HAL
archives-ouvertes.fr

Extraction, identification and antioxidant activity of the phenolic secondary metabolites isolated from the leaves, stems and fruits of two shrubs of the Ericaceae family

Oana-Crina Bujor

▶ To cite this version:

Oana-Crina Bujor. Extraction, identification and antioxidant activity of the phenolic secondary metabolites isolated from the leaves, stems and fruits of two shrubs of the Ericaceae family. Other. Université d'Avignon, 2016. English. NNT : 2016AVIG0261 . tel-01722698

HAL Id: tel-01722698
https://tel.archives-ouvertes.fr/tel-01722698

Submitted on 5 Mar 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

"Gheorghe Asachi" Technical University of Iasi
Faculty of Chemical Engineering and Environmental Protection, Romania

University of Avignon – National Institute for Agricultural Research (INRA)
UMR 408 Safety and Quality of Plant Products, Avignon, France

Extraction, identification and antioxidant activity of the phenolic secondary metabolites isolated from the leaves, stems and fruits of two shrubs of the *Ericaceae* family

- PhD THESIS -

Chemist Oana-Crina Bujor

Supervisors:
Professor emeritus Valentin I. POPA
Dr. Claire DUFOUR

IASI, April 19th 2016

The composition of the thesis jury:

President:

Professor Nicolae Hurduc
Faculty of Chemical Engineering and Environmental Protection
"Gheorghe Asachi" Technical University of Iasi, Romania

Supervisors:

Professor emeritus Valentin I. Popa
Faculty of Chemical Engineering and Environmental Protection
"Gheorghe Asachi" Technical University of Iasi, Romania

Dr. Claire Dufour
UMR408 SQPOV "Sécurité et Qualité des Produits d'Origine Végétale"
National Institute for Agricultural Research, Avignon, France

Reviewers:

Dr. Hélène Fulcrand
UMR1083 "Sciences Pour l'Oenologie"
National Institute for Agricultural Research, Montpellier, France

Professor Florin Dan Irimie
Faculty of Chemistry and Chemical Enginerring
Babes-Bolyai University of Cluj-Napoca, Romania

Professor Teodor Măluţan
Faculty of Chemical Engineering and Environmental Protection
"Gheorghe Asachi" Technical University of Iasi, Romania

To Rares, for his love and patience

To my niece and my nephew, Alesia and Denis: the chemistry is all around us!

ACKNOWLEDGEMENTS

This PhD thesis was elaborated in the joint supervision between the "Gheorghe Asachi" Technical University of Iasi, Faculty of Chemical Engineering and Environmental Protection (FCEEP), Romania and the University of Avignon and Vaucluse, France. The present work was carried out at the Department of Natural and Synthetic Polymers at the FCEEP, and in "Chemistry of antioxidants" team of UMR 408 "Safety and Quality of Plant Products (SQPOV)" at the National Institute for Agricultural Research (INRA) from Avignon. The travel costs to INRA-SQPOV Unit were financed by the Erasmus Program, which I gratefully acknowledge for the student mobility grants and particularly to *Mrs. Liliana Lazăr* and *Mrs. Claudia Haasis* for their support. I am also grateful to *Dr. Catherine Renard*, Head of the INRA-SQPOV Unit, for providing the facilities for my work in her unit.

I am most grateful to my supervisors, **Professor emeritus Valentin I. Popa** and **Dr. Claire Dufour**, for their guidance throughout the thesis and for always had the door open for all questions and discussions concerning all aspects of the work … and not only. I am very grateful to Professor Valentin I. Popa for his encouragement to start this work and for all support, kindly guidance, advices, and insightful comments. I express my special thanks to Dr. Claire Dufour for introducing me to the world of antioxidant polyphenols and lipid oxidation and more important for knowledge on mass spectrometry. I appreciated their never failing support and suggestions, and the constructive advice and criticism without the scientific results of this work would not attain this high level. Her kindness and friendship were and are also very precious to me. "Merci, Merci!".

I would like to extend my gratitude to the members of my examination committee, *Professor Nicolae Hurduc, Dr. Hélène Fulcrand, Professor Florin Dan Irimie* and *Professor Teodor Măluţan*, for their time and interest in evaluating the scientific quality of this thesis.

I wish to express my sincere acknowledgment to *Professor Olivier Dangles*, Head of Doctoral School 536 "Agricultural Sciences & Sciences" of University of Avignon, who promptly responded to my interest in collaboration with its university and give me the chance to do my research at the SQPOV Unit. I would like to thank him for his time, friendly attitude, advice and help in my grant and thesis applications.

Two persons at the Faculty of Chemical Engineering and Environmental Protection of Iasi are specially acknowledged for their contributions to this study. I am grateful to *Associate Professor Irina Volf* for the opportunity to work at her laboratory and use the facilities available there for my researches and her advice and support during my thesis. I also thank *Associate Professor Camelia Mihăilescu* for her proficiency and guidance in experimental extraction analysis.

Many sincere thanks go to the members of the “Chemistry of antioxidants” team of INRA-SQPOV Unit for kindness and warm welcome and pleasant working atmosphere in the laboratory. Special thanks go to: *Mrs. Marie- José Vallier* for her sympathy and providing me guidance and help on my lab work whenever needed; *Mrs. Michèle Loonis* for her help and guidance in UPLC/MS analyses and especially for her gentleness and good ideas; *Mr. Christian Ginies* for helping me through the hard laboratory work in the digestion: «Je sais: c’est pour la recherche!»; *Dr. Michel Carail* for his advice on lab «trucs» and for trying to learn romanian.

I would like to thank all the staff of INRA-SQPOV Unit for welcoming me as another member of their unit. Especially, I thanks to *Dr. Carine Le Bourvellec* for teaching me about the procyanidin analyses and their fruitful and pleasant contribution to this study. An extra thank is also addressed to the unit informaticians, *Dominique Loonis* and *Eric Pietri*, for helping me to solve the “everyday” IT problems.

I thank the *Forestry Direction of Neamt* for the access to Borca Mountains (Neamt, Romania) for collecting bilberry and lingonberry samples for my research. I also want to thank to *Dr. Gina Tiron* from *National Meteorological Administration - Moldova Meteorological Center of Iasi*, for providing the meteorological data.

I am grateful to all my colleagues and friends at the Faculty of Chemical Engineering and Environmental Protection of Iasi and the INRA-SQPOV Unit of Avignon for making my work easier during these years: Andrei and Cornel are thanked for their “useful advice” at the beginning of the thesis and helpfulness; Roxana is thanked for her all support and beautiful friendship; Adina for her collaboration for review, confident spirit and help while I was in France; Dan is thanked for his help with the lyophilisation of samples and friendship; my colleagues at the INRA-SQPOV Unit, Katérina, Anna-Lena, Rachel, Béa, Joseana, Dimitra, Albert, Romain, Sara, Julien, Aurélie, Djidji deserve great thanks for all their help, friendly welcome, kindness, lunches, coffee breaks and all the good moments that we have shared together during my work in Avignon.

Finally, I am heartily thankful to my family, and especial to my sister for his encouragement and unconditional support. In particular, my dearest thanks are addressed to Rares for understanding me and my work, and for his love, tireless support and patience.

Contents

INTRODUCTION......7

LITERATURE REVIEW 9

I. Phenolic compounds as secondary metabolites found in plants and foods...... 11

I.1. Structure and classification...... 12

I.1.1. Non-flavonoid polyphenols...... 12

I.1.2. Flavonoids 15

I.2. Methods for extraction and characterisation of phenolic compounds...... 18

I.3. Applications of phenolic compounds in different biological systems...... 20

I.3.1. Applications of phenolic compounds in the plants development...... 20

I.3.1.1. As plants growth bioregulators...... 20

I.3.1.2. As amendments in bioremediation 22

*I.3.2. Applications of phenolic compounds in microorganisms developmen*t...... 23

II. The antioxidant activity of phenolic compounds 25

II.1. The antioxidant action of phenolic compounds and their mechanisms...... 25

II.1.1. Through reducing effects: H-atom and/or electron transfer...... 26

II.1.2. Through non-reducing effects 28

II.1.2.1. Metal-ion chelating activity 28

II.1.2.2. Inhibition of enzymes implied in the production of ROS 28

II.1.3. Inhibition of lipid oxidation...... 29

II.2. Methods to evaluate the antioxidant activity of phenolic compounds 30

II.2.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method...... 30

II.2.2. Folin–Ciocalteu redox method...... 31

II.2.3. Other methods 32

III. Bilberry and lingonberry, two shrubs of the *Ericaceae* family as sources of phenolic secondary metabolites...... 33

III.1. Bilberry (Vaccinium myrtillus L.)...... 33

III.1.1. General description...... 33

III.1.2. Chemical composition: phenolic compounds and other constituents...... 36

III.2. Lingonberry (Vaccinium vitis-idaea L.) 42

III.2.1. General description...... 42

III.2.2. Chemical composition: phenolic compounds and other constituents 44

III.3. Extraction and analysis of bilberry and lingonberry phenolic compounds .. 48

III.4. Health benefits of bilberry and lingonberry 48

III.4.1. Cardioprotective activity... 49

III.4.2. Anti-cancer activity 50

III.4.3. Antidiabetic activity 51

III.4.4. Vision improvement activity... 52

III.4.5. Bacterial anti-adhesion activity 52

III.5. Other applications of bilberry and lingonberry extracts 53

PERSONAL CONTRIBUTION.. 54

Chapter I. PHENOLIC CONSTITUENTS IN BILBERRY (*VACCINIUM MYRTILLUS* L.): ACCUMULATION IN LEAVES, STEMS AND FRUITS AT DIFFERENT HARVEST PERIODS AND ANTIOXIDANT ACTIVITY.................. 55

1. Background 57

2. Methodology 59

2.1 Materials 59
2.1.1. Bilberry samples 59
2.1.2. Chemicals and solvents.. 59

2.2. Extraction of phenolic compounds... 60

2.3. Qualitative and quantitative analyses of phenolic compounds........................... 61
2.3.1. Identification of phenolic compounds by UPLC/MS 61
2.3.2. Quantification of phenolic compounds.. 62

2.4. Analysis of procyanidins using thioacidolysis 63
2.4.1. Freeze-dried extracts.. 63
2.4.1.1. HPLC analysis without thiolysis 63
2.4.1.2. HPLC analysis after thiolysis... 63
2.4.2. Freeze-dried fruits.. 64
2.4.2.1. HPLC analysis without thiolysis 64
2.4.2.2. HPLC analysis with thiolysis 64

2.5. Antioxidant activity by applying spectrophotometric methods 65
2.5.1. Total Phenolic Contents by the Folin Ciocalteu method 65
2.5.2. DPPH (2,2-diphenyl- 1-picrylhydrazyl) radical scavenging test............... 65

2.6. Statistical analyses.. 66

3. Results and discussion 67

3.1. Optimal extraction conditions: the preliminary test... 67

3.2. Phenolic profile and content of bilberry extracts 69
3.2.1. Caffeic acid derivatives 69

3.2.2. Coumaric acid derivatives 73
3.2.3. Flavonol glycosides 75
3.2.4. Flavanols 76
3.2.5. Anthocyanins 85

3.3. Influence of the harvest period on the phenolic composition in bilberry leaves, stems and fruits 86

3.4. Characterization of flavan-3-ol oligomers 91
3.4.1. In freeze-dried extracts 91
3.4.2. In freeze-dried fruits 92

3.5. Antioxidant activity of bilberry extracts 94

4. Conclusions 99

ANNEXES 101

Chapter II. PHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF LEAF, STEM AND FRUIT EXTRACTS OF LINGONBERRY (*VACCINIUM VITIS-IDAEA* L.) AT THREE VEGETATIVE STAGES 105

1. Background 107

2. Methodology 109

2.1. Materials 109
2.1.1. Lingonberry samples 109
2.1.2. Chemicals and solvents 109

2.2. Extraction of phenolic compounds 110

2.3. Qualitative and quantitative analyses of phenolic compounds 110

2.4. Analysis of procyanidins using thioacidolysis 111
2.4.1. Freeze-dried extracts 111
2.4.2. Freeze-dried fruits 111

2.5. Antioxidant activity by applying spectrophotometric methods 111
2.5.1. Total Phenolic Contents by the Folin Ciocalteu method 111
2.5.2. DPPH (2,2-diphenyl- 1-picrylhydrazyl) radical scavenging test 112

2.6. Statistical analyses 112

3. Results and discussion 113

3.1. Phenolic profile and content of lingonberry extracts 113
3.1.1. Hydroxycinnamic acid derivatives 116
3.1.1.1. Caffeic acid and ferulic acid derivatives 116
3.1.1.2. Coumaric acid derivatives 117
3.1.2. Flavonol glycosides 118
3.1.3. Flavanols 119
3.1.4. Other compounds in lingonberry extracts 121
3.1.5. Anthocyanins 131

3.2. Influence of the harvest period on the phenolic composition in lingonberry leaves, stems and fruits 132

3.3. Characterization of flavan-3-ol oligomers *137*
3.3.1. In freeze-dried extracts 137
3.3.2. In freeze-dried fruits 139

3.4. Antioxidant activity of lingonberry extracts.. *141*

4. Conclusions ***146***

ANNEXES ***148***

Chapter III. LIPID PROTECTION FROM OXIDATION BY BILBERRY AND LINGONBERRY PHENOLIC EXTRACTS: *IN VITRO* INVESTIGATION UNDER SIMULATED DIGESTION CONDITIONS.. **150**

1. Background ***151***

2. Methodology ***153***

2.1. Materials………… *153*
2.1.1. Chemicals and solvents.. 153
2.1.2. Antioxidants: bilberry and lingonberry samples and phenolic compounds . 154
2.1.2.1. Bilberry and lingonberry extracts... 154
2.1.2.2. Freeze-dried fruits 154
2.1.2.3. Phenolic compounds 154

2.2. Lipid oxidation in gastric model emulsions *155*
2.2.1. Preparation of emulsions.. 155
2.2.2. Preparation of lipid oxidation initiator solutions 155
2.2.3. Lipid oxidation and its inhibition by phenolic extracts and fruits 155
2.2.3.1. Bilberry and lingonberry extracts and phenolic compounds............ 155
2.2.3.2. Freeze-dried fruits 156
2.2.3.3. Determination of lipid-derived conjugated dienes 156

2.3 Simulated static in vitro digestion: preliminary study on the inhibition of lipid oxidation by a bilberry leaf extract... *157*
2.3.1. Bilberry leaf extract 157
2.3.2. Preparation of o/w emulsions 157
2.3.3. Preparation solutions of simulated digestion fluids................................ 157
2.3.4. Experimental protocol of in vitro digestion 158
2.3.4.1. Oral phase.. 159
2.3.4.2. Gastric phase 159
2.3.4.3. Intestinal phase... 159
2.3.5. Determination of lipid-derived conjugated dienes.................................. 160

3. Results and Discussion ***161***

3.1. Inhibition of lipid oxidation by lingonberry and bilberry extracts in in vitro gastric digestion models.. *161*
3.1.1. Inhibition of lipid oxidation by bilberry extracts and various phenolic compounds 161
3.1.1.1. With $MbFe^{III}$ as the initiator.. 161
3.1.1.2. With Fe^{II} as the initiator 164
3.1.2. Inhibition of lipid oxidation by lingonberry extracts.............................. 166

3.1.3 Inhibition of lipid oxidation: bilberry versus lingonberry extracts 168
3.1.4. Inhibition of lipid oxidation by bilberry and lingonberry fruits 169

3.2. Inhibition of lipid oxidation by bilberry leaf extract during digestion in an in vitro model of oro-gastro-intestinal digestion 171

4. Conclusions 175

ANNEXES 176

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES 177

References of thesis 181

Scientific publications 199

INTRODUCTION

Bilberry and lingonberry, two shrubs of the *Ericaceae* family, are known as natural sources of food, beverage and nutraceutical ingredients due to their richness in nutritional and bioactive compounds. Increased attention for these berries is associated to their phenolic composition, antioxidant activity and potential health-related benefits. The high phenolic content found in bilberry and lingonberry is thought to be linked to their biological activities.

The interest in phenolic compounds has grown over recent years, particularly because they are excellent antioxidants. Consumption of antioxidants has shown its efficiency in the prevention of cancer, cardiovascular diseases, osteoporosis, obesity, diabetes and against skin aging (Dai and Mumper, 2010). The antioxidant properties of plant phenolic compounds are relevant in the field of food (inhibition of lipid oxidation), physiology (protection against oxidative stress) and cosmetology. They reflect the UV filter and reducing properties of these compounds and their ability to interact with metal ions and proteins (Cheynier, 2005). In particular, phenolic compounds provide antioxidant activity by direct reducing of reactive oxygen species (ROS), inhibiting enzymes involved in oxidative stress, binding metal ions responsible for the production of ROS and stimulating the endogenous antioxidant defence systems (Dangles, 2012).

The quality and quantity of phenolic compounds in plants are generally influenced by the stage of growth, the parts of the plant to be used and the environmental growing conditions. Until the present, numerous previous studies have focused on the study of a single morphological part of bilberry or lingonberry plant and especially on the fruits, sometimes on leaves and never on stems.
In this context, the aim of this thesis is to simultaneously assess the seasonal variations of phenolic compounds in leaves, stems, and fruits of bilberry and lingonberry collected at different periods of vegetation during two years. This study reports the most comprehensive qualitative study ever conducted leading to the identification of a large variety of phenolic secondary metabolites isolated from the leaves, stems and fruits of bilberry and lingonberry plants. The antioxidant activity of the extracts is also evaluated. Contents in total polyphenols, assessed globally by the Folin-Ciocalteu method or specifically by UPLC, and

the antioxidant capacity in the DPPH test are also tentatively correlated. An original analysis of the oligomeric procyanidins was also proposed in order to determine the degree of polymerization and flavanol unit constitution. Additionally, the antioxidant activity of fruits, leaves and stems of bilberry and lingonberry extracts is evaluated *in vitro* towards lipid oxidation. The investigation of lipid oxidation in an *in vitro* model of gastric digestion and its inhibition by extracts of bilberry and lingonberry was evaluated using two different oil-in-water emulsion systems as models of the gastric content, the bovine serum albumin model (BSA) and phospholipids (PL) model. Finally, the protective capacity of the bilberry leaf extract against lipid oxidation is assessed in a simulated static *in vitro* digestion model (oral, gastric and intestinal phases).

