstadt, Germany), and glacial acetic acid from Merck (Fontenay Sous Bois, France). Ultrapure water (resistivity 18.2 MΩ.cm^{-1} at 25 °C) was obtained with a Milli-Q water purification system (Millipore, Bedford, MA).

2.2. Extraction of phenolic compounds

To 1 g of ground bilberry leaf, stem and fruit samples placed in an extraction vial fitted with a condenser was added 30 mL of 1% aqueous citric acid. Next, the mixture was extracted in a Milestone START S microwave oven for synthesis, at a microwave power of 300 W, for 7 min and a temperature of 40 °C (Zheng et al., 2013). Additionally, the fruit samples were extracted with 55% aqueous ethanol (*v/v*) under the same extraction conditions. The extracts were filtered and then the volume of each sample was adjusted to 30 mL prior to the determination of the total phenolic content (TPC). The extract solutions were freeze-dried and the dry extracts (DE) stored at 4 °C before use. Triplicate extractions were made for each sample. Dry matter (DM) refers to the initially ground dry sample after correction from the residual water.

2.3. Qualitative and quantitative analyses of phenolic compounds

For UPLC/MS analyses, freshly prepared solutions of leaf and stem dry extracts (10 mg/mL) in water were directly used. For the fruit dry extract, purification was first conducted to eliminate sugars and organic acids that could interfere in the analysis of phenolic compounds. Solutions of fruit extracts at 20 mg/mL prepared in 1% aqueous HCl (*v/v*) were purified by elution on C18 Sep-Pak Plus mini-columns (360 mg; Waters, Milford, MA). The C18 cartridge was first conditioned with two column volumes of 0.01% HCl in methanol followed by three volumes of 0.01% aqueous HCl (*v/v*) to remove remaining methanol. Secondly, the extracts were injected onto the mini-column and then the cartridge was washed with two volumes of 0.01% aqueous HCl. Finally, the phenolic compounds were eluted with 0.01% HCl in methanol. The phenolic fractions were immediately subjected to UPLC/MS analyses.

Separation and identification of phenolic compounds were performed using a Waters ACQUITY UPLC chromatograph (Waters, Milford, MA) coupled to a UV−Vis diode-array detector and an HCT ultra ion trap mass spectrometer equipped with electrospray ionisation (Mane, Loonis, Juhel, Dufour, & Malien-Aubert, 2011).

Polyphenol standards in MeOH, except for cyanidin-3-galactoside, which was further acidified with 1% HCl (v/v), were used for 6-point calibrations. Other phenolic compounds were quantified as follows: caffeic acid derivatives and sinapic acid hexoside as chlorogenic acid (325 nm), coumaric acid derivatives as *p*-coumaric acid (330 nm), quercetin glycosides as quercetin-3-*O*-galactoside (350 nm), A-type dimers as procyanidin A2 (280 nm), B-type dimers and cinchonains II as procyanidin B2 (280 nm), A-type and B-type trimers as procyanidin C1 (280 nm), cinchonains I as (−)-epicatechin, 3,4-dihydroxyphenylpropionic acid hexoside as (+)-catechin and anthocyanins as cyanidin-3-*O*-galactoside (520 nm). The flavanol monomers were calculated as the sum of (−)-epicatechin and (−)-epigallocatechin or cinchonain I, while the flavanol oligomers were reported as the sum of all the dimers, trimers, and cinchonains II. Injected volumes were 3 µL for phenolic compounds and 1 µL for anthocyanins. All samples were injected in triplicate from independently prepared solutions of dry extracts.

2.4. Analysis of procyanidins using thioacidolysis

Procyanidin analysis was performed after thioacidolysis in the presence of toluene-α-thiol using a method adapted from Le Bourvellec et al. (2011). Procyanidins were characterised by their subunit composition and their average degree of polymerisation (mDP). The HPLC apparatus was a Shimadzu LC-20AD equipped with a SPD-M20A DAD detector and an RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan). Separations were achieved using a Lichrocart column (250 mm × 4 mm i.d, Lichrospher PR-18 5 mm) with a guard column (Lichrospher PR-18 5 mm column; Merck, Darmstadt, Germany) operated at 30 °C (20 µL injected). (+)-Catechin benzyl thioether was quantified at 280 nm as (+)-catechin whereas (−)-epicatechin benzyl thioether was quantified as (−)-epicatechin. In samples containing anthocyanins, (+)-catechin and (−)-epicatechin were specifically identified and

quantified by their excitation-emission energy (278 nm and 360 nm), in order to avoid overlapping peaks due to anthocyanin absorbance at 280 nm.

2.5. Antioxidant activity by applying spectrophotometric methods

2.5.1. Total phenolic contents by the Folin Ciocalteu method

The total phenolic content (TPC) of the extract solutions was determined by the Folin-Ciocalteu spectrophotometric method described by Hainal, Ignat, Volf, and Popa (2011). An aliquot of 1 mL of the diluted extract (1:50 for leaves and stems, and 1:25 for fruits) was mixed with 0.5 mL of the Folin-Ciocalteu reagent (2 M), 2 mL of 10% aq. Na_2CO_3 and 5 mL H_2O. Then, the mixture was left for 90 min in the dark at room temperature. Absorbance was measured at 765 nm (CINTRA 101 UV–VIS spectrometer; GBC Scientific Equipment Ltd., Dandenong, Australia) using a mixture of water and reagents as a blank. The results were expressed as mg of gallic acid equivalents per gram of dry matter (mg GAE/g DM) after correction from residual moisture. Triplicates from independent extract solutions were analysed.

2.5.2. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging test

The DPPH test was adapted from a method developed by Goupy, Dufour, Loonis, and Dangles (2003). Small volumes (25 µL) from dry extracts freshly prepared in water (10 mg/mL) were added to 2 mL of a 0.2 mM solution of DPPH in methanol. The decay of the absorbance at 515 nm (HP 8453 diode-array spectrometer, optical path length = 1 cm) was recorded for 30 min at 25 °C under constant magnetic stirring (1250 rpm). The results were expressed as micromoles of Trolox equivalents (TE) per gram of dry extract using Trolox calibration curves. All determinations were carried out three to four times and independent extract solutions were used each time.

2.6. Statistical analyses

Results are expressed as the mean ± standard deviation (SD). Significant differences at a 95% confidence interval were assessed through the analysis of ANOVA with Tukey-Kramer post hoc test using XLStat software (version 2008.3.02; Addinsoft SARL, Paris, France).

3. Results and discussion

3.1. Phenolic profile and content of bilberry extracts from UPLC/MS analyses

In all the morphological parts of bilberry 106 phenolic compounds were tentatively identified, with 62 in leaf, 73 in stem and 40 in fruit extracts (Table 1). Additionally, 17 phenolic compounds were found only in leaves, 32 only in stems, and 9 only in fruits. Maximum absorption wavelength, molecular ion and fragmentation pattern in MS were used for structure assessment in the absence of standards.

3.1.1. Caffeic acid derivatives

In leaves, caffeic acid derivatives are present in 3- to 10-fold higher levels compared to coumaric acid derivatives, whereas there is no content difference in stems in these two hydroxycinnamic acid derivatives (Table 2). Caffeic acid derivatives were principally found in leaves as caffeic acid esterified with quinic acid, shikimic acid and monotropein or esterified/etherified with a hexose moiety.