The thesis is structured in two parts. The first part presents a literature review regarding the the abundance of phenolic compounds in plants and foods (structure, methods for extraction and characterization and applications in biological systems), the mechanisms by which phenolic compunds act as antioxidants, the methods for the evaluation of their antioxidant activity, and general aspects on the phenolic compounds of bilberry and lingonberry and their health benefits. The second part concerns the experimental results and contains three chapters. In the first two chapters (I and II), the qualitative and quantitative analysis of phenolic compounds of bilberry and lingonberry and the antioxidant activity of various extracts are presented. Chapter III is focused on the evaluation of the antioxidant activity of stems, leaves and fruits of bilberry and lingonberry extracts in lipid oxidation under *in vitro* simulated digestion conditions.

This work is the result of a PhD thesis undertaken under the joint supervision between "Gheorghe Asachi" Technical University of Iasi, Romania and University of Avignon and Vaucluse, France. The experimental work regarding the UPLC and mass spectrometry analyses, DPPH test and lipid oxidation were obtained in the laboratories of INRA-UMR 408 SQPOV Unit from Avignon in the frame of cooperation facilitated by the Erasmus Program and supervised by Dr. Claire Dufour.

LITERATURE REVIEW

This first chapter of literature review was published as a bibliographic review in the journal "Tappi Journal":

> Bujor O-B., Talmaciu I.A., Volf I., Popa I.V. (2015). Biorefining to recover aromatic compounds with biological properties. *Tappi Journal*, 14(3), 187-193.

I. Phenolic compounds as secondary metabolites found in plants and foods

Polyphenols are a widespread group of secondary metabolites found in all plants, representing the most desirable phytochemicals due to their potential to be used as additives in food industry, cosmetics, medicine and others fields. At present, there is an increased interest to recover them from plant of spontaneous flora, cultivated plant and wastes resulted in agricultural and food industry. Phenolic compounds are considered the most abundant constituents of plants and processed foods; some phenolic compounds are extremely widespread while others are specific to certain plant families or found only in certain plant organs or at certain development stages (Cheynier, 2012).

Main edible sources of phenolic compounds are fruits and vegetables, seeds, cereals, berries, beverages (wine, tea and juices), olive and aromatic plants. Particularly, in last years many studies focused a special attention to the presence of these compounds in agricultural and industrial wastes, wood and non-wood forest resources (Moure et al. 2001; Ignat et al. 2011b; Stevanovic et al. 2009). In the works carried out in our laboratory, sources such as vine stems and grape seeds and wood bark (spruce and pine bark) have been used to separate lignin and different phenolic compounds. Spruce and pine wood bark, which represent a waste in the wood industry, have been reported to contain a wide range of phenolic compounds like stilbene glycosides (Balas and Popa, 2007a), gallic acid, catechine, vanillic acid (Hainal et al. 2011). Also, grape seeds were found to contain up to 506 mg GAE/100 g of total phenolics, 193 mg GAE/100 g of total tannins, 27 mg RE/100 g of total flavonoids and 18 mg/100 g of total antocyanins (Ignat et al. 2011c). In another study on grapes, Moreno-Montoro et al. have showed that red and white grape juices gives an elevated concentration of total phenolic compounds, which were higher in the red grape juices, largely attributable to their elevated anthocyanins and flavonoids content. It should be mentioned that some

polyphenols (e.g. gallic, syringic, p-coumaric, ferulic and vanilic acids, rutin, quercitine, catechine) are present in wood bark and other forestry residues (Table I-1).

Table I-1. Concentration of phenolic compounds (mg/100 g dried plant) in different sources (Ignat et al. 2013).

Raw material	Type of extract	Gallic acid	Catechin	Vanillic acid	Syringic acid	p-coumaric acid	Ferulic acid	Rutin	Quercetin
Spruce bark	Aqueous extract	-	31	39.4	-	-	-	-	-
	Ethanolic extract	10.2	71.9	71.9	-	-	-	-	1.39
Grape seeds	Aqueous extract	6.12	44.36	-	-	-	-	-	
	Ethanolic extract	12.54	63.60	-	-	-	-	-	2.38
Crataegus monogyna	Aqueous Extract	-	23.42	-	2.14	-	-	-	
	Ethanolic extract	10.98	89.52	-	2.95	3.59	2.25	30.32	0.64
Asclepias syriaca	Aqueous extract	-	-	0.87	0.98	0.11	-	-	
	Ethanolic extract	0.65	-	2.94	1.94	0.40	-	2.25	0.14

I.1. Structure and classification

Phenolic compounds are one of the most numerous and widely distributed group of aromatic compounds in the plant kingdom, with over 8000 phenolic structures currently known, of which more than 6000 are the flavonoids (Garcia-Salas et al., 2010; Tsao et al., 2010; Vladimir-Knežević et al., 2012). From the chemical point of view, polyphenols are natural compounds with aromatic structures containing one or more aromatic rings with or without the vicinity of a heterocycle and which are grafted with hydroxyl, carboxyl, methoxyl and carbonyl functional groups. According to the biological function, polyphenols can be classified into different classes; however, two main groups of polyphenols can be identified: the flavonoids and the nonflavonoids.

I.1.1. Non-flavonoid polyphenols

In the literature, non-flavonoids can be classified according to their chemical structure into the following groups: phenolic acids with the subclasses derived from

hydroxybenzoic acids and from hydroxycinnamic acid, stilbenes, lignans and the polymeric lignins (Han et al., 2007; Vladimir-Knežević et al., 2012).

Phenolic acids. From a chemical point of view, phenolic acids containing carboxyl group with one or more hydroxyl groups grafted onto a benzene nucleus. Phenolic acids are the most abundant polyphenols in our diets (30%) and are found in different forms in plants, including aglycones (free phenolic acids), esters, glycosides, and/or bound complexes (Garcia-Salas et al., 2010; Khoddami et al., 2013). Based on position of the hydroxyl group, phenolic acids can be divided into two main types, benzoic acid (C1–C6) and cinnamic acid derivatives (C3–C6) (Figure I-1) (Tsao, 2010).

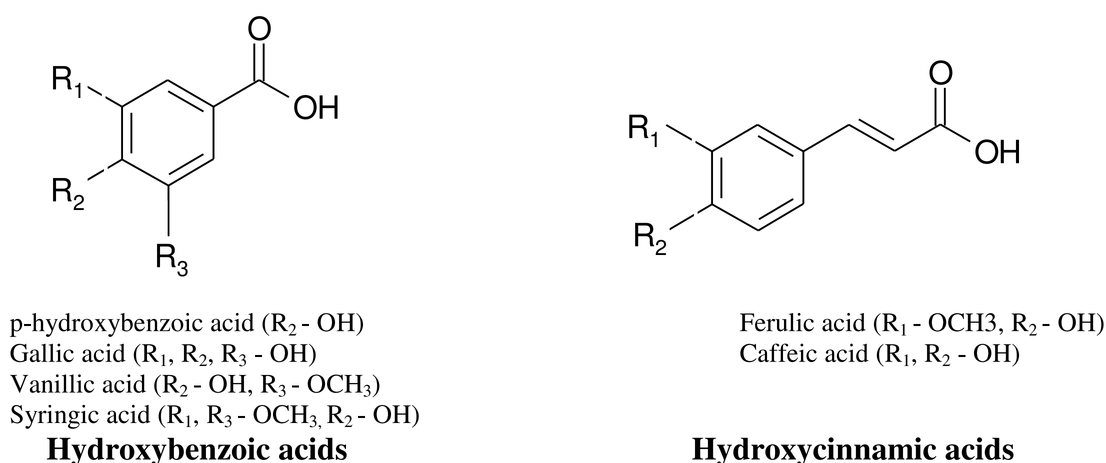


Figure I-1. Representative examples of phenolic acids.

The most common hydroxybenzoic acids are vanillic, syringic and gallic acids. Among hydroxycinnamic acids, caffeic and ferulic acids are the most abundant compounds in foods. Ferulic acid is mainly found from dietary fiber, sources of which include wheat bran and caffeic acid occurs mainly as esters (chlorogenic acid) and is largely obtained from coffee, fruits and vegetables (Ndhlala et al., 2010).

Stilbenes. Stilbenes are another class of compounds that are part of nonflavonoid polyphenols with 1,2-diphenylethylene as basic structure.

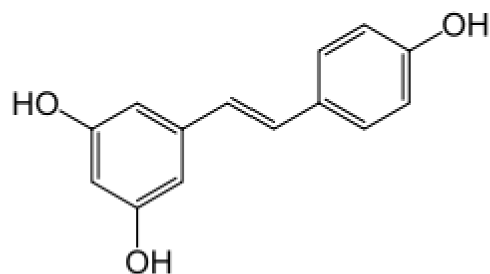


Figure I-2. Chemical structure of stilbene (resveratrol).

Resveratrol (Figure I-2) is the main representative of this group of phenolic compounds. This compound exists in two stereoisoforms with configuration *cis*- or *trans*-, the latter being the most widely studied (Giovinazzo et al., 2012). Resveratrol is found in small fruits such as grapes and *Vaccinium* berries, peanuts and in *Polygonum* species (Rimando & Suh, 2008). High interest in this compound is linked to its use in the treatment of cardiovascular diseases (Kelsey et al., 2010), but also in the fight against motor deficiencies that lead to mobility problems of old people (nutraingredients.com).

Lignans. The lignans are a group of natural phenolic compounds with carbon skeletons derived from two phenylpropane units joined together by at least one carbon-carbon bond between the two central β -carbons of the C3 chains (lignans) or by bonds other than the β-β'-carbon-carbon bond, in which case the resulting compounds are called neolignans (Ferrazzano et al., 2011). In nature, lignans are present in the aglycone forms, while their glycosides occur only in small amounts (Ignat et al, 2011b).

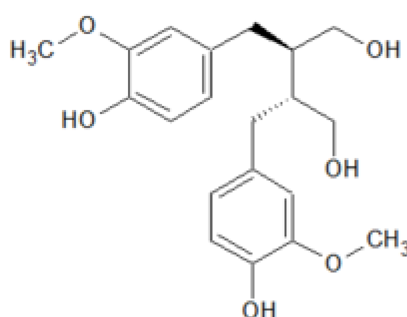


Figure I-3. Chemical structure of lignans (secoizolariciresinol).

The greatest dietary source of lignans is considered to be the flax seeds, but they are also found in appreciable quantities in sesame seed and, to a lesser degree, in a variety of grains, seeds, fruits, and vegetables (Craft et al., 2012). In general, the lignans

content of foods not exceed 2 mg/100 g with some exceptions: 335 mg/100g in flaxseed and 373 mg/100g in sesame seeds, which have lignan content a hundred times higher than other dietary sources (Peterson et al., 2010). Lignans have many biological activities, showing antiviral, anticancer, anti-inflammatory, antimicrobial, antioxidant, immunosuppressive properties and hepatoprotective and osteoporosis prevention (Cunha et al., 2012).

Lignins. Lignins are important plant polymers that comprise 16–33% of wood biomass and represent the second largest organic compound after cellulose (Mäki-Arvela et al., 2007). The chemical structure of lignin is the result of polymerization of the *p*-coumaryl, coniferyl, and sinapyl hydroxycinnamic alcohols (Yang et al., 2013). In plants, lignin strengthen the plant cell walls, aid water transport, protects polysaccharides in the plant cell walls from degrading, help plants to resist on pathogens and other threats, and provide texture in edible plants (Peterson et al., 2010). Due to its so complex structure, lignin valorization is one of the greatest challenges in biorefining being the only large-volume renewable feedstock that is composed of aromatics (Ragauskas et al., 2014). Furthermore, Gilca et al., 2014 showed that using hydroxymethylation reaction of lignin it was possible to obtain nanoparticles from Sarkanda grass lignin, which can be used as biocide in wood protection. Lignins are also important to the human health because possesses multiple properties such as antioxidant, UV-absorption antifungal, antibiotic activity, anticarcinogenic, apoptosis-inducing antibiotic, anti-HIV activities and it has been suggested that can be applied for stabilization of food and feed (Dumitriu & Popa, 2013).

I.1.2. Flavonoids

Flavonoids are a class of phenolic compounds which together with carotenoids and chlorophyll give colour to many species of flowers and fruits. Flavonoids occur only in plants where are present predominantly as glycosides (El Gharras, 2009), in which one or more hydroxyl groups of phenols are combined with reducing sugars. Flavonoids are also associated with a wide range of biological effects on health, including antibacterial, anti-inflammatory, anti-allergic and antithrombotic activities (Pyrzynska and Biesaga, 2009).
The term flavonoid is assigned to the polyphenolic compounds of the general structure C6-C3-C6 in which the two phenolic benzene rings A and C are linked by a pyran ring B (Figure I-4).

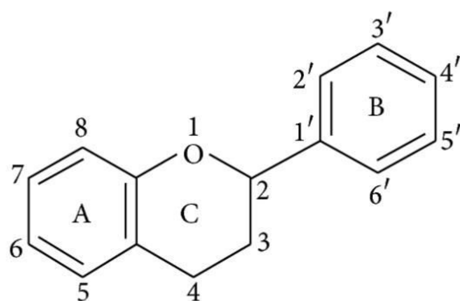
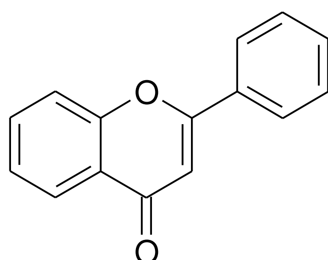


Figure I-4. Basic structure of flavonoids (2-phenyl-1-benzopyran).

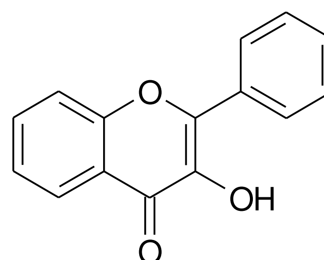
According to the oxidation state of the central C ring, flavonoids are divided into six subgroups: flavones, flavonols, flavanols, flavanones, isoflavones, and anthocyanins (Figure I-5) (Dai and Mumper, 2010). This subclass is also distinguished by the number, position and nature of the substituent existing in the phenolic ring (free hydroxyl, glycosidic and methylated groups).

Regarding the biosynthesis of flavonoids, they are derived from the aromatic amino acids, phenyalanine and tyrosine, and have three-ringed structures (Khoddami et al., 2013).



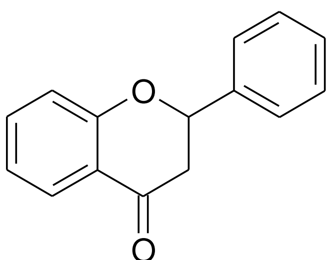
Flavone

(2-Phenyl-chromen-4-one)

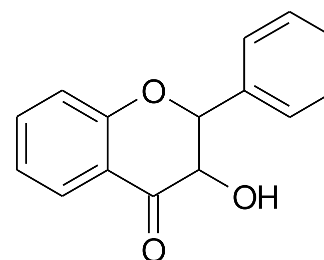


Flavonol

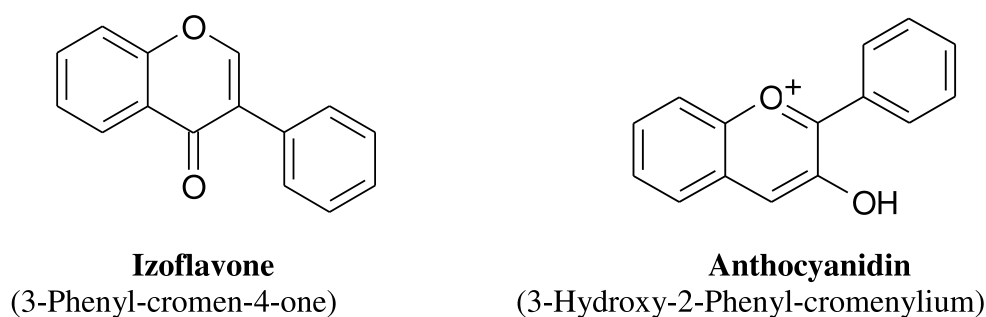
(3-Hydroxy-2-phenyl-chromen-4-one)



Flavanone
(2-Phenyl-croman-4-one)



Flavanol
(2-Phenyl-croman-3-ol)



Izoflavone
(3-Phenyl-cromen-4-one)

Anthocyanidin
(3-Hydroxy-2-Phenyl-cromenylium)

Figure I-5. Chemical structures of some representative subclasses of flavonoids (Tapas et al., 2008).

Flavones and flavonols. Flavones are characterized by the presence of a double bond between C2 and C3 in the heterocycle of the flavan skeleton (Vladimir-Knežević et al., 2012). The most studied flavones are apigenin, luteolin and their derivatives. Flavonols are hydroxylated flavone derivatives and distinguished by the presence of hydroxyl functional group in C3 position. They are found in many fruits and vegetables, including onions, apples, broccoli, and berries (Patel, 2008). Among the flavonols, kaempferol, quercetin and myricetin are most important.

Flavanones and flavanols. Compared with the flavonols and flavones, these two groups are characterized by the absence of the double bond between C2 and C3 and have the precursor 2-phenyl-benzopyrone. The main source of flavanones and flavanols are citrus fruits and juices, flavanols having an important role in generating these fruit taste (Peterson et al., 2006). Among flavanones we can mention: hesperetin, naringin, pinocembrina and eriodictyol. Naringin is present in grapefruit, oranges, grape berries epicarp. This substance has an antibacterial, antiviral, anticancer, depression and antioxidant effect (Sandhar et al., 2011). Hesperetin is known for his antibacterial, antiviral, pesticidal, cancer preventive and hepatoprotective action. Flavanols or flavan-3-ols exist as simple monomers such as (+)-catechine and (−)-epicatechine, but also oligomers or polymers are called proanthocyanidins because they release anthocyanidins when are heated in acidic solutions.