Caffeoylquinic acids grafted with a hexosyl group were represented by two diversely polar molecules (**9** and **51**). Both of them were newly identified in bilberry. Compound **9** with fragment ions at *m/z* 353 and 191 was assigned as 5-*O*-caffeoylquinic acid-4′-*O*-hexoside, based on the fragmentation pattern similar to that of 5-*O*-caffeoylquinic acid and the hypsochromic shift in the spectrum. Compound **51** displayed a major fragment ion at *m/z* 341 (caffeic acid hexoside) and fragments at *m/z* 191 and 173 (typical of 4-*O*-caffeoylquinic acid). The four compounds displaying a parent ion at *m/z* 707 (MS) and major fragments at *m/z* 353 (MS^2) and at *m/z* 191 (MS^3) are presumably caffeoylquinic acid derivatives (**20**, **22**, **24** and **29**). The presence of two further hydrogens when comparing with the structure of a caffeoylquinic acid covalent dimer as well as a λ_{max} at 282 nm suggest that the α,β-unsaturated double bond in caffeic acid is no longer present. A fragment at *m/z* 515 could be interpreted as an additional caffeoyl or hexosyl unit on caffeoylquinic acid. Related caffeoylquinic acid derivatives (**50**, **56**), sharing a parent ion at *m/z* 705 and a sole fragment ion at *m/z* 513, display a maximal absorption wavelength at 320 nm. They could be caffeoylquinic acid covalent dimers or result from the oxidation of the previous caffeoylquinic acid derivatives with *m/z* 707. Another caffeoylquinic acid derivative (**74**), with a parent ion at *m/z* 381 and fragmentation ions at *m/z* 191, 179, 161 and 135 typical for caffeoylquinic acid, was identified in leaves (Ieri, Martini, Innocenti, & Mulinacci, 2013) and newly described in fruit.

Caffeic acid can be covalently bound to glycosyl residues in two different manners through esterification or etherification. Etherification of the 4-hydroxyl group of caffeic acid led to two isomers of caffeic acid-4-*O*-*β*-D-hexoside (**9** and **17**) whose structures are supported by the lack of clear absorption at λ_{max} 320 nm (Mane et al., 2011). The first isomer was present in leaves and fruits while the second was in fruits only. By analogy with the fragmentations of *p*-coumaroyl malonylhexosides (**83** and **94** on one side, and **96** and **97** on the other side), compounds **68** and **87** were assessed as caffeoyl malonylhexosides. Caffeoyl malonylhexosides, which have been identified in all the morphological parts of bilberry, are newly named in this study (Ieri et al., 2013).

3.1.2. Coumaric acid derivatives

In leaves and stems, two *p*-coumaroylquinic acids (**30** and **44**) were assigned respectively to the *cis* and *trans* forms of 5-*p*-coumaroylquinic acid, based on their major fragment ion at *m/z* 191 (quinic acid) resulting from the loss of *p*-coumaric acid and the hypsochromic shift in the UV–Vis spectrum of **44** (Clifford, Knight, Johnston & Kuhnert, 2003). Additionally, four hexosides of *p*-coumaric acid (**7**, **8**, **14** and **21**) were detected in the various morphological parts. Compound **8** displays a λ_{max} at 295 nm, which is characteristic of the electronic density modification induced by the glycosylation at the *O*-4 position (Chanforan, Loonis, Mora, Caris-Veyrat, & Dufour, 2012). Derivatives with a λ_{max} at 310 nm are esters of *p*-coumaric acid. *p*-Coumaric acid hexosides can be further acylated by acetic acid and malonic acid. Indeed, two *p*-coumaroyl diacetylhexosides with *m/z* 409 (**78** and **92**) were tentatively identified in all the morphological parts based on fragment ions at *m/z* 325 (loss of 2 acetyl groups) and *m/z* 163 typical of *p*-coumaric acid. Also, three *p*-coumaroyl triacetylhexosides with *m/z* 451 (**98**, **102** and **105**) were only observed in leaves. Two of them were characterised by fragment ions at *m/z* 367 (loss of 2 acetyl groups) and *m/z* 245 (loss of both acetyl and *p*-coumaroyl groups), while the other isomer displayed a first fragment at *m/z* 341.

Putative malonylated derivatives comprise four *p*-coumaroyl malonylhexosides, which are present in leaves, stems, and fruits,

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Table 1
Phenolic compounds identified by UPLC/ESI-MS^n in leaf, stem and fruit extract of bilberry.

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013			2014		
						May	July	Sept.	May	July	Sept.
1	1.65	270	305	287, 261, 219, 179, 165, 125	(+)-Gallocatechin[c]	S	S	S	S	S	S
2	1.7	240	389	227, 209, 183, 165, 139	Monotropein[c]	S	S	S	S	S	S
3	1.9	288	329	167, 152; MS^3 [167]: 152, 123, 108	**Hydroxymethoxybenzoic acid – hexose**	S[a]	S[a]	S[a]	S[a]	S	S
4	2.1	278	315	153, 123	**Dihydroxybenzoic acid – hexose**	S[a]	S	S	S	S	S
5	2.45	278	451	405, 289, 245, 161	**(epi)Catechin derivative (1)**	S	S	S	S	S	S
6	2.55	290sh, 310	447	315, 271, 207, 152	**Dihydroxybenzoic acid – hexose-pentose**	S[a]	S	S	S	S	S
7	2.6	290sh, 310	325	307, 187, 163, 119	***p*-Coumaroylhexoside (1)**	L, S[a]	L[a], S, F_{H_2O}, F_{EtOH}	L[a], S	L, S	L[a], S, F_{H_2O}, F_{EtOH}	L, S
8	2.7	295, 306sh	325	163, 119	***p*-Coumaric acid-4-*O*-hexoside (1)**[h]	L	L	L	L	L[a]	L
9	2.8	290, 320sh	515	353, 191	**5-*O*-Caffeoylquinic acid-4′-*O*-hexoside**[i]	L	L	L	L[a]	L	L
10			341	179, 135	**Caffeic acid-4-*O*-β-D-hexoside (1)**[h]	L	L, F_{H_2O}, F_{EtOH}[a]	L	L	L, F_{H_2O}, F_{EtOH}	L
11	2.9		593	575, 467, 441, 423, 305, 287, 273	(epi)Gallocatechin-(epi)catechin dimer (1)	S[a]	S	S	S[a]	S	S
12	3.05	278	863	711, 575MS^3 [575]: 499, 489, 451, 289, 287, 245	A-type trimer (1)	S	S	S	S	S	S
13	3.1	279	405	289, 179	**(epi)Catechin derivative (2)**	–	L[a]	L	L[a]	–	L
14	3.3	290sh, 312	325	307, 187, 163	***p*-Coumaroylhexoside (2)**	L, S[a]	L, S	L, S	L, S	L, S	L, S
15	3.6	270	305	287, 261, 221, 219, 179, 165, 125	(−)-Epigallocatechin (std)[c]	S	S, F_{H_2O}[a], F_{EtOH}[a]	S	S, F_{H_2O}[a], F_{EtOH}[a]	S	S
16	3.7	278	577	559, 451, 425, 407, 289, 245	Procyanidin B1 (std)	S	S	S	S[a]	S[a]	S[a]
17			341	179, 135	**Caffeic acid-4-*O*-β-D-hexoside (2)**[i]		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}	
18	3.8	295sh, 324	353	191	5-*O*-Caffeoylquinic-acid (std)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
19	3.9		593	575, 467, 441, 423, 305, 287, 245	(epi)Gallocatechin-(epi)catechin dimer (2)	S	S	S	S	S	S
20	4.2	282	707	533, 515, 463, 393, 341, 323, 297	**Caffeoylquinic acid derivative (1)**	L	L	L	L	L	L[a]
				MS^3 [353]: 191		L	L	L[a]	L	L	L
21	4.3		325	163, 119	***p*-Coumaric acid hexoside (2)**	S[a]	S[a]	S	S	S	S
22	4.55	282	707	533, 515, 463, 359, 353, 323, 321, 295	**Caffeoylquinic acid derivative (2)**	L	L	L	L	L	L
				MS^3 [353]: 191		L[a]	L	L	L	L	L
23			343	298, 221, 181, 161, 137	**3,4-Dihydroxyphenylpropionic acid hexoside**[h]	S	S	S	S	S	S
24	4.65	282	707	533, 515, 463, 393, 359, 323, 297, 271, 219	**Caffeoylquinic acid derivative (3)**	L	L	L	L[a]	L	L
				MS^3 [353]: 323, 297, 289, 231, 191, 179, 173		L[a]	L	L	L[a]	L	L
25	5.15	278	577	559, 531, 451, 425, 407, 289	B-type dimer (2)	L[a], S	L, S	L[a], S	L[a], S	L[a], S	L[a], S
26			451	289, 245, 161	Cinchonain I (1)[c]	S[a]	S	S	S[a]	S	S
27	5.25		385	223	**Sinapic acid hexoside**	S	S[a]	S	S	S	S
28	5.7	278	865		B-type trimer (1)	S[a]	S[a]	S[a]	S[a]	S[a]	S[a]
29		282	707	533, 513, 489, 353, 323	**Caffeoylquinic acid derivative (4)**	L[a]	L	L	L[a]	L[a]	L
				MS^3 [353]: 191		L	L	L[a]	L[a]	L	L
30	5.75	290sh, 310	337	191, 163	5-*p*-Coumaroylquinic acid[c,g]	L, S[a]	L, S	L, S[a]	L, S	L, S	L, S
31	6.2	290sh, 312	353	191	5-Caffeoylquinic acid (cis)[h]	L	L	L	L	L	L
32	6.3	278	577	559, 451, 425, 407, 289, 245	Procyanidin B2 (std) (3)	L[a], S	L, S	L, S	S	L, S	L[a], S
33	6.4	278	865	847, 739, 713, 695, 587, 577, 575, 451, 425, 407, 289, 287,	B-type trimer (2)	L[a], S	L, S	L, S	S[a]	L[a], S	S
34	6.85	278	289	245, 205, 125	(−)-Epicatechin (std)[c,e,f,g]	L[a], S	L, S, F_{H_2O}[a], F_{EtOH}[a]	L, S	L, S, F_{H_2O}[a], F_{EtOH}[a]	L, S	L, S
35	7.0		335	179, 135	Caffeoylshikimic acid[c,e]	L	L[a]	L	L	L[a]	L
36	7.3	278	881	863, 755, 711, 593, 575, 467, 423, 305, 287	(epi)Gallocatechin–(epi)catechin-(epi)catechin trimer	S	S	S	S	S	S
37			451	289, 245	Cinchonain I (2)	–	S[a]	S[a]	S[a]	S[a]	S
38	7.35		739	721, 649, 619, 587, 497, 449, 359, 329, 287	Cinchonain II (1)[c]	S[a]	S	S[a]	S[a]	S[a]	S[a]
39	7.55	278	879	727, 709, 559, 467, 411, 305, 287, 285	A-type trimer of (epi)gallocatechin-(epi)catechin-(epi)catechin	S	S	S	S[a]	S[a]	S
40	7.60	278	576[b]	567, 500, 491, 451, 407, 289, 287, 245	B-type tetramer (1)	S	L[a], S	L[a], S[a]	S[a]	L[a], S	L[a], S[a]

(*continued on next page*)

Table 1 (*continued*)

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013			2014		
						May	July	Sept.	May	July	Sept.
41	7.80	278	863	711, 693, 573, 531, 451, 411, 289, 287	A-type trimer (2)	S	S	S[a]	S[a]	S	S
42	7.90	278	577	559, 541, 533, 451, 439, 425, 393, 329, 289, 245	B-type dimer (4)	S	S	S[a]	–	S	S
43	8.10		576[b]	567, 500, 491, 451; 407, 289, 287, 245	B-type tetramer (2)	S	L, S	L, S	L[a], S	L[a], S	L[a], S
44	8.25	285, 305sh	337	191, 163	**5-*p*-Coumaroylquinic acid (cis)**	L	L	L[a]	L	L	L
45	9.05	278	863	711, 693, 573, 559, 531, 451, 411, 289, 285	A-type trimer (3)	S	S	S	S	S	S
46	9.50	278	865	847, 739, 713, 695, 577, 543, 451,449, 425, 407, 287	B-type trimer (3)	L[a], S	L, S	L, S	L[a], S	L, S	L[a], S
47	9.60		575	413, 395, 377, 351, 287, 266, 204, 165	A-type dimer (1)	L, S[a]	L[a], S[a]	L[a], S[a]	L, S[a]	L[a], S[a]	L[a], S[a]
48	9.70	278	593	575, 467, 441, 423, 305, 287	(epi)Gallocatechin-(epi)catechin dimer (3)	S	S	S	S	S	S
49	9.80		577	559, 451, 425, 407, 289, 287	B-type dimer (5)	S	S	S[a]	S[a]	S	S
50	10.00	320	705	513	**Caffeoylquinic acid derivative (5)**	L	L	L[a]	L	L[a]	L[a]
51	10.35	278	515	341, 323, 297, 281, 255, 191, 173	**Caffeoylquinic acid hexoside (2)**	L[a]	L	L	L[a]	L[a]	L[a]
52	10.45	278	575	520, 499, 490, 452, 423, 289, 245	A-type dimer (2)	S	S	S[a]	S	S	S
53	10.60	320	705	513	**Caffeoylquinic acid derivative (6)**	L	L[a]	L[a]	L	L[a]	L[a]
54		278	359	197, 153	**Syringic acid hexoside**		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}	
55	10.65	278	576[b]	559, 521, 500, 491, 451, 413, 289, 287, 245	B-type tetramer (3)	S	L, S	L, S	L[a], S	L[a], S	L[a], S
56	10.95		595	475, 463, 445, 343, 300, 271, 255	**Quercetin pentosyl hexoside**	S	S	S	S	S	S
57	11.00	295sh, 324	551	507, 389, 371, 345, 327, 179	Caffeoyl monotropein[e]	L	L[a], F_{EtOH}	L[a]	L	F_{H_2O}, F_{EtOH}	–
58	11.05	278	865	847, 739, 713, 695, 577, 575, 451, 407, 287, 245	B-type trimer (4)	S[a]	S	S[a]	S[a]	S[a]	S[a]
59	11.3	278	739	721, 629, 587, 569, 435, 417, 339, 289	Cinchonain II (2)	L[a], S	L, S	L, S	L[a], S	L, S	L, S
60	11.7	278	451	341, 217	Cinchonain I (3)	L[a], S	L, S	L, S	L[a], S	L, S	L, S
61	11.95	254, 350	477	301	Quercetin hexuronide (1)	L, S	L, S	L, S	L, S	L, S	L, S
62			463	301	Quercetin-3-*O*-galactoside (std)[g]	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
63			535	491, 371, 329, 311, 267, 191, 163	*p*-Coumaroyl monotropein (1)[c,e,g]	L, S	L[a], S, F_{H_2O}, F_{EtOH}	L[a], S[a]	L[a], S[a]	L[a], S, F_{H_2O}, F_{EtOH}	L[a], S
64	12.25	255, 352	477	301	Quercetin hexuronide (2)[g]	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
65	12.50	279, 307	455	309, 291, 163, 145	*p*-Coumaric acid derivative[e]	S	S	S	S	S	S
66	12.70	254, 354	477	301	Quercetin hexuronide (3)	L	L[a]	L	L	L	L
67			463	301	Quercetin-3-*O*-glucoside (std)	L	L, F_{H_2O}, F_{EtOH}	L	L	L, F_{H_2O}, F_{EtOH}	L
68			427	323, 179, 161, 135	**Caffeoyl malonylhexoside (1)[e,f]**	L, S[a]	L, S[a]	L[a], S[a]	L, S	L, S	L[a], S[a]
69	12.9	280, 310sh	697	535, 371	***p*-Coumaroyl monotropein hexoside**	L[a]	F_{H_2O}, F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}	–
70	13.00	285sh, 312	535	491, 371, 329, 311, 267, 191, 163	*p*-Coumaroyl monotropein (2)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L[a], S
71	13.25	306	537	493, 373, 331, 313, 193, 163	***p*-Coumaroyl dihydromonotropein (1)**	S[a]	L[a], S[a], F_{H_2O}, F_{EtOH}[a]	L[a]	S[a]	L[a], F_{H_2O}[a], F_{EtOH}[a]	–
72		354	493	331, 316	3′-*O*-Methylmyricetin hexoside[d]	–	F_{H_2O}, F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}	–
73	13.40	254, 352	433	301	Quercetin pentoside (1)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
74	13.55	324	381	191,179, 161, 135	**Caffeoylquinic acid derivative (7)[e]**	L	L, F_{H_2O}, F_{EtOH}	L[a]	L	F_{H_2O}, F_{EtOH}	–
75			577	559, 451, 425, 407, 289, 287, 245	B-type dimer (6)	S	L, S, F_{H_2O}, F_{EtOH}[a]	L, S	L, S	L[a], S, F_{H_2O}[a], F_{EtOH}	L[a], S
76	13.75	312	537	493, 373, 331, 313, 193, 163	***p*-Coumaroyl dihydromonotropein (2)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L[a], S	L, S	L, S, F_{H_2O}, F_{EtOH}	S
77	13.8		433	301, 271	Quercetin pentoside (2)	–	F_{H_2O}, F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}[a]	–
78	13.95		409	325, 307, 217, 187, 163, 159, 145	***p*-Coumaroyl diacetylhexoside (1)[e]**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
79			461	285	Kaempferol hexuronide[c,e,f]	L	L, F_{H_2O}, F_{EtOH}	L	L	L, F_{H_2O}, F_{EtOH}	L
80	14.05	278	865	739, 713, 695, 577, 561, 543, 525, 407, 285	B-type trimer (5)	S	S	S[a]	S[a]	S	S[a]