Anthocyanins. Anthocyanins are the main class of flavonoids that are responsible for cyanotic colours ranging from pink, red and purple to dark blue of most fruits, flowers and leaves (Andersen and Markham, 2006). Chemically anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts,

the only differences between individual anthocyanins being the number of hydroxyl groups in the molecule, the degree of methylation of these hydroxyl groups, the nature and number of sugars attached to molecule and the position of the attachment, and the nature and number of aliphatic or aromatic acids attached to the sugars in the molecule (Mazza and Miniati, 1946). The aglycones of anthocyanins, *the anthocyanidins*, consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also bonded by a carbon–carbon bond to a third aromatic ring B (Ignat et al., 2011b). The diversity of anthocyanins are due to the number and position of hydroxyl and methoxy groups, the identity, number, and positions at which sugars are attached, and the extent of sugar acylation and the identity of the acylating agent, but only six are ubiquitously spread and of great importance in human diet: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. The most commonly types of sugars linked to anthocyanidins are monosaccharides (glucose, galactose, rhamnose and arabinose), and di- or tri-saccharides formed by combination of the four monosaccharides (Ignat et al., 2011b; Ienaşcu et al., 2009).

Isoflavones. Found abundantly in vegetable, isoflavones are a group of compounds derived from flavanones. The main factor that differentiates them from other isoflavones is given by the orientation of the C3 position of the benzene ring C (Andersen and Markham, 2006). Isoflavones are also called phytoestrogens because their structure is analogous to the structure of estrogen (Ignat et al., 2011b). The most representative compounds of this class are daidzein, genistein, biochanin A and formononetin. Natural sources of isoflavones are soy and products thereof, being found in dry peas, alfalfa seeds and grain / seed of clover, green beans, chickpeas, lima beans and sunflower seeds.

I.2. Methods for extraction and characterisation of phenolic compounds

Due to the structural diversity and complexity of phenolic compound in plants, extraction is the first and the most important step in the separation and characterization of these compounds. The most common liquid/liquid and solid/liquid extractions are frequently employed to separate phenolic compounds. The phenolic nature of polyphenols makes them relatively hydrophilic, thus free phenolic compounds, including aglycones, glycosides, and oligomers, are extracted using water, polar organic

solvents such as ethyl acetate, methanol, ethanol, chloroform, dietyl ether, acetonitrile and acetone, or their mixture with water (Ignat et al., 2011).

At present, regarding the overall environmental impact of an industrial extraction, the concept of the green extraction is introduced to protect both the environment and consumers, and in the meantime to enhance competition of industries to be more ecologic (the use of co-products, biodegradability), economic (less energy and solvent consumption) and innovative (Chemat et al., 2012). In agreement with this green extraction approach, the unconventional extraction methods such as microwave (Mandal et al., 2007), ultrasound-assisted extractions (Ghitescu et al., 2015), and techniques based on the use of compressed fluids as extracting agents, such as subcritical water extraction (SWE) (Dai and Mumper, 2010) supercritical fluid extraction (SFE) (Herrero et al., 2010), pressurized fluid extraction (PFE) or accelerated solvent extraction (ASE) (Kaufmann and Christen, 2002) are applied actually to separate phenolic compounds.

For the quantification and characterization of phenolic compounds from plant extracts different spectrophotometric and chromatographic methods have been developed. As spectrophotometric method, Folin–Ciocalteu assay is widely used for determining total phenolics content, the vanillin and proanthocyanidin assays have been used to estimate total proanthocyanidins, pH differential method are used for the quantification of total anthocyanins, and the total flavonoids content can be determined using a colorimetric method based on the complexation of phenolic compounds with Al(III) (Ignat et al. 2011b). Among them, the Folin-Ciocalteu method is most commonly used. The mentioned spectrophotometric assays gives an estimation of the total phenolic contents, while various chromatographic techniques are employed for separation, identification and quantification of individual phenolic compounds (Vladimir-Knežević et al., 2012).

To identify phenolic compounds, the most common technique is high performance liquid chromatography (HPLC). The HPLC method is also used for the quantitative analysis of phenolic metabolites from different plant extracts (Lee et al., 2008; Kim et al., 2014). Identification and analysis of phenolic compounds are usually achieved by using a combination of UV– visible spectrophotometry (diode array detector – DAD), mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) (Fulcrand et al., 2008; Cheynier, 2012).

I.3. Applications of phenolic compounds in different biological systems

The biological activities of phenolic compounds from natural sources was demonsted in many *in vitro* and *in vivo* studies that showed their application as antioxidants, antibacterial and anticarcinogenic agents; amendments in bioremediation, allelochemicals, and plants growth regulators, and also in chelation of metal ions process (Popa, 2000; Popa et al., 2008; Balas et al., 2008). In addition, polyphenols are partially responsible for the organoleptic properties of food plants (colour, taste, smell, astringency, bitterness, oxidative stability) due to the interaction between phenolics, mainly proanthocyanidins, and glycoprotein in saliva (Dai and Mumper, 2010).
In this section some examples concerning the role of phenolic compounds in the fields of vegetal and in microorganisms development are presented.

I.3.1. Applications of phenolic compounds in the plants development

I.3.1.1. As plants growth bioregulators

Researches carried in the recent years have shown that natural compounds with aromatic structure, such as phenolic compounds have extremely complex roles in plant physiological processes. Phenolic compounds are important for the physiology of plants contributing to the resistance to microorganisms, insects and herbivorous animals. All these compounds help to preserve the integrity of plant with continuous exposure to environmental stressors, including ultraviolet radiation, relatively high temperatures, presence of heavy metals (Stingu et al., 2012) and dehydration, being also involved in plant growth and development cycle, as they have been shown to regulate the transport of polar auxins (Castillo et al., 2012; Ignat et al., 2013).
The phenolic compounds as bioregulators have an important role in the growth and development of different parts of plants: roots, stems, buds, leaves (Tanase et al., 2013a). Data from the literature provide information concerning the influence of phenolic compounds in the processes of seed germination and plants development, either as individual compounds or global extracts obtained from different plant sources. Depending on extraction procedure, concentration and the nature of extracted compounds containing the same bioactive phenolic compounds may act as stimulators as well as inhibitors (Table I-2) in plant growth (Popa et al., 2002; Ignat 2009).

Table I-2. Effects of polyphenolic extracts in the processes of seed germination (Stingu et al., 2009; Tanase al al., 2013).

Extract type	Tested plant	The effects of the polyphenolic aqueous extracts					
		Roots length	Stems length	Leaves area	Roots dry mass	Stems dry mass	Leaves dry mass
Chestnuts shell extract	Oat seeds	_	_	~	+	_	~
	Rapeseeds	+	+	~	~	+	_
Spruce bark extract	Maize seeds	+	_	~	+	_	~

+: stimulation effect. ~: no visible effect. -: inhibition effect.

Assessing the action of polyphenols of spruce bark extracts on tomato seeds a positive influence on the rate and capacity of germination, seedlings growth and biomass amount accumulated after germination experiment was observed. The stimulating effect of primary root elongation and hipocotiles, found in the presence of polyphenolic extracts at concentrations of 40 and 200 mg / L in the growth medium, can be compared with those of auxins or citokinins (Balas et al., 2005; Balas and Popa, 2007a). The same effect of spruce bark extracts it was also demonstrated on the maize callus tissue developed in culture medium containing also deuterium depleted water (Tanase et al., 2013a; Tanase et al., 2013b).

In a study carried out by Ignat et al., 2009 was observed both stimulating effect on the radicles elongation of bean plants (*Phaseolus vulgaris*) in the presence of aqueous extracts from spruce bark, *Asclepias syriaca* plant and grape seeds obtained by sonication, and inhibitory effect on the amount of fresh biomass accumulated. In the studies carried out in our laboratory different lignins and polyphenols extracted from phytomass sources (old wood and stems of *Vitis species)* have been tested in model experiments to follow their actions as allelochemicals. The results of investigations allow to appreciate that lignin have a biostimulating effect on mitotic division, in the radicular meristems of *Phaseolus vulgaris*. It was concluded that this effect is induced as a result of the improvement of micromedia conditions at plants' roots level, correlated with the beneficent influence of lignin on the microflora present in soil (Popa et al., 2008).

I.3.1.2. As amendments in bioremediation

Bioremediation refers to the use of green plants to remove, contain, or render harmless environmental contaminants such as organic solvents, PCBs, heavy metals, polyaromatic hydrocarbons, explosives and energetics, or nutrients (Bodarlau et al., 2002).

There are known two possibilities to deal with heavy metal contaminated soil: phytoextraction and phyto-stabilisation. Phytoextraction (phyto-accumulation) is a nondestructive technique, aesthetically pleasing by its nature, developed to remove the trace elements from the soil through their uptake and accumulation by plants. Phyto-stabilisation (phyto-immobilisation) aims at establishing a vegetation cover and at promoting *in situ* inactivation of trace elements by combining the use of metal tolerant plants and soil amendments that help reduce the mobility and toxicity of pollutants and, at the same time, may increase soil fertility and improve plant establishment (Stingu et al., 2011a).

In the studies developed in our group individual phenolic compounds (catechine) and phenolic extracts from spruce bark (*Picea abies*), *Asclepias syriaca* plant, chestnuts shell (*Castanea sativa*) and grape seeds (*Vitis vinifera*) were used as modulators of copper (Figure I-6) and cadmium bioaccumulation in rape, bean (*Phaseolus vulgaris*), oat (*Avena sativa*) and maize (*Zea mais*) plants (Stingu et al., 2009b; Stingu et al., 2011a; Stingu et al., 2011b; Stingu et al., 2012; Volf et al., 2012).

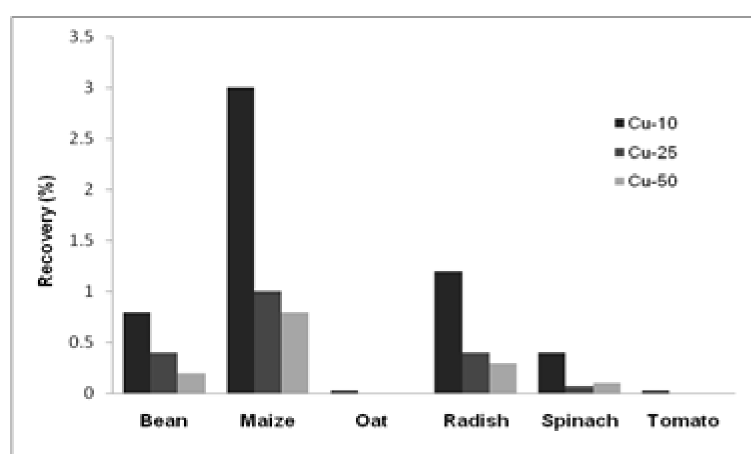


Figure I-6. Utilization of different vegetal biomass resources in copper bioremediation Cu-10, Cu-25, Cu-50 - copper ions concentration in testing solution - 10, 25, 50 mg/L (Stingu et al., 2009; Stingu et al., 2010).

All extracts could properly be used in bioremediation as an alternative to synthetic chelators determining *in situ* inactivation of heavy metal ions and being suitable in phytostabilisation and improving the phytoextraction process (Stingu et al., 2012). However, it is important to

note that the bioaccumulation process depends on heavy metal concentrations and polyphenolic extracts compositions determined by raw materials (Volf et al., 2012).

I.3.2. Applications of phenolic compounds in microorganisms development

Phenolic compounds have various defensive functions in plants, such as cell wall strengthening and repair or antimicrobial and antifungal activities (Hainal et al., 2010; Ferrazzano et al, 2011). Phenolic compound as catechine act on different bacterial strains belonging to different species (*Escherichia coli*, *Bordetella bronchiseptica*, *Serratia marcescens*, *Klebsiella pneumonie*, *Salmonella choleraesis*, *Pseudomonas aeruginosa*, *Staphilococcus aureus*, and *Bacillus subtilis*) by generating hydrogen peroxide and by altering the permeability of the microbial membrane (Ferrazzano et al., 2011). Ignat et al., 2013 have been evaluated antibacterial activities of several types of phenolics extracted from spruce bark, grape seeds, *Crataegus monogyna* (hawthorn) and *Asclepias syriaca* against *Gram-positive* and *Gram-negative* pathogen bacteria. The obtained results showed that spruce bark, *Crataegus monogyna* and grape seed ethanol extracts exerted antibacterial activity (the largest inhibition zones, 12-15 mm) against Gram-positive pathogenic bacteria (*Staphylococcus aureus*) (Table I-3). Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) are less susceptible (smaller inhibition zones) to herbal ethanolic extracts obtained from spruce bark and *Asclepias syriaca* and not susceptible to aqueous extracts.

Table I-3. Antimicrobial activity of spruce bark, *Crataegus monogyna, Asclepias syriaca* and grape seed extracts. Addapted from Ignat et al. (2013).

Inhibition zones (mm)			
Reference microbial strainss / Plant extracts	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***
Asclepias syriaca aqueous extract	-	6	6
Asclepias syriaca ethanolic extract	-	12	6
Spruce bark aqueous extract	-	-	-
Spruce bark ethanolic extract	15	10	10
Crataegus monogyna aqueous extract	-	-	-
Crataegus monogyna ethanolic extract	15	-	-
Grape seed aqueous extract	-	-	-
Grape seed ethanolic extract	12	-	-

Moreover, phenolic extracts from grape seeds, spruce bark, red grape seeds and *Asclepias syriaca* plant were tested on the cultivation of different species of *Rhodotorula spp.* yeast. It was showed that phenolic compounds existing in all extracts influence the development of yeast species in terms of biomass yield resulting behind fermentation process and of the carotenoid pigment biosynthesis. It was also found out that the yeasts used the phenolic compounds as a carbon and energy source, their concentration being reduced when increasing the duration of cultivation; when these extracts were the only carbon source yeasts have shown the ability to metabolize them. (Danaila et al., 2007; Hainal et al., 2011; Hainal et al., 2012a). Another natural phenolic compound, lignin, can be used as a carbon source for the cultivation of *Rhodotorula spp*. yeast species. The results obtained are in agreement with the result mentioned above, namely that the lignins introduced in the culture medium for the cultivation of *Rhodotorula spp*. yeasts are convenient for increasing the biomass yield and for the biosynthesis of carotenoid compounds (Hainal et al., 2012b).

II. The antioxidant activity of phenolic compounds

II.1. The antioxidant action of phenolic compounds and their mechanisms

The interest in phenolic compounds has grown in recent years, particularly because they are excellent antioxidants (Vladimir-Knežević et al., 2012). Antioxidants consumption has shown its effectiveness in the prevention of cancer, cardiovascular disease, osteoporosis, obesity, diabetes and protection against UV irradiation and aging of the skin (Dai & Mumper, 2010).

There are three important systems where phenolic compounds can express their antioxidant activity: in plants, in foods and in humans. The antioxidant properties of these compounds are of particular interest for foods through the inhibition of lipid oxidation while the protection against oxidative stress is central in plant and human physiology). These properties are reflecting the reducing properties of phenolic compounds and their ability to interact with metal ions and proteins (Cheynier, 2005). In particular, phenolic compounds exert their antioxidant activity by direct scavenging of reactive oxygen species (ROS), inhibition of enzymes involved in oxidative stress, regeneration of other antioxidant (α-tocopherol), chelation of metal ions that are responsible for ROS production and, finally, stimulation of endogenous antioxidant defense systems.

ROS are generated as a result of partial reduction of oxygen which leads to the formation of radical oxygen species such as $O_2^{\bullet-}$ (anion superoxide), $HO^{\bullet}$ (hydroxyl radical), $NO^{\bullet}$ (nitric oxide), as well as $RO^{\bullet}$ (oxyl) and $ROO^{\bullet}$ (peroxyl) radicals that are generated during lipid peroxidation (specifically from polyunsaturated fatty acid (PUFA) oxidation) (Quideau et al., 2011; Dangles, 2012). Other reactive species, such as H_2O_2 (hydrogen peroxide), $^1O_2$ (singlet oxygen), O_3 (ozone), $ONOO^-$ (peroxynitrite), HOCl (hypochlorous acid), HOBr (hypobromous acid), are also ROS which can cause biological damage. Although they are nonradical oxygen species, they are oxidizing agents and/or are easily converted into radicals (Halliwell, 2006). For example, H_2O_2 is a precursor of hydroxyl radical and hypervalent iron complexes formation in the presence of transition metal ions with low oxidation state or heme proteins (Dangles,

2012). To explain the mechanisms by which phenolic compounds act as antioxydants, two main approaches will be discussed below, including their 1) reducing and 2) non-reducing effects.

II.1.1. Through reducing effects: H-atom and/or electron transfer

The substitution whith electron donor groups (hydroxyl group) gives reducing character to phenolic compounds. Due to these properties, the capacities to transfer a hydrogen atom and/or electrons to ROS are the main chemical mechanisms of the antioxidant activity of phenolic compounds (Dangles, 2012).

In the case of H-atom transfer (HAT) (Figure II-1), a phenol antioxidant donates an H-atom to an unstable free radical ($R^{\bullet}$) whith formation of the corresponding phenoxyl radical which is stabilized by delocalization of the unpaired electron throughout the aromatic ring (Craft et al., 2012; Dangles, 2012). In the electron transfer (ET), the phenoxyl radical is produced by single-electron oxidation of the phenol antioxidant, followed by rapid deprotonation of the resultant radical cation (Figure II-1). These reducing effects are characterized by two important physicochemical parameters, the bond dissociation energy (BDE) of the O–H bond and the ionization potential (IP) of the phenolic compounds that quantify the HAT and ET, respectively. The lower the BDE and the IP, the stronger is the reducing activity of a phenolic compound.