Table 1 (*continued*)

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013			2014		
						May	July	Sept.	May	July	Sept.
81	14.15	254, 352	447	301	Quercetin-3-*O*-rhamnoside (std)[c,e,f]	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
82	14.20		535	491, 371, 355, 329, 311, 191, 163	*p*-Coumaroyl monotropein (3)	L[a], S[a]	S[a], F_{H_2O}, F_{EtOH}	L[a], S[a]	L[a], S[a]	L[a], S[a] F_{H_2O}, F_{EtOH}	–
83	14.25		411	307, 163, 145, 119, 117	***p*-Coumaroyl malonylhexoside (1)**[e,f,g]	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
84	14.35	285sh, 312	535	491, 373, 355, 329, 311, 201, 163	*p*-Coumaroyl monotropein (4)	L, S[a]	S, F_{H_2O}, F_{EtOH}	L[a], S	S[a]	S[a], F_{H_2O}, F_{EtOH}	–
85	14.50		451	341, 217	Cinchonain I (4)	–	F_{EtOH}[a]	–	–	L, F_{H_2O}[a], F_{EtOH}	L
86	14.55	254, 350	579	475, 447, 429, 355, 300, 271	**Quercetin pentosyldeoxyhexoside**	S	S	S	S	S	S
87	14.60	290, 324	427	265, 179, 161, 135	**Caffeoyl malonylhexoside (2)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
88			573	411, 393, 249, 163	***p*-Coumaroyl malonyldihexoside (1)**	L[a], S	L, S	L, S	L[a], S	L[a], S	L[a], S[a]
89	14.65		507	387, 343, 329, 301, 273, 179, 163, 151	***p*-Coumaric acid derivative**	–	F_{H_2O}, F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}	–
90	14.70		521	345, 329	Syringetin hexuronic acid[d]	–	F_{H_2O}, F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}	–
91	15.0		435	273	**Phloretin-2-*O*-hexoside**	–	F_{H_2O}, F_{EtOH}[a]	–	–	F_{H_2O}, F_{EtOH}	–
92	15.15	290sh, 306	409	325, 307, 217, 187, 163, 159, 145	***p*-Coumaroyl diacetylhexoside (2)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
93	15.25		573	411, 393, 163	***p*-Coumaroyl malonyldihexoside (2)**	S	S	S	S	S	S
94	15.3	286, 306	411	307, 163, 145, 119	***p*-Coumaroyl malonylhexoside (2)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
95	15.35	280, 306	543	411, 163	***p*-Coumaroyl malonylpentosylhexoside**	S[a]	S	S	S	S	S
96	15.55	285sh, 310	411	249, 163, 145, 119	***p*-Coumaroyl malonylhexoside (3)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
97	15.65	286, 304	411	249, 163, 145, 119	***p*-Coumaroyl malonylhexoside (4)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S
98	15.8	290sh, 312	451	341, 307, 229, 187, 163	***p*-Coumaroyl triacetylhexoside (1)**[c,e]	L	–	–	L	–	–
99		280	451	341, 217	Cinchonain I (5)	–	L, S, F_{H_2O}, F_{EtOH}	L, S	S	L, S, F_{H_2O}, F_{EtOH}	L, S
100	15.9	254, 350	591	529, 489, 447, 301	Quercetin-3-*O*-(4″-HMG)-α-rhamnoside[e,f,j]	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L[a], S	L, S, F_{H_2O}, F_{EtOH}	L, S
101			425	179, 135	**Caffeoyl derivative**	–	F_{H_2O}[a], F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}	–
102	15.95	290sh, 312	451	367, 349, 307, 245, 203, 187, 159, 145	***p*-Coumaroyl triacetylhexoside (2)**	L	–	–	L	–	–
103			451	341, 299	Cinchonain I (6)	S[a]	L, S[a]	L, S[a]	S[a]	L, S[a]	L, S[a]
104		284, 314	445	179, 135	Caffeoyl derivative[g]	–	F_{H_2O}, F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}	–
105	16.15	290sh, 312	451	367, 349, 307, 245, 203, 187, 159, 145	***p*-Coumaroyl triacetylhexoside (3)**	L	L[a]	L[a]	L	L	L[a]
106	16.6	286, 310	249	163, 145	Malonyl *p*-coumaric acid[e]	S	S	S	S	S	S

L: leaf extract; S: stem extract; F_{H_2O}: aqueous fruit extract; F_{EtOH}: ethanolic fruits extract; underlined: major fragment; –: not present; std: compounds were identified by comparison with standards.

[a] Not fragmented.
[b] Doubly-charged ion. Compounds in bold are newly described or identified.
[c] Hokkanen, Mattila, Jaakola, Pirttilä, and Tolonen (2009).
[d] Lätti, Jaakola, Riihinen, and Kainulainen (2010).
[e] Ieri, Martini, Innocenti, & Mulinacci (2013).
[f] Liu, Lindstedt, Markkinen, Sinkkonen, Suomela, & Yang (2014).
[g] Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic (2015).
[h] Chanforan, Loonis, Mora, Caris-Veyrat, & Dufour (2012).
[i] Mane, Loonis, Juhel, Dufour, & Malien-Aubert (2011).
[j] HMG = 3-hydroxy-3-methylglutaryl.

two *p*-coumaroyl malonyldihexosides and one *p*-coumaroyl malonylhexosylpentoside, the last two molecules being mostly present in stems. *p*-Coumaroyl malonylhexosides (**83**, **94**, **96** and **97**; *m/z* 411) display fragment ions at *m/z* 307 (loss of malonic acid) or 249 (loss of hexose), 163, 145, and 119. Newly identified *p*-coumaroyl malonyldihexosides (**88** and **93**) and *p*-coumaroyl malonylpentosylhexoside (**95**) have parent ions at *m/z* 573 and 543, respectively, and a common major fragment ion at *m/z* 411. When accessible, absorption spectra are showing dual λ_{max} at *ca.* 286 and 310 nm as found for the last compound to be eluted (**106**). This apolar compound has a parent ion at *m/z* 249 and its structure could be attributed to 4-*O*-malonyl *p*-coumaric acid. As a matter of fact, this large family of glycosides linked to both *p*-coumaric acid and malonic acid may encompass three main

Table 2
Phenolic composition in bilberry leaves, stems and fruits at three different periods of vegetation and for two different years.