The presence, number, and relative position of the hydroxyl groups, intramolecular hydrogen bonds and electronic effects, and polarity are determinant factors that can affect the BDE and IP (Quideau et al., 2011). Regarding the chemical structure of flavonoids (see Figure I-4) several structure-reactivity relationships are involved: the catechol (*ortho*-dihydroxyphenyl) structure in the B-ring increases the stability of oxidized flavonoid radicals through H-bonding or electrondelocalization; the 2,3-double bond, in conjugation with the 4-oxo function, function, enhances the electron delocalization; the presence of both 3- and 5-OH groups, enable the formation of stable quinonic structures as a result of flavonoid oxidation (Han, Zhang & Skibsted, 2012). Bendary et al. (2013) showed that the *ortho* position is more active, due to its intramolecular hydrogen bonding, followed by *para* position and then *meta* position of compounds. Other phenolic compounds, including tyrosol, hydroxytyrosol, gallic, and caffeic acids, resveratrol, epicatechin,

kaempferol, and cyanidin were evaluated for their antioxidant activity by calculations of BDE and IP in gas and solution (water and benzene) phases (Leopoldini et al., 2004). It was found that hydroxytyrosol, gallic acid, caffeic acid, and epicatechin act particularly as H-atom donors because of the small values of BDE, whereas kaempferol and resveratrol act better by electron-transfer mechanism showing small values of IP and very negative values of ΔIP referred to phenol. Cyanidin is less active as an electron donor than phenol with a positive value of ΔIP in the gas phase and benzene, but more active in water solution.

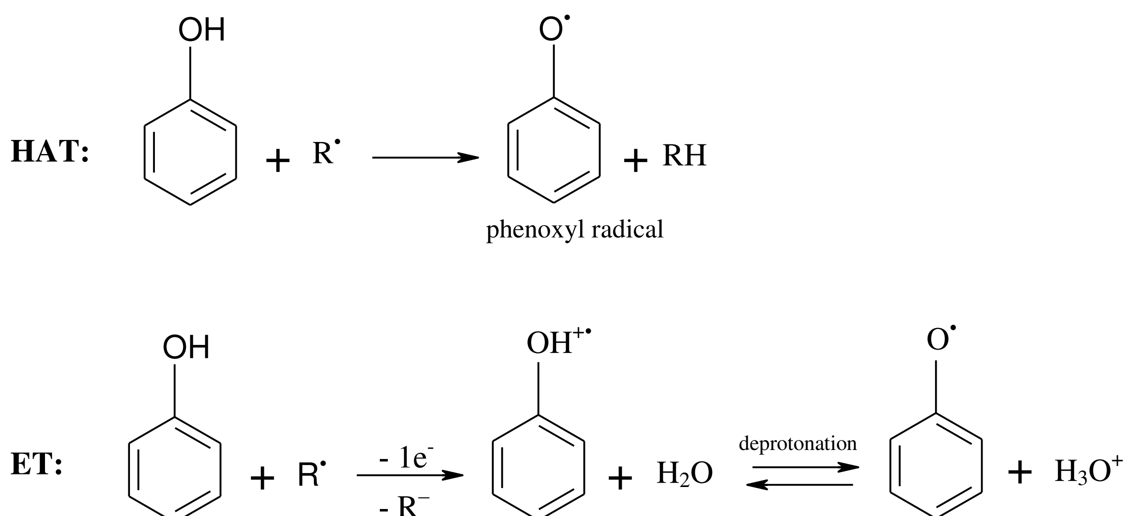


Figure II-1. Hydrogen-atom transfer (HAT) and single-electron transfer (SET) mechanisms in the antioxidant activity of phenolic compounds.

Phenolic compounds also manifest reducing activity in the regeneration of other antioxidants. Interaction between them and tocopherols is the most relevant example of synergism among antioxidants. When *α*-tocopherol donates hydrogen to radicals, it leads to the *α*-tocopheroxyl radical (reaction II-1). The phenolic compounds (ArOH) regenerates *α*-tocopherol by reducing the *α*-tocopheroxyl radical (reaction II-2).

$$\alpha\text{-TOH} + R^{\bullet} \longrightarrow \alpha\text{-TO}^{\bullet} + RH \qquad (II\text{-}1)$$

$$\alpha\text{-TO}^{\bullet} + ArOH \longrightarrow \alpha\text{-TOH} + ArO^{\bullet} \qquad (II\text{-}2)$$

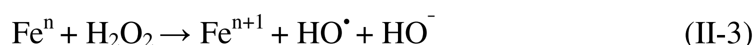
It was found that flavonoids quercetin, (-)-epicatechin and (+)catechin with a catechol group led to substantial increase of α-tocopherol concentrations in blood plasma and liver tissue of rats (Frank et al., 2006) while the green tea phenolic compunds, (−)-

epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, (−)-epigallocatechin gallate, and gallic acid, have been found to reduce the α-tocopheroxyl radical to regenerate alpha-tocopherol (Zhou et al., 2005).

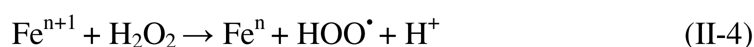
II.1.2. Through non-reducing effects

II.1.2.1. Metal-ion chelating activity

Phenolic compounds are known to act as antioxidants by inhibiting the prooxidative action of metal ions by a chelation action. In this process metal ions like iron(II)/copper(I) and iron(III)/copper(II) ions are involved in the conversion of $O_2^{-\bullet}$ and H_2O_2 into $HO^{\bullet}$ via the Fenton reaction (Quideau et al., 2011):



$$Fe^{n} + H_2O_2 \rightarrow Fe^{n+1} + HO^{\bullet} + HO^{-} \qquad \text{(II-3)}$$



$$Fe^{n+1} + H_2O_2 \rightarrow Fe^{n} + HOO^{\bullet} + H^{+} \qquad \text{(II-4)}$$

The chelating activity of phenolic compounds is influenced by the number and position of hydroxyl groups in their structure. The 5-OH and/or 3-OH group with a 4-oxo group in the A/C ring structure or a large number of hydroxyl groups are important for the binding/chelation of metal ions while hydroxyl groups covalently linked to methyl groups or a carbohydrate moiety are not involved in the complexation of metal ions (Craft et al., 2012).

II.1.2.2. Inhibition of enzymes implied in the production of ROS

Phenolic compounds are also implied in the reduction of ROS through the inhibition of prooxidative enzymes such as xanthine oxidase, lipoxygenases, NADPH oxidase and myeloperoxidase. Xanthine oxidase is an enzyme that catalyzes the ultimate step in purine metabolism (conversion of hypoxanthine into xanthine and of xanthine in uric acid), lipoxygenases (mammalian 15-lipoxygenase) catalyzes the conversion of arachidonic acid into eicosanoids such as leukotriene B_4 and myeloperoxidase, that is a heme enzyme, reduces H_2O_2 into H_2O while being converted in a twoelectron oxidized intermediate (Dangles, 2012).

II.1.3. Inhibition of lipid oxidation

Lipid peroxidation is free radical chain reaction initiated by the hydrogen abstraction or addition of an oxygen radical from/to PUFAs. Mechanims of lipid oxidation involves three steps: initiation, propagation and termination (Berton-Carabin et al., 2014).

- **Initiation -** unsaturated fatty acids (LH) lose a hydrogen atom (H) in an allylic position relative to a fatty acid double bond with the formation of a lipoyl or alkyl free radical ($L^{\bullet}$):

$$RH \rightarrow H^{\circ} + L^{\bullet} \quad \text{(II-5)}$$

This reaction occurs by different way:

- direct reaction between LH and transition metal ions (M):

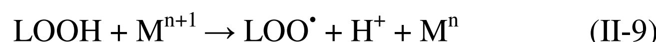
$$LH + M^{n+1} \rightarrow L^{\bullet} + H^{+} + M^{n} \quad \text{(II-6)}$$

- reaction between LH and oxygen radicals from metal autoxidation (reaction II-3):

$$HO^{\bullet} + LH \rightarrow H_2O + L^{\bullet} \quad \text{(II-7)}$$

- oxidation or reduction of hydroperoxides (LOOH) catalyzed by metals:

$$LOOH + M^{n} \rightarrow LO^{\bullet} + HO^{-} + M^{n+1} \quad \text{(II-8)}$$



$$LOOH + M^{n+1} \rightarrow LOO^{\bullet} + H^{+} + M^{n} \quad \text{(II-9)}$$

- thermal decomposition of hydroperoxides:

$$LOOH \rightarrow LO^{\bullet} + HO^{\bullet} \quad \text{(II-10)}$$

$$2LOOH \rightarrow LO^{\bullet} + H_2O + LOO^{\bullet} \quad \text{(II-11)}$$

- **Propagation** – the alkyl radicals ($L^{\bullet}$) (reaction II-5) react quickly with triplet oxygen to generate peroxyl radicals ($LOO^{\bullet}$):

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad \text{(II-12)}$$

Next, the unstable peroxyl radicals abstract hydrogen atoms from other unsaturated fatty acids to form hydroperoxides and other alkyl radicals:

$$LOO^{\bullet} + L'H \rightarrow LOOH + L'^{\bullet} \quad \text{(II-13)}$$

- **Termination -** in this step the radicals react together to form stable nonradical compounds:

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow \text{nonradical products} \quad \text{(II-14)}$$

The most popular methods used for the quantification of lipid oxidation products are the conjugated dienes assay and the thiobarbituric acid-reactive substances (TBARS) test (Lorrain et al., 2010; Lorrain et al., 2012; Toda et al., 2011; Gobert et al., 2014). Both

methods evaluate spectrophotometrically the extent of lipid oxidation. Conjugated dienes method is based on the strong UV absorbance of the CD moiety at λ_{max} of 234 nm whereas the TBARS test quantifies malonaldehyde and malonaldehyde-type products (*trans,trans*-2,4- heptadienal, *trans*-2-heptenal, *trans*-2-hexenal, and hexanal) with a λ_{max} of 532 nm (Craft et al. 2012).

II.2. Methods to evaluate the antioxidant activity of phenolic compounds

II.2.1. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method

The DPPH method is the most frequently used assay for the evaluation of the free radical-scavenging capacity of plant extracts. The reaction mechanism involves the H-transfer from a phenolic compound to the DPPH radical (Figure II-2). Interaction of the DPPH radical (purple-coloured) with a phenolic compound, which is able to neutralize its free radical character, leads to the formation of yellow colorless hydrazine and the resulting effect can be quantified spectrophotometrically at 515 nm (Vladimir-Knežević et al., 2012).

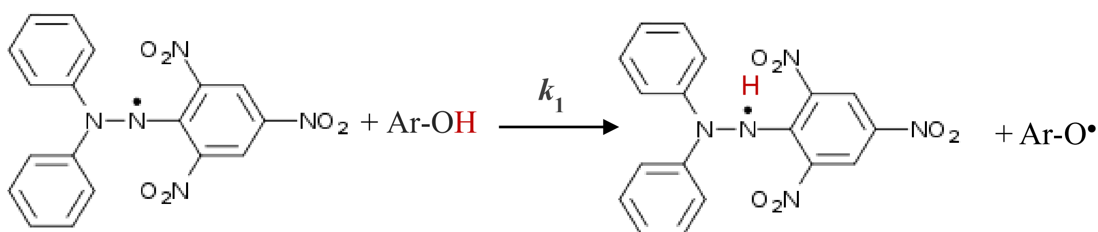
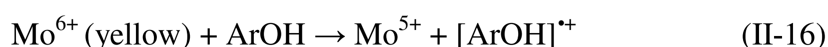


Figure II-2. The H-transfer reaction from a phenolic compound (AR-OH) to DPPH.

In the DPPH test, the antioxidant activity of phenolic compounds is generally quantified by their EC50 values (concentration necessary to reduce 50% of DPPH) or their stoichiometry (number of DPPH molecules reduced by one molecule of antioxidant) (Goupy et al., 2003; Goupy et al., 2009; Vučić et al., 2013) but also as micromoles Trolox equivalents (Faria et al., 2005).

II.2.2. Folin–Ciocalteu redox method

The Folin-Ciocalteu (FC) method is based on a single electron transfer mechanism and is used to quantify the contents in total phenolic compounds in plant extracts using gallic acid as a standard. Since its mechanism is an oxidation/ reduction reaction, the FC method can be considered also a method for quantification of the antioxidant capacity. The FC method involves the reduction of the molybdenum component in the phosphotungsticphosphomolybdic complexing reagent according to the following reaction (Craft et al., 2012):



$$Mo^{6+} \text{ (yellow)} + ArOH \rightarrow Mo^{5+} + [ArOH]^{\bullet +} \qquad \text{(II-16)}$$

The Total Phenolic Compounds are estimated after reaction of the sample with diluted Folin-Ciocalteu reagent (a mixture of sodium molybdate, sodium tungstate and other reagents) and sodium carbonate. The reaction with phenolic compounds produces a blue color which typically absorbs at 765 nm.

Numerous reducing compounds could interfere in the quantification of polyphenols by the FC method, vitamin C being supposed to have the major contribution, but other reducing substances such as some sugars and amino acids could also interfere (George et al., 2005; Everette et al. 2010; Ma et al. 2007). Consequently vitamin C quantification should be concomitantly performed in this method to get appropriate values for polyphenol contents.

II.2.3. Other methods

Other popular methods for evaluation of the antioxidant activity of phenolic compounds are oxygen radical absorbance capacity (ORAC), cupric ion reducing antioxidant capacity (CUPRAC), ferric ion reducing antioxidant power (FRAP), and Trolox equivalent antioxidant capacity (TEAC) assays (Craft et al., 2012).

ORAC reflects radical chain breaking antioxidant activity by H atom transfer mechanism. In the basic protocol of ORAC assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product which is quantitated by fluorescence (Prior et al., 2005). Antioxidant capacity is mesured by a decreased rate and amount of product formed over time (Figure II-3). The advantage of this method compared to other assays is than it can be adapted to detect both hydrophilic and hydrophobic antioxidants by altering the radical source and solvent (Prior et al., 2005).

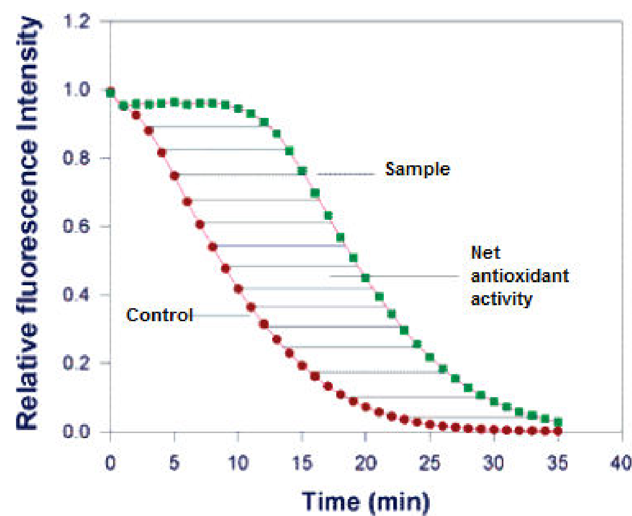


Figure II-3. ORAC antioxidant activity of tested sample expressed as the net area under the curve (AUC) (adapted from Prior et al. (2005)).

CUPRAC, FRAP and TEAC methods are ET-based assays which changes color when bis(neocuproine)$Cu^{2+}Cl_2$, Fe^{3+}(2,4,6-tripyridyl-*s*-triazine)$_2Cl_3$, and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS·+) probes are reduced, respectively (Dai & Mumper, 2010).

III. Bilberry and lingonberry, two shrubs of the *Ericaceae* family as sources of phenolic secondary metabolites

Bilberry (*Vaccinium myrtillus L.*) and Lingonberry (*Vaccinium vitis-idaea* L.) are two wild shrubs of the *Ericaceae* family, genus *Vaccinium.* There are currently more than 450 species of shrubs in the genus *Vaccinium,* found throughout the Northern Hemisphere and extending south along tropical mountain ranges (www.britannica.com). Both species, *Vaccinium myrtillus* L. and *Vaccinium vitis-idaea* L. are distributed throughout Europe, Asia and North America. Fruits and aerial parts of bilberry and lingonberry are consumed as dietary supplements for health benefits.

III.1. Bilberry (*Vaccinium myrtillus* L.)

III.1.1. General description

Bilberry (Figure III-1), also known as European blueberry, whortleberry, and huckleberry is a shrubby perennial plant which can be found in the mountains and forests of Europe and the northern United States (Thorne Research, 2001). In France and Romania bilberry is called “myrtille” and “afin”, respectively (Blamey and Grey-Wilson, 2003; Fischer, 2000). Bilberry grows in alpine areas, et an altitude of 1000-2500 m, especially on shaded and humid mountain slopes, in coniferous forests, alone or in association with lingonberry. The worldwide distribution of bilberry is given in Figure III-2.

Figure III-1. Bilberry (*Vaccinium myrtillus* L.). Photo by Oana-Crina Bujor.

Bilberry is occasionally mistaken for blueberry, but bilberry is native of Europe whereas the true blueberry is native of North America (Chu et al., 2011) and is referred to the most common cultivated species *Vaccinium corymbosum* L. (Bunea et al., 2011).

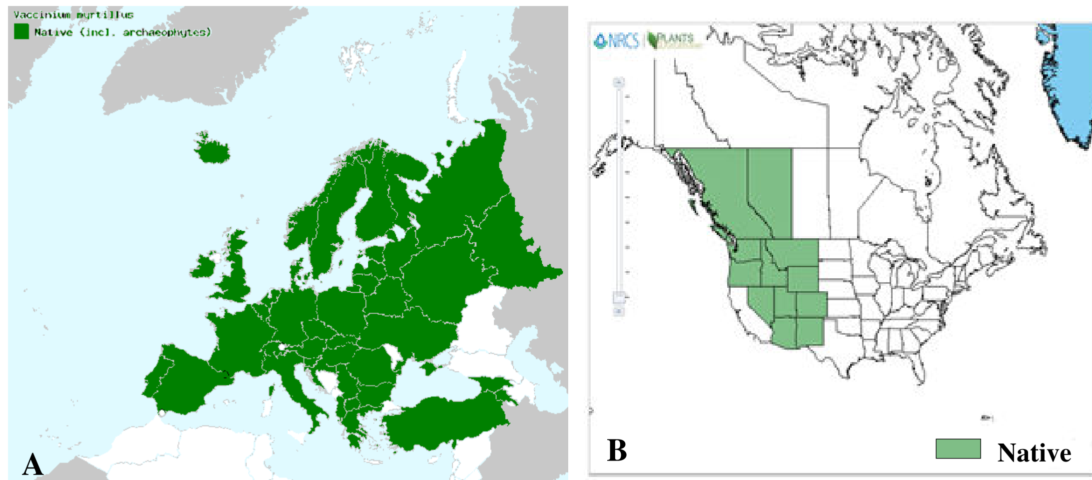


Figure III-2. Distribution of bilberry in Europe and the Mediterranean region (A) and in North America (B). http://ww2.bgbm.org/EuroPlusMed.