Extract/ Period of vegetation	Caffeic acid derivatives (mg/g DE)	Coumaric acid derivatives (mg/g DE)	Flavonol glycosides (mg/g DE)	Flavanol monomers (mg/g DE)	Flavanol oligomers (mg/g DE)	Anthocyanins (mg/g DE)	Sum of phenolic compounds (mg/g DE)	Total Phenolic Content (mg GAE/g DE)	Total Phenolic Content (mg GAE/g DM)
Leaves									
May 2013	65.2 ± 5.6 (A)	21.6 ± 2.3 (A)	10.6 ± 0.5 (A)	–	–	–	97.4 ± 7.9 (A)	–	54.7 ± 3.9 (A)
May 2014	*124.6 ± 3.5 (a)**	*35.8 ± 1.4 (a)**	*10.4 ± 3.7 (a)*	–	–	–	*170.8 ± 4.4 (a)**	*118.7 ± 2.4 (a)*	*75.1 ± 1.6 (a)**
July 2013	98.0 ± 10.6 (B)	8.83 ± 0.78 (B)	15.8 ± 3.2 (A,B)	1.12 ± 0.22 (A)	1.10 ± 0.14 (A)[b]	–	124.9 ± 14.4 (B)	–	105.7 ± 6.0(B)
July 2014	*100.5 ± 0.6 (b)*	*10.2 ± 0.0 (b)**	*22.5 ± 0.5 (b)**	*1.36 ± 0.15 (a)*	*1.33 ± 0.34 (a)*[b]	–	*135.9 ± 1.9 (b)*	*166.1 ± 4.4 (b)*	*106.9 ± 2.9 (b)*
September 2013	72.1 ± 4.4 (A)	7.48 ± 0.25 (B)	17.9 ± 2.0 (B)	0.53 ± 0.21 (B)	1.87 ± 0.08 (B)[b]	–	99.9 ± 6.7 (A,B)	–	102.4 ± 5.3(B)
September 2014	*72.3 ± 0.7 (c)*	*7.91 ± 0.21 (c)*	*14.0 ± 0.3 (a)**	*1.01 ± 0.28 (a)**	*1.37 ± 0.35 (a)*[b]	–	*96.6 ± 0.6 (c)*	*142.9 ± 19.2 (a,b)*	*87.1 ± 11.7 (a)*
Stems									
May 2013	7.16 ± 0.18 (A)	9.54 ± 0.29 (A)	11.5 ± 1.4 (A)	4.39 ± 1.49 (A)	40.0 ± 2.9 (A)	–	71.0 ± 5.9 (A)	–	72.4 ± 14.4(A)
May 2014	*9.79 ± 0.53 (a)**	*11.5 ± 0.2 (a)**	*14.5 ± 0.1 (a)**	*7.24 ± 0.77 (a)**	*49.7 ± 0.7 (a)**	–	*92.7 ± 1.2 (a)**	*136.6 ± 4.1 (a)*	*73.1 ± 2.2 (a)*
July 2013	7.58 ± 0.33 (A)	11.5 ± 0.6 (B)	9.63 ± 0.89 (A,B)	6.31 ± 1.63 (A)	49.1 ± 6.2 (A)	–	79.3 ± 1.7 (A)	–	78.8 ± 9.3 (A)
July 2014	*10.2 ± 0.1 (a)**	*13.3 ± 0.3 (b)**	*16.4 ± 1.0 (b)**	*10.6 ± 0.80 (b)**	*71.3 ± 5.3 (b)**	–	*121.8 ± 4.8 (b)**	*174.3 ± 2.8 (b)*	*98.7 ± 4.6 (b)**
September 2013	6.90 ± 0.36 (A)	9.26 ± 0.29 (A)	8.71 ± 0.38 (B)	10.9 ± 4.0 (A)	48.7 ± 2.0 (A)	–	80.8 ± 0.3 (A)	–	81.2 ± 4.8 (A)
September 2014	*5.87 ± 0.05 (b)**	*11.7 ± 0.2 (a)**	*9.22 ± 0.38 (c)*	*11.9 ± 1.7 (b)*	*57.1 ± 0.4 (a)**	–	*95.8 ± 1.9 (a)**	*140.0 ± 18.8 (a)*	*81.8 ± 11.0 (a)*
Fruits with H_2O									
July 2013	2.57 ± 0.42	1.12 ± 0.08	0.96 ± 0.16	–	–	22.3 ± 1.0	26.9 ± 1.7	–	31.8 ± 1.2
July 2014	*2.44 ± 0.25*	*1.45 ± 0.14**	*0.99 ±±0.16*	*0.11 ± 0.06*[a]	–	*29.6 ± 5.8*	*34.7 ± 5.6*	*38.6 ± 2.2*	*30.5 ± 1.7*
Fruits with EtOH 55%									
July 2013	3.54 ± 0.43	1.49 ± 0.14	1.39 ± 0.23	0.13 ± 0.05[a]	–	34.5 ± 10.3	41.1 ± 11.1	–	41.9 ± 1.7
July 2014	*2.36 ± 0.07* *	*1.47 ± 0.07*	*0.94 ± 0.03* *	*0.12 ± 0.07*[a]	–	*25.7 ± 4.0*	*30.6 ± 4.1*	*33.1 ± 0.9*	*34.7 ± 1.0**

Values represented mean ± SD ($n = 3$). Sum of phenolic compounds is obtained from the different columns on the left (UPLC). Total Phenolic Content is obtained by the Folin-Ciocalteu method. DE: dry extract. DM: dry matter. – Means below quantification limit or not present. Different letters indicate a significant difference between the three periods of vegetation at $p < 0.05$; capital and small letters are used to compare the samples from 2013 and 2014, respectively.

* Means a significant difference between the two years ($p < 0.05$).

[a] Flavanol monomers in fruits contain only a cinchonain I isomer.

[b] Flavanol oligomers contain B-type and A-type oligomers in stems, only cinchonains I + II in leaves.

structures. Malonic acid could be grafted on the 4-OH group of *p*-coumaric acid or on the hexose unit when *p*-coumaric acid can be either etherified or esterified by the hexose unit, in agreement with the earlier identification of *p*-coumaric acid-4-*O*-hexosides and *p*-coumaroylhexosides, respectively. Compounds with parent ions at *m/z* 409, 411 and 451 were already found in bud and leaf extracts of bilberry by Ieri et al. (2013), Liu et al. (2014), and Mikulic-Petkovsek et al. (2015), although they were only named as *p*-coumaroyl derivatives.

Several iridoid glycosides acylated by *p*-coumaric acid (*m/z* 535) were identified in all the morphological parts and assigned as *p*-coumaroyl monotropeins (**63**, **70**, **82** and **84**). *p*-Coumaroyl dihydromonotropein isomers (**71** and **76**) were newly identified in bilberry similarly to a compound with a parent ion at *m/z* 697, which was attributed to *p*-coumaroyl monotropein hexoside (**69**) through major fragments at *m/z* 535 (*p*-coumaroyl monotropein) and *m/z* 371 (subsequent loss of coumaric acid). The latter was found in trace amounts in fruits and leaves.

3.1.3. Flavonol glycosides

In stems and leaves, quercetin glycosides were present in considerable amounts from May to September (Table 2), whereas in fruits from July they appeared in lower concentrations. Quercetin-3-*O*-galactoside (**62**), quercetin-3-*O*-glucoside (**67**), quercetin hexuronides (**61**, **64** and **66**), quercetin pentosides (**73** and **77**), and a quercetin rhamnoside (**81**) were observed (Table 1). The berry characteristic quercetin-3-*O*-(4″-(3-hydroxy-3-methyl glutaryl))-α-rhamnoside (**100**) was identified in all the morphological parts. This compound is newly described in bilberry fruit while it was evidenced in bilberry buds and leaves by Hokkanen et al. (2009), and Ieri et al. (2013). It was quantified in leaves in July and September (4th most abundant flavonol) and in stems from all seasons.