The differences between the bilberry and blueberry species like wild-growing low bush blueberries (*Vaccinium augustifolium*), Northern high bush blueberry (*Vaccinium corymbosum*) and rabbit eye blueberry (*Vaccinium ashei Reade*) (Routray and Orsat, 2011) are the blackish-blue colour of the flesh (Figure III-3) compared to the whitish fruit flesh of the others and also its fruits are not produced in clusters, but only as single (Martinussen et al. 2009; www.mirtoselect.info).

Figure III-3. Bilberry red flesh pulp (left) vs blueberry white pulp (right). http://www.mirtoselect.info/bilberry-vs-blueberry.

From a botanical point of view, bilberry is a highly branched shrub with stems to 30-50 cm high, wiry, branched glabrous and green in color (Mckenna et al. 2002). The leaves *(Myrtilli folium)* are 1-3 cm long and 0.6-2 cm wide, shortly petiolate, oval or eliptic, green

on both sides, slightly dentate, falling each autumn, alternate and with short petiole (Bojor, 2003). The flowers are small, actinomorphic, greenish-reddish, with white or rose petals bell-shaped, placed one or two in the leaf axils. Bilberry flourishes in May, after leaf emergence, until July (Fischer, 2000). The fruit (*Myrtilli fructus*) is a bacca, 0.5-0.9 cm in diameter, blue-black in color, sweet and sour taste.

Bilberry fruits are consumed fresh, frozen or processed as ingredient in desserts, dried whole berries (in cereals), as well as in the form of preserves, jams, fruit puree, juices and powdered concentrates (Chu et al., 2011). Aerial parts of bilberry, leaves and stems, are considered a waste byproduct by the industries (Zhu et al. 2013) and are popularly used as infusion and herbal teas in traditional herbal medicine. During the last years the market of dietary supplement ingredients was practically “invaded” by bilberry supplements from fruits and aerial parts which are available as powder, tablets, capsules and liquid extracts. In Europe are known several standardized extracts of bilberry (*Vaccinium myrtillus* L.) such as: MyrtiPROTMEuropean Bilberry Extract of BGG (Beijing Gingko Group) extracted from the wild bilberry found in Northern Europe; Mirtoselect® and Myrtocyan® of Indena (Italy), Bilberry Fruit Dry Extract (EP, USP) and Bilberry Ethanol Extract of Linnea (Swiss), NutriPhy® Bilberry of Chr. Hansen SAS (Danemark) and 600761 Bilberry Extract produced by Kaden Biochemicals GmbH (Germany) (Juadjur et al., 2015).

In Romania, the activity of collection and valorization of forest fruits is controlled and monitored by the Forestry Direction of each county. Increasing concerns for the forest fruits has known a great interest in the last 50 years when their request for export has become significant. At the same time, fruits and leaves of bilberry are collected from the natural habitats by the poor population of rural mountain areas for domestic use and sold in markets or "ont the side of the road".

III.1.2. Chemical composition: phenolic compounds and other constituents

- **Phenolic compounds**:

The main classes of phenolic compounds present in the fruit and aerial parts (leaves and stems) of bilberry are flavonoids, phenolic acids and proanthocyanidins. High amounts of anthocyanins (cyanidin, delphinidin, malvidin, petunidin and peonidin glycosides), hydroxycinnamic acid derivatives and low amounts of flavonols (quercetin and myricetin glycosides), monomers and oligomers flavanols and iridoid derivatives of coumaric acid were identified in bilberry fruits (Juadjur et al. 2015; Mikulic-Petkovsek et al., 2015). Due to the important level of anthocyanins, bilberry was called "wild superberry from Europe" by Jaakola, Uleberg & Martinussen (2013). In contrast, leaves are known to contain predominantly phenolic acids (mainly chlorogenic acid) and flavonol glycosides (mainly quercetin and kaempferol glycosides) but also cinchonains, iridoids, cinnamic acid derivatives and proanthocyanidins in smaller amounts (Hokkanen et al., 2009; Martz et al., 2010; Ieri et al., 2013; Liu et al., 2014). The comparative study conducted by Teleszko & Wojdyło (2015) showed that phenolic compounds were found in significantly higher contents in the leaves than in the fruits. The antioxidant potential of leaves (79.30 and 59.58 mM TE/100 g dm in ABTS and FRAP tests) is also stronger than that fruits (35.34 and 26.81 mM TE/100 g dm in ABTS and FRAP tests as well). A summary of the studies of literature regarding the various phenolic compounds identified in bilberry, *Vaccinium myrtillus* L. species, is shown in Table III-1.

Table III-1. Phenolic compounds identified in bilberry (*Vaccinium myrtillus* L.)

Class	Phenolic compounds	References/Origin
Catechins	catechin, epicatechin gallocatechin epigallocatechin	Hokkanen et al., 2009/Finland[a,L] Može et al., 2011/Slovenia[a,F] Szakiel et al. 2011/Poland [S,R] Ieri et al., 2013/Italy[a,L] Díaz-García et al., 2013/Austria[b, F] Liu et al., 2014/ Finland[a,L] Prencipe et al., 2014/Italy[a,F] Stefkov et el., 2014/Macedonia[a,L] Juadjur et al. 2015/ Germany[b,F] Mikulic-Petkovsek et al., 2015/Slovenia[a,F] Teleszko & Wojdyło, 2015/Poland[a,L,F]
Cinchonains	cinchonain Ix isomer cinchonain IIx isomer	
Proanthocyanidins	B-type dimer B-type trimer, B-type tetramer B-type pentamer A-type trimer	
Phenolic acids	3,4-dihydroxybenzoic acid p-coumaroyl quinic acid isomers p-coumaroyl malonic acid p-coumaroyl derivatives p-coumaroyl glucose coumaroyl iridoid p-coumaric acid feruloyl quinic acid isomer caffeoyl quinic acid isomers caffeic acid ethyl ester caffeoyl shikimic acid caffeoyl glucose caffeoyl iridoid 5-hydroxyvanillic acid chlorogenic acid caffeic acid ferulic acid ellagic acid vanillic acid synapic acid syringic acid gallic acid	

Class	Phenolic compounds	References/Origin
Flavonols	quercetin-3-O-(4“-HMG)-α-rhamnoside quercetin-3-O-galactoside quercetin-3-O-glucoside quercetin-3-O-glucuronide quercetin-3-O-arabinoside quercetin-3-O-α-rhamnoside quercetin-3-O-rutinoside quercetin, quercetin derivative kaempferol-hexoside kaempferol-rhamnoside kaempferol-3-glucuronide kaempferol-3-O-rutinoside kaempferol-O-pentoside myricetin myricetin-3-O-galactoside myricetin-3-O-arabinoside	
Stilbenes	resveratrol 3-O-glucoside trans-resveratrol	
Lignans	lyoniside (9-O-b-D-xylopyranosyl(+)lyoniresinol)	
Anthocyanins	cyanidin, peonidin, petunidin malvidin, delphinidin delphinidin-3-O-galactoside delphinidin-3-O-glucoside delphinidin-3-O-arabinoside cyanidin-3-O-galactoside cyanidin-3-O-glucoside, cyanidin-3-O-arabinoside cyanidin-3-O-xyloside cyanidin-3-O-arabinopyranoside petunidin-3-O-galactoside petunidin-3-O-glucoside petunidin-3-O-arabinoside petunidin-3-O-rutinoside, peonidin-3-O-galactoside peonidin-3-O-glucoside, peonidin-3-O-arabinoside	Kähkönen et al., 2003/Finland[b,F] Faria et al., 2005[a,F] Burdulis et al., 2007/Lithuania[a,F] Lätti et al., 2008/ Finland[a,F] Ienaşcu et al., 2009/ Romania[b,F] Burdulis et al., 2009/Lithuania[a,F] Jovančević et al., 2011/Montenegro[a,F] Može et al., 2011/Slovenia[a,F] Müller et al., 2012/Germany[b, F] Díaz-García et al., 2013/Austria[b, F] Oancea et al., 2013/Romania[a,F] Paes et al., 2014/Brazil[b,F] Prencipe et al., 2014/Italy[a,F]
Anthocyanins	peonidin 6-acetyl-3-glucoside malvidin-3-O-galactoside malvidin-3-O-glucoside malvidin-3-O-arabinoside malvidin-3-O-xyloside malvidin-3-O-arabinopyranoside	

[a]natural habitats, [b]commerce or industry, [F]fruits, [L]leaves, [S]stems, [R]rhizomes.

The quality and quantity of phenolic compounds in *Vaccinium myrtillus* L. are generally influenced by the stage of growth, the country of origin, the parts of the plant to be used, the environmental conditions (temperature, sunlight, soil nutrients, the latitude and altitude of the location where the plants are growing, geographical locations) and genetic factors (Harris et al., 2007; Åkerström et al., 2010; Martz et al., 2010; Uleberg et al., 2012; Mikulic-Petkovsek et al., 2015). As described by Jovančević et al. (2011), the total phenolic content of bilberry harvested from localities exposed to the sun was higher compared with plants grown in shadow. In the same study it was shown that at altitude higher than 1500 m the amount of total phenolics is higher. Differences in fruit phenolic concentrations were also evidenced, i.e. samples from Norway have total anthocyanins of 275 mg/100g and total phenols of 612 mg/100g, whereas fruits from other countries contain 364 mg/100g and 472 mg/100 g, respectively (Nestby et al., 2011). According to the USDA Database, the quantity of each anthocyanidin from the bilberry fruit varies between 20 and 98 mg/100 g (Table III-2).

Table III-2. The Flavonoid Content in raw bilberry fruit. Source: USDA Database, 2013.

Flavonoid class	Flavonoid	Flavonoid content (mg/100 g)
Anthocyanidins	Cyanidin	85.26
	Delphinidin	97.59
	Malvidin	39.22
	Peonidin	20.45
	Petunidin	42.69
Flavonols	Kaempferol	0.00
	Myricetin	1.09
	Quercetin	3.04

- **Other constituents:**

Apart from antioxidant phenolic compounds bilberry contains vitamins A, K, C and E, carotenoids as well as minerals like calcium, potassium, magnesium and phosphorus (Table III-3). The study carried out by Bunea et al. (2012) on *Vaccinium myrtillus* and *Vaccinium corymbosum* species shows that their fruits contain carotenoids such as lutein, β-cryptoxanthin and β-carotene. Other carotenoids like neoxanthin, violaxanthin, antheraxanthin and zeaxanthin have been identified in the fruits of bilberry (Lashmanova et al, 2012).

Bilberry is also an important source of fatty acids. Research carried out on the different species of bilberry have shown that along with linoleic and linolenic acids, bilberry may also contain other fatty acids, but in lower quantities: palmitic, stearic, oleic, elaidic, arachidic, cis-11-eicosenoic and lauric acids (Bunea et al., 2012; Dulf et al., 2012).

Table III-3. Nutrient content in bilberry fruits (values/100 g). Source: Fineli-Finnish Food Composition Database.

Nutrient	Content	Nutrient	Content
Macro-components		**Minerals**	
Energy, kcal	184	Calcium, mg	19.0
Carbohydrate, g	6.4	Iron, mg	0.6
Fat, g	0.6	Iodine, mg	1.0
Protein, g	0.5	Potassium, mg	110.0
Carbohydrate components		Magnesium, mg	9.0
Fibre, g	3.3	Sodium, mg	0.3
Organic acid, g	1.4	Salt, mg	0.8
Sugars, g	6.4	Phosphorus, mg	20.0
Fructose, g	2.9	Selenium, mg	0.1
Glucose, g	3.0	Zinc, mg	0.2
Sucrose, g	0.5	**Vitamins**	
Polysaccharides, g	0.5	Folate, µg	11.5
Fibre, g	2.6	Niacin equivalents, mg	0.6
Fat		Niacin (nicotinic acid + nicotinamide), mg	0.4
Fatty acids, g	0.3		
Fatty acids - polyunsaturated, g	0.2	Vitamers pyridoxine, mg	0.06
Fatty acids - monounsaturated cis, g	< 0.1	Riboflavine, mg	0.07
Fatty acids - saturated, g	< 0.1	Vitamin B1, mg	0.04
Fatty acids - n-3 polyunsaturated, g	0.1	Vitamin C, mg	15.0
Fatty acids - n-6 polyunsaturated, g	0.1	Vitamin A, µg	3.9
Fatty acids 18:2 (linoleic acid), mg	123.012	Vitamin E, mg	1.9
Fatty acids 18:3 (linolenic acid), mg	116.832	Vitamin K, µg	9.0
Sterols, mg	26.4	**Carotenoids, µg**	**310.5**
Nitrogen components			
Tryptophan, mg	10.0		

III.2. Lingonberry (*Vaccinium vitis-idaea* L.)

III.2.1. General description

Lingonberry, like bilberry, is part of the *Ericaceae* family, genus *Vaccinium*. The scientific name of lingonberry is *Vaccinium vitis idaea* L. and it is also known as cowberry (Andersen, 1985; Pyka et al., 2007; Lee and Finn, 2012). In France and Romania lingonberry is called "airelle rouge" and "merisor", respectively (Blamey and Grey-Wilson, 2003; Fischer, 2000). Lingonberry is native (Figure III-4) to Scandinavia, Europe, Alaska, the U.S., Canadian Pacific Northwest and northeastern Canada (Penhallegon, 2006). In Europe, lingonberry are very popular in Nordic countries, the Baltic states, Germany, Austria, Switzerland, Czech Republic, Poland, Slovenia, Slovakia, Romania, Russia, and Ukraine (http://en.wikipedia.org/wiki/Vaccinium_vitis-idaea). Regarding Romania lingonberry is found throughout the Carpathian mountain chain, mostly in the counties of Suceava, Bistrita-Nasaud, Harghita, Neamt, Brasov, Prahova, Dimbovita, Arges, Sibiu, Vâlcea, Gorj and Hunedoara.

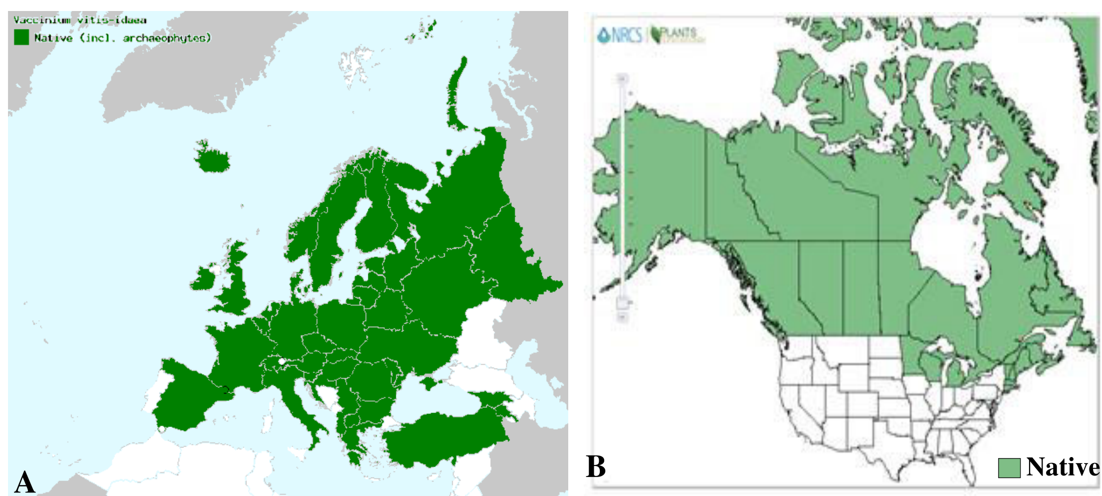


Figure III-4. Distribution of lingonberry in Europe and the Mediterranean region (A) and in North America (B). Source: http://ww2.bgbm.org/EuroPlusMed.

Lingonberry is a small wild shrub with a height of up to 10-30 cm that grows in acidic soils. Lingonberry is a perennial evergreen plant as it keeps its leaves during winter, covering the ground like a carpet. The leaves (*Vitis idaea* Folium) of the lingonberry are

alternate, dark green upper surfaces and light green lower leaf surfaces with black spots on the back. The stem is cylindrical and branched. Campanulate white corollas flowers (Figure III-5A), of 5 to 7 mm long, bloom from May until July. Lingonberry fruits grow in clusters (Figure III-5B) and are dark red in colour, 4 to 10 mm in diameter, with a sour taste due to the arbutin content (Bojor, 2003). Similar to lingonberry is cranberry that refers to the species *Vaccinium oxycoccus* or the European cranberry, *Vaccinium microcarpum* or the small cranberry and *Vaccinium macrocarpon* or the large cranberry, industrially cultivated in North America (Baroffio et al., 2012; Lee and Finn, 2012).

Figure III-5. Lingonberry (*Vaccinium vitis-idaea* L.), with flowers (A) and fruits (B). Photo by Oana-Crina Bujor.

Because of its high nutritional value, lingonberries are used as food source being considered as a health promoting natural fruit. The fruits have a variety of applications such as jams, jellies, syrups, purees, sauces, fruit juices, beverage concentrates as well ingredient for chocolates, tarts, cookies, ice cream, cereals and yogurt. Dried fruits are commercially available in whole, powder or ingested in supplemental form (NutriPhy® Bilberry extract of Chr. Hansen SAS, Denmark). Leaves and stems are consumed as infusions and herbal teas.

III.2.2. Chemical composition: phenolic compounds and other constituents

Lingonberry is known as a natural source of food, beverage and dietary supplements due to its richness in nutritional and bioactive compounds. Although lingonberry constituents have multiple biological activities, most of the research has focused on the polyphenolic compounds (Mane et al., 2011; Kylli et al., 2011).