The last two quercetin glycosides presented similar fragmentation pathways with the loss of 132 and 150 amu characteristic of a pentose unit. Quercetin pentosylhexoside (**56**, *m/z* 595) and quercetin pentosyldeoxyhexoside (**86**, *m/z* 579) were newly identified in stems.

Finally, a dihydrochalcone was newly identified in bilberry fruit and assigned as phloretin hexoside (**91**), displaying a parent ion at *m/z* 435 and a fragment ion at *m/z* 273 (loss of hexose).

3.1.4. Flavanols

In stems, flavanols were present from May to September (Table 1) although they tended to be more abundant in July and September (Table 2). Epicatechin or catechin-based oligomeric flavanols encompass a large variety with various B-type dimeric (**16**, **25**, **32**, **42**, **49** and **75**), trimeric (**28**, **33**, **46**, **58** and **80**), and tetrameric forms (**40**, **43** and **55**). A-type dimers (**47** and **52**) and trimers (**12**, **41** and **45**) were also present, the latter resulting from an intramolecular two-electron oxidation of the B-type corresponding structures. Additionally, (−)-epigallocatechin (**15**) and (−)-epicatechin (**34**) were present in quantifiable amounts, whereas (+)-gallocatechin (**1**) was only detected by mass spectrometry. (Epi)gallocatechin was further identified in three mixed B-type dimers with (epi)catechin (**11**, **19** and **48**), a mixed B-type trimer (**36**) and a mixed A-type trimer (**39**). Coupling between caffeic acid and monomeric or dimeric flavanols led to five cinchonain I isomers (**26**, **37**, **60**, **99** and **103**) and two cinchonain II isomers (**38** and **59**), respectively. Two main fragmentation pathways were observed for cinchonains I with isomers giving major fragment ions at *m/z* 289 and 245 and others at *m/z* 341 and 217. None of them were quantified, being either minor compounds in co-eluted peaks or present below the limit of quantification.

In leaves, eight B-type dimers (**25**, **32** and **75**), trimers (**33** and **46**), and tetramers (**40**, **43** and **55**) and one A-type dimer (**47**) were identified but not quantified, when only one B-type dimer (**75**) was identified in fruits. (−)-Epicatechin (**34**) was only present in leaves from July and September, representing less than 1% of the phenolic pool (Table 3). Lastly, cinchonains I (**60**, **85**, **99** and **103**) and II (**59**) were identified in leaves when only two cinchonain I (**85** and **99**) were present in fruits. Cinchonains were only quantified in leaves

Table 3
Relative content of major phenolic compounds in bilberry leaves and stems at three different periods of vegetation and for two different years.

Morphological parts	Major phenolic compounds[a]	Relative content (%)[b]					
		2013			2014		
		May	July	Sept.	May	July	Sept.
Leaf extracts	5-*O*-Caffeoylquinic-acid (18)	55.6	74.6	68.3	67.9	70.0	70.3
	5-*O*-Caffeoylquinic acid (cis) (31)	0.8	1.3	1.1	0.7	1.2	1.6
	Caffeoyl malonylhexoside (87)	1.5	0.9	1.2	1.1	0.9	1.2
	Quercetin-3-*O*-galactoside (62)	1.4	4.6	7.8	1.1	3.3	1.9
	Quercetin hexuronide (64)	8.9	6.2	8.1	4.8	11.5	11.7
	Quercetin pentoside (73)	0.5	1.4	1.7	0.2	1.2	0.6
	5-*p*-Coumaroylquinic acid (30)	2.0	0.6	0.4	2.0	0.4	0.4
	p-Coumaroyl monotropein (70)	5.1	0.5	0.3	4.5	0.5	0.1
	p-Coumaroyl diacetylhexoside (78)	5.3	1.5	1.4	5.6	1.6	1.0
	p-Coumaroyl malonylhexoside (83)	1.2	1.0	1.3	0.8	1.4	2.4
	p-Coumaroyl malonylhexoside (96)	2.6	1.8	2.3	2.8	1.9	2.4
	(−)-Epicatechin (34)	–	0.9	0.5	–	1.0	1.0
Stem extracts	A-type trimer (45)	16.9	18.2	15.4	14.6	14.1	19.5
	B-type trimer (46)	14.3	13.3	13.9	15.8	18.1	11.3
	Procyanidin B2 (32)	7.8	9.2	9.4	9.7	10.4	7.7
	5-*O*-Caffeoylquinic-acid (18)	6.9	7.5	6.4	8.0	7.0	4.6
	(−)-Epigallocatechin (15)	2.9	2.7	3.3	2.9	2.6	5.0
	(−)-Epicatechin (34)	3.1	4.7	7.3	4.9	6.1	7.4
	Quercetin-3-*O*-galactoside (62)	4.8	2.8	3.6	2.4	1.8	2.2
	Quercetin hexuronide (64)	4.4	3.2	2.4	7.1	6.2	2.2
	p-Coumaroyl malonylhexoside (96)	6.4	5.9	6.2	6.4	4.8	7.0
	p-Coumaroyl monotropein (70)	2.5	2.9	2.0	2.0	2.2	1.5

[a] Values in parentheses correspond to compound number in Table 1.
[b] Mean for $n = 3$.

from July and September (**59** and **60**) and fruits (**99**), as found for (−)-epicatechin. They thus appear to be specifically biosynthesised from spring to summer.

3.1.5. Anthocyanins

Both aqueous and ethanolic fruit extracts contained high levels of anthocyanins (Table 2). Fifteen anthocyanins were assessed through their major fragment ions at *m/z* 303, 287, 317, 301, and 331, which are characteristic of the aglycones delphinidin, cyanidin, petunidin, peonidin, and malvidin, respectively (Table 5, Supplementary material). Delphinidin-3-*O*-galactoside and delphinidin-3-*O*-glucoside are predominant anthocyanins in fruit extracts (Moze et al., 2011; Prencipe, Bruni, Guerrini, Benvenuti, & Pellati, 2014).

3.2. Influence of the harvest period on the phenolic composition in bilberry leaves, stems and fruits

Relatively similar phenolic profiles were found in bilberry leaves and stems, harvested at the three different periods during the two years. Qualitative analysis showed the presence of caffeic acid and coumaric acid derivatives, quercetin glycosides, and (−)-epicatechin in leaves, whereas in stems (−)-epigallocatechin and epicatechin-derived oligomers were additionally identified (Table 1).

In leaves, caffeic acid derivatives were the most representative group of phenolic compounds, as found earlier by Martz et al., 2010. Whatever the period and the year of harvest, their level ranged between 67 and 79% of the dry extract weight (Table 2). Their seasonal evolution differed between years 2013 and 2014. Chlorogenic acid (**18**) contributed more than half and its relative content varied between 55% (May 2013) and 75% (July 2013) (Table 3).

Flavonol glycosides were present in lesser contents in May leaves compared to *p*-coumaric acid derivatives although the opposite was observed in July and September. As a matter of fact, flavonol glycosides markedly increased in July and this high level remained steady (2013) or decreased (2014) in September. Liu et al. (2014) also observed no flavonol variation between July and September 2013 for leaves collected in Finland, while Martz et al. (2010) observed a slight increase for leaves exposed to light also in Finland (2006). The second most important contributor to the leaf extract was a quercetin hexuronide (**64**) except for May 2014, with relative levels ranging between 5% (May 2014) and 12% (September 2014). Ranking third was quercetin-3-*O*-galactoside (**62**) from July to September in year 2013 and July 2014 (3–8%). The second and third leaf contributors are thus variable: ranking second in May 2014 and third in May 2013 is *p*-coumaroyl diacetylhexoside (**78**) (5%) while *p*-coumaroyl malonylhexosides (**83** and **96**) are equally placed third in September 2014 (2.4%).

With *p*-coumaric acid derivatives at their highest levels in May, another important contributor is *p*-coumaroyl monotropein (**70**) ranking 4th in May 2013 and May 2014, although this ranking largely decreased in July and September in both 2013 and 2014.

Finally, flavanol monomers and oligomers, which were mainly composed of (−)-epicatechin, cinchonain I (**60**) and cinchonain II (**59**), became quantifiable in July and September although in trace amounts.

In stems, flavanol oligomers were the major group, representing between 54 to 62% of the Sum of phenolic compounds (w/w of DE). The major contributor to the stem extract was an A-type trimer (**45**) in May, July, and September 2013 as well as in September 2014 (15–20%) or a B-type trimer (**46**) in May and July 2014 (16–18%). Ranking second was the same B-type trimer (**46**) for May, July, and September 2013 as well as for September 2014 (11–14%) while the A-type trimer (**45**) was favored for May and July 2014 (14–15%). Ranking third was dimer B2 (**32**) for all seasons over the two years of study with contents varying between 8 and 10%. Furthermore, 5-caffeoylquinic acid (**18**) was the fourth more abundant compound from May to July whatever the year with levels between 7 and 8% when (−)-epicatechin (**34**) dominated in September (7%). Finally, the next compounds highly present were: *p*-coumaroyl malonylhexoside (**96**), quercetin hexuronide (**64**), quercetin-3-*O*-galactoside (**62**), *p*-coumaroyl

monotropein (**70**), and (−)-epigallocatechin (**15**). The recurrence for the three most abundant contributors over the seasons and years points to a flavanol oligomer biosynthesis under genetic control.

By contrast, seasonal and inter-annual variations were observed for contents in most phenolic groups in stem as in leaf (Table 2). The year effect was even higher for stems than for leaves, suggesting that abiotic stress clearly influences polyphenol biosynthesis. This can be attributed to contrasted weather conditions like air and soil temperature, hours of sunshine, and level of precipitations (Table 6, Supplementary material) as observed earlier by Martz et al. (2010) and Uleberg et al. (2012). Biotic stress was recently shown to elicit the biosynthesis of leaf phenolic compounds whose structures differed after attack by a fungal endophyte or *B. cinerea* (Koskimäki et al., 2009).

In fruits, the sum of phenolic compounds was lower than in leaves and stems and this could be attributed to the high sugar concentration in fruits. In both fruit extracts, anthocyanins clearly dominated with levels ranging from 22 to 35 mg/g DE and representing 83–85% of the extract weight (Table 2). The other classes ranked as follows in a decreasing order: caffeic acid derivatives (2.4–3.5 mg/g) > coumaric acid derivatives (1.1–1.5 mg/g) > flavonol glycosides (0.9–1.4 mg/g) > flavanol monomers (0–0.1 mg/g). The contents of these various phenolic classes remained relatively unaffected between 2013 and 2014.

3.3. Characterization of flavan-3-ol oligomers

In leaves, flavanol oligomers could appear as the second most abundant class of phenolic compounds when considering data from thioacidolysis (Table 4), ranging between 13 and 32 mg/g DE in July and September 2013 and 2014. This is rather contradictory with data from UPLC (Table 2) where no A-type or B-type oligomers, but only cinchonain II (**59**) were quantified in leaves (1.1–1.9 mg/g DE) for the same periods. mDP ranging from 2.9 to 4.5 is however in agreement with the presence of the eight B-type dimers and trimers identified by MS. Cinchonains II are expected to react in thioacidolysis partly releasing quantifiable (epi)catechin terminal units along with new benzylthioether adducts escaping quantitation. Of note, (−)-epicatechin was the only constituting unit of flavanol oligomers in leaves.

In stems, the contents in flavanol oligomers were similar whatever the method used, ranging between 48 and 70 mg/g DE after thioacidolysis and between 40 and 71 mg/g DE by UPLC (Tables 2 and 4). Moreover, seasonal variations for flavanol oligomers were close in 2013 and 2014, independently of the methods. However, the oligomer contents appear to be underestimated after thioacidolysis in accordance with A-type oligomers being incompletely degraded as well as (epi)gallocatechin units escaping quantification. Indeed, the major contributor to stems is an A-type trimer (**45**). mDP ranging between 2 and 3 are consistent with the predominance of two A-type and B-type trimers (**45** and **46**) by UPLC. Lastly, catechin appeared as both terminal units (2–8%) and extension units (1–3%).

In fruits, low amounts of flavanol oligomers (2–5 mg/g DE) were determined by the thioacidolysis method when no oligomers were quantified by UPLC, mostly because of co-elution with anthocyanins. (+)-Catechin appeared only as a terminal unit when (−)-epicatechin was present both as terminal and extension units, accounting for more than 90% of the total units. mDP remained low (2–3). As expected, 55% aqueous ethanol was twice as efficient at extracting oligomeric flavanols.

3.4. Antioxidant activity of bilberry extracts

The antioxidant activity of the bilberry extracts was determined by two complementary methods. The DPPH test relies on the ability of reducing molecules to transfer an electron or a hydrogen atom to the nitrogen-centred DPPH radical. As for the Folin-Ciocalteu method, it measures the ability of a sample to reduce transition metal ions as in the complex between sodium phosphomolybdate and phosphotungstate, giving access to the total phenolic content (TPC).

The TPC was reported in weight per dry matter for years 2013 and 2014 and in weight per dry extract in 2014 (Table 2). The difference is due to the extraction yield of the DM (ca. 58, 52, and 85% *w/w* for leaves, stems and fruits, respectively). When expressed in

Table 4
Flavan-3-ol composition and mDP in bilberry leaves, stems and fruits using thioacidolysis.

Morphological part extracts	Period of vegetation	Oligomeric flavanol characterization				Flavanol oligomers (mg/g DE)	mDP
		Terminal units (%)		Extension units (%)			
		CAT	*EC*	*CAT*	*EC*		
Leaf extracts							
	May 2013	–	34.7 ± 4.5	–	65.3 ± 16.6	4.25 ± 0.99(A)	2.9 ± 0.5(A)
	May 2014	**–**	**34.5 ± 5.5**	**–**	**65.5 ± 2.2**	**2.11 ± 0.56(a)***	**3.0 ± 0.4(a)**
	July 2013	1.98 ± 0.03	27.9 ± 5.8	–	70.1 ± 2.3	32.4 ± 2.2(B)	3.4 ± 0.4(A)
	July 2014	**–**	**22.1 ± 0.3**	**–**	**77.9 ± 1.2**	**25.5 ± 1.4(b)***	**4.5 ± 0.1(b)***
	September 2013	–	26.3 ± 2.3	–	73.7 ± 0.8	23.8 ± 0.6(C)	3.8 ± 0.2 (A)
	September 2014	**–**	**28.0 ± 3.7***	**–**	**72.0 ± 1.7***	**12.7 ± 0.6(c)***	**3.6 ± 0.3(a)**
Stem extracts							
	May 2013	2.17 ± 0.11	36.1 ± 1.8	–	61.7 ± 3.0	51.7 ± 2.1(A)	2.6 ± 0.0(A)
	May 2014	**–**	**32.6 ± 0.1***	**1.61 ± 0.37**	**65.8 ± 0.4***	**60.0 ± 4.3(a)***	**3.1** ± 0.0 **(a)***
	July 2013	3.10 ± 0.87	37.5 ± 1.1	–	59.4 ± 0.4	63.6 ± 0.3(B)	2.5 ± 0.1(B)
	July 2014	**–**	**33.7 ± 0.6***	**1.20 ± 0.17**	**65.1 ± 0.9***	**69.6 ± 2.3(b)***	**3.0 ± 0.0(b)***
	September 2013	5.86 ± 1.06	36.5 ± 1.0	–	57.6 ± 1.9	60.3 ± 0.3(C)	2.4 ± 0.0 (C)
	September 2014	**7.90 ± 0.28**	**38.1 ± 0.9**	**2.66 ± 2.60**	**51.4 ± 2.0***	**47.6 ± 1.0(c)***	**2.2 ± 0.1(c)***
Fruit extracts							
H_2O	July 2013	9.48 ± 2.61	34.6 ± 2.0	–	55.9 ± 23.5	2.21 ± 0.68	2.3 ± 0.6
	July 2014	**9.63 ± 0.62**	**37.6 ± 0.3***	**–**	**52.8 ± 0.9**	**2.45 ± 0.06**	**2.1 ± 0.0**
EtOH 55%	July 2013	8.99 ± 2.50	33.6 ± 4.5	–	57.4 ± 28.0	4.18 ± 1.50	2.3 ± 0.5
	July 2014	**5.75 ± 0.33**	**27.1 ± 1.0**	**–**	**67.2 ± 2.1**	**5.42 ± 0.25**	**3.0 ± 0.0***