- **Phenolic compounds:**

The most abundant polyphenols in the fruit of lingonberry (*Vaccinium vitis-idaea* L.) are glycosides of quercetin, monomers and oligomers of catechin and epicatechin, caffeic acid derivatives and anthocyanins, all known to be powerful antioxidants that act by direct trapping of reactive oxygen species, binding of transition metal ions and inhibition of enzymes involved in the oxidative stress (Lorrain et al., 2012; Goupy et al., 2009; Volf et al., 2014). Many phenolic compounds identified in *Vaccinium vitis-idaea* L. are presented in Table III-4.

Table III-4. Phenolic compounds identified in the lingonberry (*Vaccinium vitis-idaea* L.)

Class	Phenolic compounds	References/Origin
Catechins	(+)-catechin	Zheng and Wang, 2003/Canada[a,F]
	(-)-epicatechin	Ek et al., 2006/ Finland[a,L,F]
		Hokkanen et al., 2009/Finland[a,L]
		Mane et al., 2011/Danemark[a,F]
Cinchonains	cinchonain Ix isomer	Jungfer et al., 2012/Europe and China[a,F]
	cinchonain IIx isomer	Ieri et al., 2013/Italy[a,L]
		Liu et al., 2014/Finland[a,L]
Proanthocyanidins	B-type dimer,	
	B-type trimer	
	B-type tetramer	
	A- type dimer	
	A- type trimer	
	procyanidin A2	
	procyanidin B1	
	procyanidin B2	
	procyanidin B5	
	procyanidin C1	

Class	Phenolic compounds	References/Origin
Phenolic acids	caffeoyl quinic acid isomers	
	caffeoyl shikimic acid	
	caffeic acid hexoside	
	4-Glc-caffeic acid	
	caffeoyl acetyl arbutin	
	caffeoyl arbutin	
	acetyl arbutin	
	caffeic acid	
	arbutin	
	coumaroyl quinic acid isomers	
	4-Glc-p-coumaric acid	
	p-coumaroyl derivatives	
	feruloyl quinic acid isomer	
	coumaroylarbutin	
	coumaroyl iridoid	
	p-coumaric acid	
	chlorogenic acid	
	5-Caffeoylquinic acid	
	cryptochlorogenic acid	
	4-O-caffeoyl-quinic acid	
	fraxetin-8-O-glucoside	
	dihydroferulic acid	
	gentisic acid derivative	
	ferulic acid hexoside	
	(E)-isoferulic acid	
	ferulic acid	
Flavonols	quercetin	
	quercetin-3-O-(4“-HMG)-α-rhamnoside	
	quercetin-3-O-β-galactoside	
	quercetin-3-O-glucoside	
	quercetin-3-glucuronide	
	quercetin-3-O-α-arabinoside	
	quercetin-3-O-α-rhamnoside	
	quercetin-7-O-rhamnoside	
	quercetin-3-O-rutinoside	
	quercetin-3-O-β-xyloside	
	quercetin-3-O-α-arabinofuranoside	
	quercetin-acetylglucoside	
	quercetin 3-(6”-ethylglucuronide)	
	quercetin 3-O-rhamnosyl-(1→2)-arabinoside	
	kaempferol-O-(hexose-deoxyhexoside)	
	kaempferol-3-O-(4″-3-hydroxymethylglutaryl)-rhamnoside	
Flavonols	kaempferol-deoxyhexoside	
	kaempferol-3-glucoside	
	kaempferol pentoside	
	kaempferol rhamnoside	
	kaempferol	

Class	Phenolic compounds	References/Origin
Anthocyanins	delphinidin 3-galactoside delphinidin 3-glucoside delphinidin 3-arabinoside cyanidin 3-galactoside cyanidin 3-glucoside, cyanidin 3-arabinoside petunidin 3-galactoside petunidin 3-glucoside petunidin 3-arabinoside peonidin 3-galactoside peonidin 3-glucoside peonidin-3-arabinoside malvidin 3-galactoside malvidin 3-glucoside malvidin 3-arabinoside	Andersen, 1985/Norway[a,F] Zheng and Wang, 2003/Canada[a,F] Mane et al., 2011/Danemark[a,F] Lee & Finn, 2012/USA[c,F]

[a]wild habitats, [b]commerce and industry, [c]cultivars, [F]fruits, [L]leaves.

- **Other constituents:**

Lingonberries contain also carotenoids, terpenoids, fatty acids, vitamins C, A and E, minerals, citric (18.2 g/l), malic (4.2 g/l) and benzoic (0.7 g/l) acids (Viljakainen et al., 2002; Bojor, 2003; Radulović et al., 2010; Seeram, 2008). Benzoic acid is known to contribute to the acidity of the lingonberries as well to prevent the fermentation of lingonberry juice due to its microbiocidal properties (Viljakainen et al., 2002). Among vitamins, niacin (0.6 mg/100 g), vitamin C (7.5 mg/100 g), vitamin A (0.8 µg/100 g), vitamin E (1.5 mg/100 g) and vitamin K (9.0 µg/100 g) are mainly found in fruits (Fineli Database, www.fineli.fi). Lingonberry fruits also contain minerals in small concentrations: calcium – 22 mg/100 g, potassium – 80 mg/100 g, phosphorus – 17 mg/100g and magnesium – 9 mg/100 g.

As carotenoids, Lashmanova et al., (2012) found in the fruits neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene in the concentration range of 6 to 76 mg/100 g dry weight.

Both the leaves and the seeds are rich in oil. Seed oil content is approximately 15% of the dry matter. The oils from seeds and leaves have quite different fatty acid compositions. The seed oil is rich in linoleic (46.4%), α-linolenic (27.3%), oleic (15.1%), palmitic (6.7%), stearic (1.9%) and myristic (2.6%) acids (Yang et al., 2003), whereas the oil from leaves is rich in α-terpineol (17.0%), pentacosane (6.4%), (*E,E*)-α-farnesene (4.9%), linalool (4.7%) and (Z)-hex-3-en-1-ol (4.4%) (Radulović et al., 2010). Comparative studies of the triterpenoid content of fruit and leaves of *Vaccinium vitis-idaea* from Finland and Poland showed the presence of the following compounds: α-β-amirin, amirin, betulin, campesterol, cicloartanol, eritrodiol, fern-7-en-3-β-ol, friedelin, lupeol, sitosterol, stigmasterol, stigmasta-3,5-dien-7-one, swert-9 (11)-en-3-ol, β-taraxasterol, bear-12-en-29-al, uvaol, ursolic and oleanolic acids (Szakiel et al., 2012). Ursolic acid was identified as the main triterpenoid in fruits, while sitosterol is the major compound in leaves.

III.3. Extraction and analysis of bilberry and lingonberry phenolic compounds

For the extraction of bilberry and lingonberry phenolics are used methods that are generally applied for the analysis of phenolic compounds and that are presented in chapter II.1.2. Generally the most common extraction methods use methanol (Hokkanen et al., 2009; Ek et al., 2006; Burdulis et al., 2009; Jovančević et al. 2011) or acetone (Zheng and Wang, 2003; Kylli et al., 2011; Jungfer et al., 2012; Lee and Finn, 2012; Liu et al., 2014;) as extraction agents of bilberry/lingonberry phenolic compounds but in terms of the utilisation for food and cosmetic industry ethanol and water are prefered (Ignat et al., 2011; Denev et al., 2010; Oancea et al., 2012; Ieri et al., 2013; Aaby et al., 2013).

III.4. Health benefits of bilberry and lingonberry

Fruits and aerial parts of bilberry and lingonberry constitute natural sources of food and beverage and are consumed as dietary supplements or pharmaceutical products for health benefits. Bilberry fruit extracts have been studied for the prevention and treatment of chronic pathologies such as cardiovascular diseases, cancer and vision-related diseases (Karlsen et al., 2010; Tumbas Šaponjac et al., 2014; Song et al., 2010). Bilberry leaves are used as herbal tea and have also been shown to exhibit antibacterial and antioxidant activity (Vučić et al., 2013). Similarly to bilberry, lingonberry has different biological properties such as antioxidant, antimicrobial, antiadhesive and antiinflammatory properties. These benefits are largely attributed to the high content in phenolic compounds in bilberry and lingonberry, compounds which are recognized to have multiple biological activities. In many research papers it was shown that the antioxidant activity of phenolic compounds is correlated whith their health benefits. Regarding this approach of the action of polyphenols as antioxidants in humans, Dangles (2012) clarified very well that the cardioprotective effects of phenolic compounds involve their anti-inflammatory rather than their antioxidant properties. Additionally, the issue of the limited bioavailability of phenolic compounds and their *in vivo* metabolism must be taken into consideration. At the same time, in the case of complex mixtures, such as plant extracts, other interfering constituents and non-phenolic antioxidants (vitamins and transition metals) may also be partly responsible for their activities.

III.4.1. Cardioprotective activity

Atherosclerosis, a chronic inflammatory disorder associated to oxidative processes, is the major cause of cardiovascular disease (CVD) including myocardial infarction (MI), heart failure, stroke and claudication (Frostegård, 2013). Other important risk factors of CVD comprise obesity, diabetes, hypertension, high levels of lipids and uric acid (Chu et al., 2011).

A study on apolipoprotein E-deficient (apo $E^{-/-}$) mice model of atherosclerosis exhibited that the dietary supplementation with bilberry anthocyanin-rich extract (Antho 50 from FERLUX S.A - France) containing 52% of pure anthocyanins for 2 weeks reduced plasmatic total cholesterol (-20%) and hepatic triglyceride levels (-30% in the liver), whereas the plasma antioxidant capacity remained unchanged (Mauray et al., 2010). In a following study, these bilberry extract showed action on the modulation of gene expression involved in angiogenesis in the aortas of apo $E^{-/-}$ mice (Mauray et al., 2012).

The potential beneficial effects of bilberry have also been studied on the development of obesity in mice fed with a high-fat diet (HFD) (Mykkänen et al., 2014). Mice fed with 5% or 10% (w/w) of whole bilberries in HFD for three months had lower glucose, blood pressures levels compared to mice fed HFD alone. Also the addition of bilberries to HFD was also found to reduce the levels of several parameters of inflammation. The levels of insulin were not affected by the addition of bilberries to HFD. Regarding lingonberry, its fruit juice moderately decreased low-grade inflammation caused by high salt diet (a risk for cardiovascular disease) in young rats (Kivimäki et al., 2014).

Human studies were also reported regarding the cardioprotective activity of bilberry. In a human study of 35 volunteers, Erlund et al. (2008) investigated the effects of daily consumption of mixed bilberries (100 g) and nectar containing 50 g crushed lingonberries on well-established risk factors of CVD, such as platelet function, HDL cholesterol and blood pressure for 2 months. Additionally, the subjects consumed black currant or strawberry puree and cold-pressed chokeberry and raspberry juice on the alternating days during the study. No changes were seen in plasma biomarkers of platelet activation, coagulation, or fibrinolysis, but the systolic blood pressure was

significantly decreased, and the serum HDL-cholesterol concentrations increased. In a recent study, intake of bilberry in conjunction with wholegrain and fish caused significant changes in lipid metabolites in subjects with risks for coronary heart (Lankinen et al., 2014).

Cardioprotective actions of products (extracts, juice) of other *Vaccinium* species have been also reported. For example, in a placebo-controlled, double-blind, parallel-arm, human study of 56 healthy adults, Novotny et al. (2015) demonstrated that low-calorie cranberry juice, rich in phenolic compounds (173 mg/240 mL juice), lowered factors of cardiovascular disease (CVD) including serum triglycerides, serum C-reactive protein, glucose, insulin resistance and diastolic blood pressure.

III.4.2. Anti-cancer activity

Several studies have investigated the effect of bilberry and lingonberry products on cancers. Cell lines, *in vitro* model systems, animals or human subjects have been used to test their anti-cancer activity through its antiproliferative and apoptotic effects.

Procyanidin-rich extract (almost A-type and B-type dimers) from lingonberry (McDougall et al., 2008) and flavonoids and phenolic acids fractions from bilberry fruits (Tumbas Šaponjac et al., 2014) are effective in preventing the proliferation of human cervical and colon cancer cells *in vitro*. The aqueous extract of bilberry press residue after juice production has also shown stronger inhibitory effect on cell proliferation of three colon cancer cell lines (Caco-2, HT-29, and HCT 116) (Aaby et al., 2013). This extract was also found to contain the highest total phenolic content (1447 mg GAE/100 g of press residue) and total monomeric anthocyanins (458 mg/100 g of press residue) and a correlation has been found between phenolic concentration of extract and its antiproliferative effect but it is possible that other compounds (e.g. vitamins) not quantified in this study may also contribute to the antiproliferative effect of bilberry extract. Recently, in a research article published in *Scientific Reports*, anthocyanin bilberry extract Antho 50 was studied for its apoptotic effect in chronic lymphocytic leukemia cells (Alhosin et al., 2015). This effect has been induced through generation of ROS and was attributed to activation of caspase 3 and down-regulation of UHRF1 and Bcl-2. In one study, other researchers have proven the inhibitory effect of lingonberry

methanolic extract and of its anthocyanin-rich and phenolic-rich fractions against apoptosis induced by ischemia-reperfusion (Isaak et al., 2015).

III.4.3. Antidiabetic activity

Hypoglycemia and hyperglycemia are the most common symptoms of diabetes. It is well known that obesity is another important risk factor for the occurrence of type 2 diabetes. Non-alcoholic steatohepatitis (NASH), which represents the accumulation of lipids in the liver, is the highest prediction factor for the occurrence of type 2 diabetes. With reference to the symptoms of diabetes, bilberry and lingonberry products have shown positive effects.

In a human study, dietary supplementation of 31 slightly overweight women with a mixture of lingonberry and bilberry berry products and other berries (163 g of berries daily) for 20 weeks showed a decrease by 23% of alanine aminotransferase level which is known as a common liver disease marker and an important risk factor of diabetes (Lehtonen et al., 2010). No differences were seen in plasma antioxidant capacity measured as ORAC and inflammation marker hs-CRP.

A study conducted in Japan with type 2 diabetic mice found that dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity and that the effects were due to activation of AMP-activated protein kinase (Takikawa et al., 2010). A standardised bilberry extract Mirtoselect containing 36% anthocyanins attenuated hepatic steatosis induced in mice fed a Western-type diet supplemented with 1% cholesterol for 20 weeks (Morrison et al., 2015). The decrease of macro- and microvesicular hepatocellular lipid accumulation, no increase of hepatic triglyceride levels and reduction of hepatic cholesteryl ester content, hepatic inflammation and hepatic fibrosis were observed in Mirtoselect-treated mice. In an *in vivo* human study, the incorporation of commercial lingonberry powder in fat-free yoghurt meal supplemented with glucose has attenuated the glycemic reponse of the sugars present in the berries and it was indicated that the fibers and/or polyphenols present in lingonberries are responsible for this effect (Linderborg et al., 2012).

III.4.4. Vision improvement activity

For several decades, the consumption of bilberry has been associated with the improvement of human vision in reduced light. A review of 30 clinical trials regarding action of anthocyanoside-rich extracts of *Vaccinium myrtillus* has been published in order to clarify the positive or negative effects of bilberry on night vision (Canter & Ernst, 2004). The results of studies discussed in this review are somewhat contradictory. Of these studies reviewed, from 12 placebo-controlled trials 4 randomized controlled trials showed no significant effects on vision in reduced-light conditions. It was suggested that the negative outcome are confounded by several factors including dose, possible geographical variations in anthocyanoside composition, and choice of subject. The fifth randomized controlled trials and 7 non-randomized trials reported positive effects on vision improvement in reduced light.

Nevertheless, according to the most recent research, beneficial effects of bilberry and lingonberry in vision improvement were evidenced in cell-based *in vitro* studies. For example, bilberry extract and lingonberry extract and their phenolic constituents (cyanidin, delphinidin, malvidin, trans-resveratrol, and procyanidin B2) appear to exert protective effects against retinal damage induced by blue light-emitting diode (LED) light via inhibition of ROS production and activation of pro-apoptotic proteins (Ogawa et al. 2014). Another study by Song et al. (2010) showed that a bilberry extract containing 25% total anthocyanins could promote physiological renewal and homeostasis of human corneal limbal epithelial cells (HCLEC). In a study of 23 patients with asymptomatic ocular hypertension, 24 weeks of dietary supplementation with Mirtogenol®, a combination of two phenolic extracts from bilberry (Mirtoselect®) (standardized to 36% anthocyanins) and French maritime pine bark (Pycnogenol®) (standardized to 70% procyanidins, was reported to lower the intraocular pressure up to 24 % and improve the diastolic ocular blood flow (Steigerwalt et al., 2010).

III.4.5. Bacterial anti-adhesion activity

Several studies demonstrated the antibacterial activity of *Vaccinium myrtillus* L. and *Vaccinium vitis idaea* L. Antibacterial effects of bilberry and lingonberry phenolic extracts from fruits and leaves have been shown especially in the prevention of urinary tract infections (Davidson et al., 2014). *In vitro*, lingonberry fruit extracts containing

mainly type-A proanthocyanidins may be bactericidal against *Staphylococcus aureus* or inhibit the hemagglutination of *Escherichia coli* (Kylli et al., 2011). Similar antimicrobial effect of flavonol glycosides, anthocyanins, procyanidins, and flavan-3-ols fractions purified from lingonberry juice was reported against other two pathogens, *Streptococcus mutans* and *Fusobacterium nucleatum (*Riihinen et al., 2014). Other researchers investigated the antibacterial activity of water, ethanol and ethyl acetate extracts from fruits and leaves of bilberry on strains of *Escherichia coli*, *Enterococcus faecalis* and *Proteus vulgaris* and found that all extracts were more effective against *E. faecalis* and *P. vulgaris* (Vučić et al., 2013).

III.5. Other applications of bilberry and lingonberry extracts

Lyoniside, a lignan glycoside, purified from ethanol extracts of the rhizomes and stems of *Vaccinium myrtillus* L., has been shown to possess allelopathic and antifungal activities *in vitro* by inhibiting seed germination and seedling growth in lettuce, cress, pine, spruce and larch and influencing mycelial growth of fungi strains of Ascomycota (Szakiel et al. 2011). Dried bilberries and their anthocyanins were found to ameliorate the induced acute and chronic colitis in mice in a study by Piberger et al. (2011).

PERSONAL CONTRIBUTION

Samplig region: Borca, Neamt, Romania

Chapter I.

PHENOLIC CONSTITUENTS IN BILBERRY (*VACCINIUM MYRTILLUS* L.): ACCUMULATION IN LEAVES, STEMS AND FRUITS AT DIFFERENT HARVEST PERIODS AND ANTIOXIDANT ACTIVITY

1. Background

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, beverage and nutraceutical ingredients due to their richness in nutritional and bioactive compounds and are 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 (Rouanet et al., 2010; Lehtonen et al., 2010; Erlund et al. 2008; Karlsen et al., 2010; Mykkänen et al., 2014). For example, a bilberry anthocyanin-rich extract reduced plasmatic total cholesterol and hepatic triglyceride levels in apolipoprotein E-deficient (apo $E^{-/-}$), a mice model of atherosclerosis while down-regulating the expression of pro-inflammatory genes (Mauray 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-glycerol-alcoholic extract in traditional herbal medicine and have also been shown to exhibit antibacterial and antioxidant activities (Vučić, Petković, Rodić-Grabovac, Stefanović, Vasić, & Čomić, 2013). These benefits are attributed to the high content in polyphenols (flavonoids, phenolic acids and proanthocyanidins) in bilberry leaves (Martz et al., 2010). The *in vitro* and *in vivo* biological activities of phenolic compounds from natural sources involve application as antioxidants, antibacterial and anticarcinogenic agents, amendments in bioremediation, allelochemicals, and plants growth regulators (Bujor et al., 2015).

The quality and quantity of phenolic compounds in *Vaccinium myrtillus* L. are generally influenced by the parts of the plant to be used, the stage of growth, the environmental conditions (temperature, sunlight, soil nutrients, latitude and altitude of the growth location) and genetic factors (Åkerström et al., 2010; Martz et al., 2010; Jovančević et al., 2011; Uleberg et al., 2012; Mikulic-Petkovsek et al., 2015). It may appear that higher phenolic contents are favored by northern latitudes, altitude and a sunny environment. In bilberry fruits high amounts of anthocyanins (cyanidin, delphinidin,

malvidin, petunidin and peonidin glycosides), hydroxycinnamic acid derivatives and low amounts of flavonols (quercetin and myricetin glycosides), proanthocyanidin and coumaroyl iridoids were identified (Mikulic-Petkovsek et al., 2015). Due to the important level of anthocyanins, bilberry was called "wild superberry from Europe". In contrast, leaves are known to contain, in decreasing levels, hydroxycinnamic acids (mainly chlorogenic acid), flavonol glycosides (mainly quercetin and kaempferol glycosides) and proanthocyanidins but also cinchonains and iridoids in nondetermined amounts (Martz et al., 2010; Liu et al., 2014).

A comparative study carried out by Teleszko & Wojdyło (2015) showed that phenolic compounds were found in a markedly higher content in the leaves than in the 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 the most commonly, leaves and stems sometimes. In this context, the primary aim of this study is to simultaneously assess the seasonal variations of phenolic compounds in leaves, stems, and fruits of bilberry collected at three different periods of vegetation. This study reports the most comprehensive qualitative study ever conducted leading to the identification of 106 phenolic compounds, with 25 of them being reported for the first time. Additionally, an original analysis of the oligomeric proanthocyanidins is proposed addressing both the degree of polymerization and flavanol unit constitution. Finally, contents in total polyphenols, assessed globally by the Folin-Ciocalteu method or specifically by UPLC, and the antioxidant capacity in the DPPH test are tentatively correlated.

2. Methodology

2.1 Materials

2.1.1. Bilberry samples

Fruits, leaves and stems of wild bilberry (*Vaccinium myrtillus* L.) were collected from mountains near Borca (Neamt, Romania, coordinate: 47° 11′ 34″ N and 25° 47′ 8″ E) in May, July and September during the years 2013-2014. The collect site is characterized by an altitude ranging between 1300 m and 1800 m, covered by coniferous forest and exposed to sun.

Fresh bilberry fruits were frozen at -24 °C, then lyophilized in a Christ Alpha 1-4 LSC (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, the leaves were manually separated from the stems, ground (using the same mill as for fruits) and sieved through a standard sieve to a final particle size < 0.315 mm. Grinded samples were kept in a dessicator to prevent adsorption of moisture from the air until extraction. Before extraction, the residual moisture of grinded samples was determined using a RADWAG MAX 50/1 moisture analyzer (RADWAG Balances & Scales, Poland). Residual moisture between 7% and 9.5% were found for all plant materials.

2.1.2. Chemicals and solvents

Standard phenolic compounds and reagents: gallic acid, chlorogenic acid and *p*-coumaric acid, (+)-catechin, (-)-epicatechin, 37% hydrochloric acid, anhydrous sodium carbonate, Folin & Ciocalteu's phenol reagent, DPPH (1,1-diphenyl-2-picrylhydrazyl) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France); isoquercitrin (quercetin-3-glucoside), quercitrin (quercetin-3- rhamnoside), hyperoside (quercetin-3-galactoside), procyanidin B2, procyanidin A2, (-)-epigallocatechin and cyanidin-3-O-galactoside (ideain chloride), were purchased from Extrasynthese (Genay, France); Procyanidin C1 (Epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin trimer) was extracted from apple

fruits (*Malus domestica* Borkh.) of the Kermerrien variety (PhD thesis of Katerina Asprogenidi).

Solvents: 96% ethanol was purchased from Chemical Company (Iasi, Roumania); HPLC-MS grade methanol and acetonitrile from Fisher Scientific (Illkirch, France); formic acid from Merck (Darmstadt, Germany); glacial acetic acid from Merck (Fontenay Sous Bois, France); toluene-α-thiol from Sigma–Aldrich. 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

Preliminary optimization of the solid-to-liquid ratio and extraction time led to optimum extraction conditions close to those of Zheng, Xu, Liu, Sun, Lin & Liu (2013). They were applied for the preparation of extracts.

To 1 g of ground bilberry samples placed in an extraction glass vial fitted with a condenser were 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. Additionally, the fruit samples were extracted with 55% aqueous ethanol (EtOH) (v/v) under the same extraction conditions as for the aqueous extracts. The extracts were filtered through filter paper and then the volume of each sample was adjusted to 30 mL with the extraction solvent prior to the determination of the Total Phenolic Content (TPC) (Figure I-1). Then the solutions of extracts were freeze–dried and the Dry Extracts (DE) were stored at 4 °C in a fridge before analysis and use in antioxidant tests. Triplicate extractions were made for each morphological part. Dry matter (DM) refers to the initially ground dry sample after correction of the residual water. Dry extract (DE) refers to the dry matter after extraction and freeze-drying.

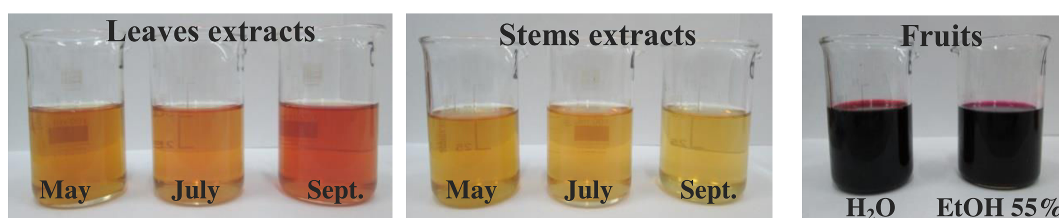


Figure I-1. Leaf, stem and fruit bilberry extracts.

2.3. Qualitative and quantitative analyses of phenolic compounds

2.3.1. Identification of phenolic compounds by UPLC/MS

For UPLC/MS analyses, freshly prepared solutions of bilberry Dry Extract leaf and stem extracts in water (10 mg/mL) were directly used. For the Dry Extract of bilberry fruit extracts, 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). In a first step, the C18 cartridge was 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 to remove compounds not adsorbed. 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 monitored by using Ultra Performance Liquid Chromatography coupled with Electrospray Ionization - Mass Spectrometry (UPLC/ESI-MS) using the conditions and methods described by Mane et al. (2011). A Waters ACQUITY UPLC chromatograph (Waters, Milford, MA) coupled to an UV−vis diode-array detector and a HCT ultra ion trap mass spectrometer equipped with an electrospray ionization source (Bruker Daltonics, Bremen, Germany) was used for identification of phenolic compounds. Separation was carried out using a reverse-phase Acquity BEH C18 column (50 mm x 2.1 mm i.d., 1.7 µm; Waters) at 30 °C.
For phenolic compounds, a binary solvent system was used with solvent A (0.05% formic acid in water, v/v) and solvent B (acetonitrile) at a flow rate of 1 mL/min and with the following elution gradient: 0-2 min, linear 0-3% B; 2-3 min, isocratic 3% B; 3-6 min, linear 3-5% B; 6-7 min, linear 5-6% B; 7-12.5 min, linear 6-10% B, 12.5-19.5 min, linear 10-30% B; 19.5-20.5 min, linear 30-60% B; 20.5-21 min, linear 60-100% B; 21-22 min, linear 100-0% B; 22-24 min, isocratic 0% B. For anthocyanins, a binary solvent system was used with solvent A (1% formic acid in water, v/v) and solvent B (1% formic acid in acetonitrile) at a flow rate of 0.17 mL/min and with the following

elution gradient: 0-15 min, linear 0-20% B; 15-20 min, linear 20-40% B; 20-20.5 min, linear 40-100% B; 20.5-20.6 min, linear 100-0% B; 20.6-23.6 min, isocratic 0% B. The volume of extract injected was 3 μL for phenolic compounds and 1 μL of anthocyanins.

Mass detection was conducted in both negative (for phenolic compounds) and positive (for anthocyanins) electrospray ionization modes from *m/z* 100 to 1000. MS conditions in the negative ion mode were as follows: capillary voltage of 2 kV, nitrogen flow rate at 12 L/min; desolvation temperature at 365 °C and nebulization pressure at 60 psi. MS conditions in the positive ion mode were: capillary voltage of 1.8 kV, nitrogen flow rate at 9 L/min; desolvation temperature at 350 °C and nebulization pressure at 40 psi.

2.3.2. Quantification of phenolic compounds

The separation was performed as described above using a Waters ACQUITY UPLC chromatograph (Waters, Milford, MA) coupled to an UV−vis diode-array detector. Chlorogenic acid, (-)epicatechin, (-)epigallocatechin, procyanidin B2, procyanidin A2, procyanidin C1, quercetin-3-glucoside (isoquercitrin), quercetin-3-galactoside (hyperoside), quercetin-3-rhamnoside (quercitrin) and cyanidin-3-galactoside (ideain chloride) were used for 6 point-calibrations. All the standards were prepared in methanol except cyanidin-3-galactoside that was prepared in methanol acidified with 1% HCl (v/v). The other phenolic compounds were quantified as follows: caffeic acid derivatives, 3,4-dihydroxyphenylpropionic acid hexoside and sinapic acid hexoside as chlorogenic acid (325 nm), coumaric acid derivatives as *p*-coumaric acid (330 nm), quercetin glycosides as quercetin-3-galactoside, quercetin-3-glucoside or quercetin-3-rhamnoside (350 nm) depending on the sugar unit in the glycosides, 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 (280 nm) and anthocyanins as cyanidin-3-galactoside (520 nm). The flavanol monomers were calculated as the sum of (–)-epicatechin and (–)-epigallocatechin, while the flavanol oligomers were reported as the sum of all dimers, trimers, cinchonains I and cinchonains II. Injected volumes were 3 μL for phenolic compounds and 1 μL for anthocyanins. All samples were injected in triplicate after independent sample extraction.

2.4. Analysis of procyanidins using thioacidolysis

Procyanidin analysis was performed by High-Performance Liquid Chromatography (HPLC)/Diode Array Detection (DAD)/Fluorimetric detection after thioacidolysis using a method adapted from Le Bourvellec et al. (2011). Procyanidins were characterized by their subunit composition and their average degree of polymerization (mDP). The mDP of procyanidins was measured by calculating the molar ratio of all the flavan-3-ol units (thioether adducts plus terminal units) to (−)-epicatechin and (+)-catechin corresponding to terminal units. After thioacidolysis, no distinction can be made between native catechins and catechins coming from the terminal units of procyanidins (Guyot et al., 2001). For this reason, HPLC-DAD analyses of methanolic extracts which were not submitted to thioacidolysis were also performed in order to separately assay monomeric and oligomeric procyanidins.

2.4.1. Freeze-dried extracts

Solutions of Dry Extracts in dry methanol were prepared at the following concentrations:

- 15 mg/mL for leaves and stems of bilberry extracts,
- 20 mg/mL for ethanolic extract of bilberry fruits,
- 40 mg/mL for aqueous extract of bilberry fruits.

2.4.1.1. HPLC analysis without thiolysis

A volume of 25 µL of the above solutions was mixed with 75 µL of dry methanol acidified by acetic acid (1% v/v). After vortexing until complete dissolution, the reaction medium was directly injected (20 µL) into the HPLC system. Analyses were done for independent triplicates.

2.4.1.2. HPLC analysis after thiolysis

A volume of 25 µL of the above solutions was mixed with 50 µL of a 5% toluene-α-thiol solution (v/v in dried methanol) and 25 µL of dried methanol acidified by concentrated HCl (0.4 N). After vortexing, the mixture was incubated in a water bath at 40 °C for 30 min, cooled in an ice bath for at least 5 min, and directly injected (20 µL) into the HPLC system. Analyses were done for independent triplicates.

2.4.2. Freeze-dried fruits

2.4.2.1. HPLC analysis without thiolysis

Bilberry fruit powder (25 mg) was dispersed in 1200 µL of dried methanol acidified by acetic acid (1% v/v) in a 1.5 mL Eppendorf vial. The reaction was carried out in an ultrasonic bath during 15 minutes. After filtration (PTFE, 0.45 µm), the reaction medium was directly injected (20 µL) into the HPLC system.

Analyses were done for independent triplicates.

2.4.2.2. HPLC analysis with thiolysis

Bilberry fruit powder (25 mg) was dispersed with 400 µL of dried methanol acidified by concentrated HCl (3 N) in a 1.5 mL Eppendrof vial and 800 µL of a 5% toluene-α-thiol solution (v/v in dried methanol) were added. The reaction was carried out at 40 °C for 30 min with vortexing every 10 min. Then, the vials were cooled in an ice bath for at least 5 min. After filtration (PTFE, 0.45 µm), the reaction medium was directly injected (20 µL) into the HPLC-DAD system. Analyses were done for independent triplicates.

HPLC/DAD analyses were performed using an Ultra Fast Liquid Chromatography Shimadzu Prominence system (Kyoto, Japan) including two pumps LC-20AD Prominence liquid chromatograph UFLC, a DGU-20A5 Prominence degasser, a SIL-20ACHT Prominence autosampler, a CTO-20AC Prominence column oven, a SPD-M20A Prominence diode array detector, a RF-10AXL Fluorescence detector, a CBM-20A Prominence communication bus module and controlled by a LC Solution software (Shimadzu, Kyoto, Japan).

Separations were achieved as in Le Bourvellec et al. (2011) using a (250 mm x 4 mm i.d.) Licrocart (Licrospher PR-18 5µm) column (Merck, Darmstadt, Germany) with a guard column (Licrospher PR-18 5 µm column, Merck, Darmstadt, Germany) operated at 30 °C.

The mobile phase consisted of water/acetic acid (97.5:2.5, v/v) (eluent A) and acetonitrile (eluent B). The flow rate was 1 mL/min. The elution program was as follows: 3–9% B (0–5 min); 9–16% B (5–15 min); 16–50% B (15–45 min); 50–90% B (45–48 min); 90–90% B (48–52 min); 90–3% B (52–55 min); 3–3% B (55–60 min). Samples and standard solutions were maintained in 4°C before injection. Phenolic compounds were identified by comparison of their retention time and their UV-visible

spectra with those of standards. Quantification was achieved by injection of standard solutions of known concentrations. Individual compounds were quantified in mg/g Dry Extract or fruit powder with external standards at 280 nm for (+)-catechin, (-)-epicatechin, (+)-catechin benzyl thioether (quantified as (+)-catechin), and (-)-epicatechin benzyl thioether (quantified as (-)-epicatechin). In the samples containing anthocyanins, (+)-catechin and (-)-epicatechin were specifically identified and quantified by their emission-excitation 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 of the extract solutions was determined by the Folin-Ciocalteu spectrophotometric method described by Hainal et al. (2011). An aliquot of 1 mL of diluted extract (1:50 for leaves and stems and 1:25 for fruits) was mixed with 0.5 mL of Folin-Ciocalteu reagent (2 M), 2 mL of 10% Na_2CO_3 solution 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) 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) using the calibration curve, $y = 0.0088x + 0.0158$, $R^2 = 0.9984$, for five different concentrations of gallic acid solutions ranging from 5-200 µg/mL. For ground samples, triplicate measurements of residual moisture and the mean values were used to correct the Dry Matter. Triplicates of independent extract solutions were analyzed.

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

The DPPH test was adapted from a method developed by Goupy et al. (2003). Small volumes (25 µL) from Dry Extracts freshly prepared in water (10 mg/mL and 5 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 during 30 minutes at 25 °C under constant magnetic stirring (1250 rpm). Chlorogenic acid at 1 mM in methanol was used as an antioxidant reference.

The results were expressed as micromoles of Trolox Equivalents (TE) per gram of dry extract (μM TE/g DE). Trolox calibration curves were obtained from 4-5 concentrations. 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 honestly significant difference (HSD) post hoc test of Multiple Comparisons using the XLStat software (version 2008.3.02, Addinsoft SARL, Paris, France).

3. Results and discussion

3.1. Optimal extraction conditions: the preliminary test

According to the literature, to extract phenolic compounds from *Vaccinium* species, solvents such water, methanol, ethanol and acetone, and aqueous mixtures are used. Leaves and stems of bilberry are known to be consumed as herbal tea, which are aqueous extracts, in traditional medicine for many centuries. Furthermore, aqueous extracts are interesting in the field of food and pharmaceuticals and water is also an environmentally friendly and inexpensive extraction solvent (Denev et al., 2010; Wang and Liu, 2012). On the other hand, since phenolic compounds are strongly oxidizable, their extraction is performed generally under acidic conditions because of their higher stability at low pH (Del Rio et al., 2010). Actually, in terms of food preservation, citric acid is one of the most natural preservative for foods and soft drinks (ethicalfoods.com). Based on this, we chose 1% citric acid in H_2O as an extraction solvent for all the bilberry samples. Considering the large number of samples, the extracts were obtained by microwave-assisted extraction due to its many benefits: shorter extraction time, lower amount of solvent and multiple samples analyzed at the same time (Mahugo Santana et al., 2009).