CAT: (+)-catechin. EC: (−)-epicatechin. mDP: average degree of polymerization of monomeric and oligomeric flavan-3-ols. Values represented mean ± SD ($n = 3$). – Means not present. Different letters indicate a significant difference between the three different periods of vegetation at $p < 0.05$: capital letters are used to compare the samples from 2013 and small letters are used to compare the samples from 2014. * Means a significant difference between the two years ($p < 0.05$).

mg GAE/g DE (year 2014), the TPC values were in the same range or higher than those found by summing all the phenolic compounds quantified by UPLC. As a matter of fact, correlation plots with stem, leaf and fruit data showed that TPC (*w/w* of DM) were well correlated to the sum of phenolic compounds (*w/w* of DE) with R^2 of 0.73 and 0.62 except for leaves from May 2013 and 2014 (Fig. 2, Suppl. material). The removal of the May data markedly increased the correlation (R^2 0.96 and 0.98). This suggests a high correlation between these two methods when assaying bilberry phenolic compounds in all the morphological parts. May leaves presented unexpectedly low TPC when compared to TPC of the samples from July and September. It is worth noting that *p*-coumaric acid derivatives contribute to 20% of the phenolic pool in May and only 7% in July and September. Besides, *p*-coumaric acid derivatives remained at low levels in stems (11–14%) and fruits (4–5%) from May to September. As a matter of fact, *p*-coumaric acid was 2.5-fold less reactive than caffeic acid with the Folin Ciocalteu reagent (Ma & Cheung, 2007). Moreover, the relative reactivity of gallic acid (1.0), caffeic acid (0.96), chlorogenic acid (1.36), and rutin (1.53) was higher than that of salicylic acid (0.26) and tyrosine (0.38), which are structurally related to *p*-coumaric acid (Everette et al., 2010).

The DPPH-scavenging activities of bilberry extracts showed contrasting seasonal variations between 2013 and 2014 (Fig. 1). A significant increase in leaf antioxidant activity is exhibited in July and September 2013 when this increase was only observed in July 2014. The antioxidant activity of the stem extracts was less affected by the season. As to fruits, the use of ethanol-containing solvents significantly improved the recovery in phenolic compounds and the antioxidant activity by both TPC and DPPH tests in 2013 while this effect was modest in 2014. Finally, the annual effect on the DPPH antioxidant activities is similar to that exhibited for the TPC values. Additionally, the activity in the DPPH test and the sum of phenolic compounds (*w/w* of DE) were highly correlated, with R^2 of 0.70 and 0.77 in 2013 and 2014 for all eight samples (Fig. 2, Suppl. material). Moreover, the correlation plots are similar to the ones observed for sum of phenolic compounds *vs* TPC. The lower reactivity of May leaves is likely linked to its large content in *p*-coumaric acid derivatives. Phenolic compounds displaying a dihydroxyphenyl moiety are generally more antioxidant than those containing a monohydroxyphenyl moiety.

Finally, TPC strongly correlated with the DPPH radical scavenging activity with R^2 of 0.91 and 0.94 for samples from 2013 and 2014, respectively (Fig. 2, Suppl. material). This suggests that phenolic compounds with mono- and dihydroxyphenyl moieties as well as other reducing substances present in the extract display the same reducing ability towards transition metal ions as in the Folin Ciocalteu method and N-centred radicals as in the DPPH test.

4. Conclusions

This study reports the most comprehensive qualitative analysis ever conducted on bilberry leaves, stems, and fruits, leading to the identification of 106 phenolic compounds. In particular, structures were proposed for 46 new compounds.

Quantitative analysis allowed the accurate determination of the ten to twelve most important contributors belonging to the groups caffeoyl derivatives, *p*-coumaroyl derivatives, flavonol glycosides, anthocyanins, and flavanol monomers and oligomers. In general, the ranking of the major contributors in leaf and stem showed low or no seasonal variations, respectively. Some variations were however outlined between May and July suggesting the appearance (flavanol oligomers in leaves, cinchonains I and II in stems from July and September) or disappearance of minor compounds (*p*-coumaroyl derivatives in leaves from May). The intra-annual variations for the content in the various phenolic groups generally differed between 2013 and 2014, in agreement with biotic and abiotic stresses. Finally, the phenolic content was highly correlated to the antioxidant activity in leaf, stem and fruit extracts of bilberry.

All the morphological parts of bilberry proved to be suitable for valorisation as sources of natural phenolic compounds. Regarding the period of harvest, leaves and stems should be better collected in July or September to be valuable feedstocks for the production of food supplements. May leaves are of interest if the health benefit of *p*-coumaroyl derivatives is sought. The stability of the collected dry material remains however to be assessed, to determine its optimal shelf life.

Conflict of interest statement

The authors declare no conflict of interest.

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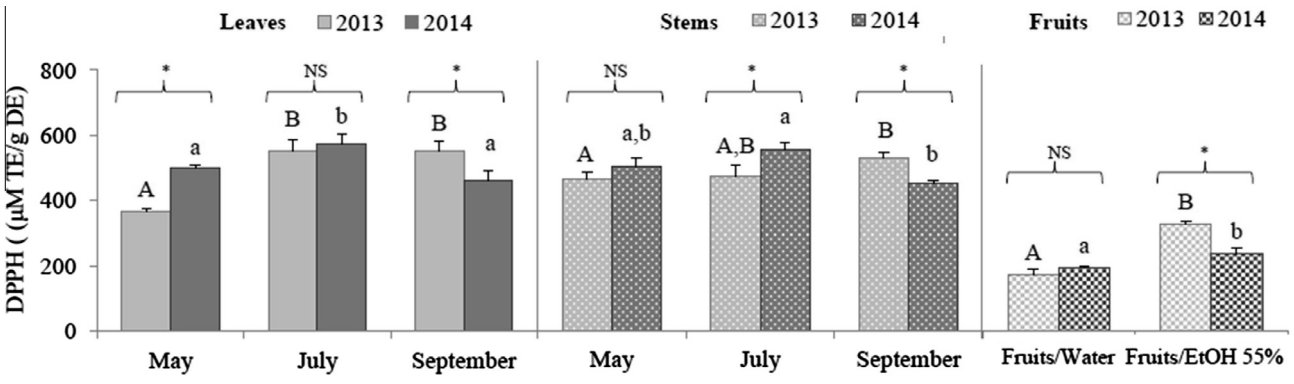

Fig. 1. Influence of different harvest periods on the DPPH radical scavenging activity in leaf, stem and fruit extracts of bilberry (mean ± SD, n = 3-4). Different letters indicate a significant difference between the three periods of vegetation at $p < 0.05$: capital letters are used to compare the samples from 2013 and small letters those from 2014. * Means a significant difference between the two years with $p < 0.05$. NS: not significant.

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Appendix A. Supplementary data

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