In the preliminary test, the extraction yield was evaluated from bilberry leaves collected in May 2013 for different solid to liquid ratios (1:20, 1:30 and 1:40 (w/v)) and extraction time (1, 3, 5, 7 and 9 minutes). The experiment was performed at a fixed temperature and power extraction of 40 °C and 300 W, respectively. The optimal extraction conditions are determined in terms of total polyphenols content (mg GAE/g Dry Matter). The variation of total polyphenol contents as a function of the extraction time for different solid to liquid ratii is presented in Figure I-2. A solid to liquid ratio of 1:30 and an extraction time of 7 min were established to be the optimum conditions.

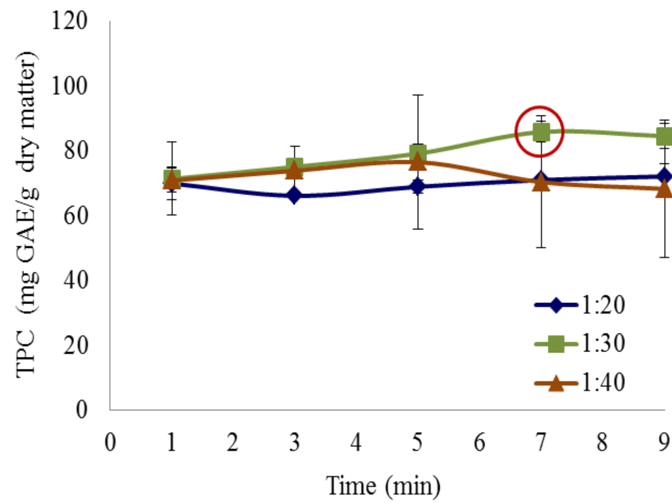


Figure I-2. Variation of total polyphenol contents as a function of the extraction time for different solid to liquid ratii (1:20, 1:30, 1:40, w/v) at 40 °C and 300 W.

3.2. Phenolic profile and content of bilberry extracts

The identification of the phenolic compounds in leaf, stem and fruit extracts of wild bilberry at three different periods of vegetation and for two different years was performed using mass fragmentation, retention times (t_R), and UV-VIS spectra. In all the morphological parts of bilberry, 106 phenolic compounds were tentatively identified (Table I-1), 62 in leaf extracts, 73 in stem extracts and 40 in fruit extracts. Additionnaly, 17 were found only in leaves, 32 only in stems, and 9 only in fruits. The presence of (–)-epicatechin, (–)-epigallocatechin, procyanidin B1, procyanidin B2, 5-O-caffeoylquinicacid, quercetin-3-O-galactoside, quercetin-3-O-glucoside and quercetin-3-O-rhamnoside was confirmed with available standards. The identity of some phenolic structures was assessed by comparing with the existing literature and for others proposed based on their characteristics and presence in other plant materials. Chromatographic profile of phenolic compounds separated from leaf, stem and fruit of bilberry are presented in Figure I-3 (A and B) and Figure I-4 (A and B).

3.2.1. Caffeic acid derivatives

In leaves, caffeic acid derivatives are present in 3 to 10-fold higher levels compared to coumaric acid derivatives and only in 1 to 2-fold higher levels in fruits. Interestingly, there is no difference in stems in the contents in these two hydroxycinnamic acid derivatives. 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. Caffeoyl quinic acid derivatives involve caffeoylquinic acid hexoside (**9** and **51**), 5-O-caffeoylquinic acid (chlorogenic acid, **18**), (*Z*)-5-O-caffeoylquinic acid (**31**), four caffeoylquinic acid derivatives displaying a *m/z* at 707 (**20, 22, 24** and **29**) and two caffeoylquinic acid derivatives with a *m/z* at 705 (**50** and **53**).

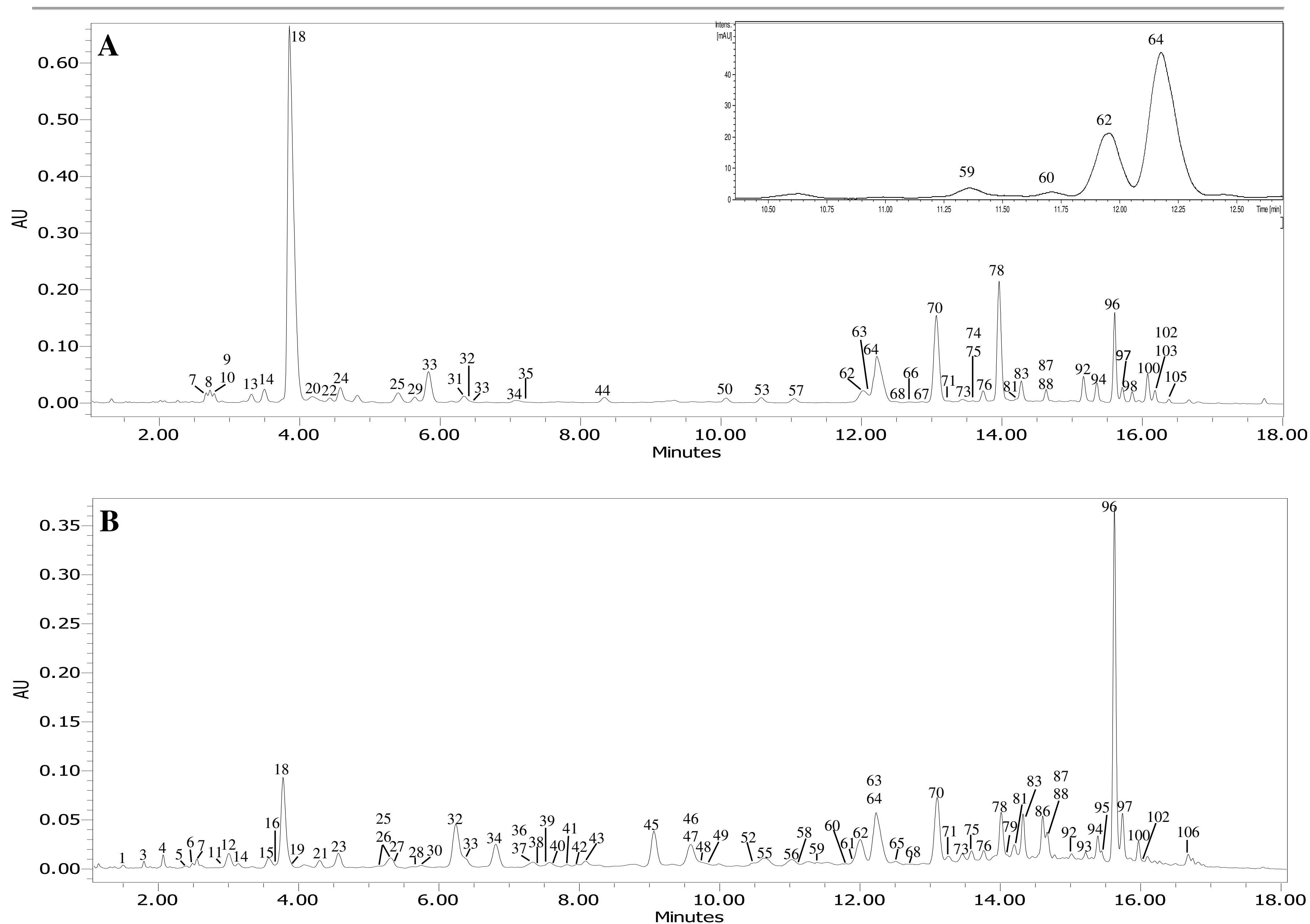


Figure I-3. Chromatographic phenolic profile of leaf (A) and stem (B) extracts of bilberry at 280 nm

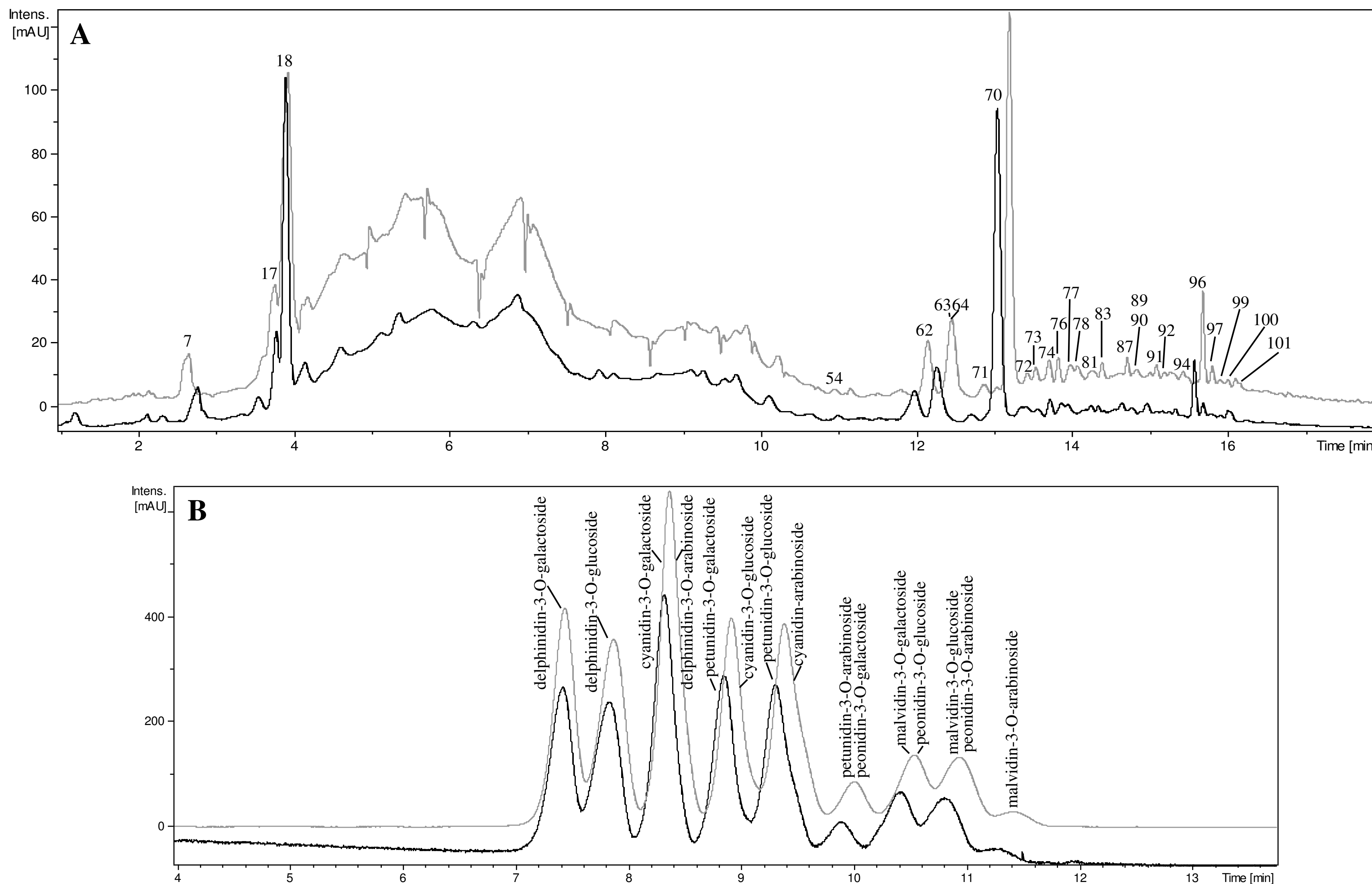


Figure I-4. Chromatographic profile at 280 nm (A) and at 520 nm (B) of aqueous (black) and ethanolic (grey) fruit extracts

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. The 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. The compound **51** displayed a major fragment ion at *m/z* 341 (caffeic acid hexoside) and fragments at *m/z* 191 and 173 (typical for 4-O-caffeoylquinic acid). The *cis* isomer of 5-O-caffeoylquinic acid was identified according to its λ_{max} at 312 nm and a shoulder at 290 nm as well as a similar fragmentation pattern as the *trans* isomer. 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 or 513 could be interpreted as an additional caffeoyl or hexosyl unit on caffeoylquinic acid. Related caffeoylquinic acid derivatives, 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 compound (**74**) belonging to the caffeoylquinic acid family presents a parent ion at *m/z* 381 and a fragmentation (*m/z* 191, 179, 161 and 135) typical for caffeoylquinic acid. This caffeoylquinic acid derivative was observed in bilberry or lingonberry buds and leaves by Ieri et al. (2013) who named it caffeoyl derivative. In this study, it was identified in fruit **for the first time**.

A single caffeoyl shikimic acid isomer (**35**) was identified, in leaves only. Last, a caffeoyl monotropein (**57**) whose fragmentation pattern in mass is analogous to the one observed for *p*-coumaroyl monotropein (**82**), although with *m/z* ratios higher by 16 units, was observed in leaves and fruits.

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 (**10** 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 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).

Two caffeoyl derivatives, only observed in fruits, remained ill-defined (**101** and **104**). Interestingly, these two apolar compounds with parent ions at *m/z* 425 and 445 exhibited similar fragmentations with a major fragment ion at *m/z* 179 (caffeic acid) and another fragment at *m/z* 135 (decarboxylated caffeic acid). Owing to its polarity and the presence of several *p*-coumaroyl diacetylhexosides, compounds with *m/z* 425 could be proposed to be a caffeoyl diacetylhexoside.
O-Methylation of the caffeic acid unit was only observed once in stems with sinapic acid hexoside (**27**). Finally, several phenolic acids were identified in stems only and assessed as hexosides of hydroxymethoxybenzoic acid (**3**), dihydroxybenzoic acid (**4**), and 3,4-dihydroxyphenylpropionic acid (**23**). Syringic acid hexoside appeared only in fruits (**54**). Only dihydroxyphenylpropionic acid and sinapic acid hexosides could be quantified.

<u>*3.2.2. Coumaric acid derivatives*</u>
In leaves and stems, two *p*-coumaroylquinic acids (**30** and **44**, *m/z* 337) were evidenced based on their major fragment ion at *m/z* 191 (quinic acid) resulting from the loss of *p*-coumaric acid (164 amu) in agreement with the reporting of Hokkanen et al. (2009). Base peak at *m/z* 191 are only produced by 3- and 5-*p*-coumaroylquinic acids (Clifford et al., 2005). Additionally, four hexosides of *p*-coumaric acid (**7, 8, 14** and **21**, *m/z* 325) were detected in the various morphological parts of bilberry, displaying a fragment ion at *m/z* 163. Compound **8** displays a maximal absorption wavelength (λ_{max}) at 295 nm which is characteristic of the electronic density modification induced by the glycosylation at the O-4 position (Chanforan et al., 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 while three *p*-coumaroyl triacetylhexosides with *m/z* 451 (**98, 102** and **105**) were only observed in leaves. Their common major

fragment ion was *m/z* 187. Two of the *p*-coumaroyl triacetylhexosides were characterized 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. The putative *p*-coumaroyl diacetylhexosides (*m/z* 409) display fragment ions at *m/z* 325 (loss of 2 acetyl group) and *m/z* 163 typical of *p*-coumaric acid hexose and *p*-coumaric acid, respectively.

Potential malonylated derivatives comprise four *p*-coumaroyl malonylhexosides, which are present in leaves, stems, and fruits, 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**) display a parent ion at *m/z* 411 and 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. We propose that some coumaroylated and malonylated glycosides could formally be (4-O-malonyl-*p*-coumaroyl)glycosides in agreement with the earlier identification of *p*-coumaroylhexosides. Compounds not displaying the *m/z* 249 fragment could be *p*-coumaric acid-4-O-malonylglycosides in agreement with the presence of *p*-coumaric acid-4-O-hexosides. For compounds with λ_{max} 310 nm and a shoulder at 295 nm, the possible structure could be (*p*-coumaroyl)malonylglycosides. Compounds with *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, 537 and 697) were also identified in bilberry. The iridoid glucoside part has been proposed to be monotropein by Hokkanen et al. (2009) as reported earlier in some *Ericaceae* species (cranberry, lingonberry and bilberry juices). Monotropein (**2**) was identified in bilberry stems displaying a parent ion at *m/z* 389 and major fragments at *m/z* 227 (loss of hexose) and 183 (further decarboxylation). Compounds with parent ions at *m/z* 535 and 537, largely present in all the morphological parts of bilberry, were

assigned as *p*-coumaroyl monotropein (**63, 70, 82** and **84**) and *p*-coumaroyl dihydromonotropein isomers (**71** and **76**), respectively. Similar fragmentation patterns were reported by Hokkanen et al. (2009) and Mikulic-Petkovsek et al. (2015) who identified two *p*-coumaroyl monotropeins in lingonberry and bilberry juices and bilberry leaves, respectively. The six isomers reported in our study could be assessed to (*E*) and (*Z*) stereoisomers although three of them were shown to maximally absorb at 312 nm and one at 306 nm.The presence of glucose and galactose moieties should be considered as well acylation of the sugar unit. *p*-Coumaroyl dihydromonotropein isomers were newly identified in bilberry similarly to a compounds 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.

Finally, two *p*-coumaroyl derivatives remained unidentified. The first one (**65**) had a parent ion at *m/z* 455 and fragment ions at *m/z* 309 and 163 (loss and presence of *p*-coumaric acid, respectively). The other compound (**89**) displayed a parent ion at *m/z* 507, with major fragments at *m/z* 343 and 163 (loss and presence of *p*-coumaric acid, respectively). This last compound was **newly reported** in stems and fruits.

3.2.3. Flavonol glycosides

Eleven different glycosides of quercetin were identified in bilberry extracts. In stems and leaves, quercetin glycosides were present in considerable amounts from May to September (Table I-3), whereas in fruits they appeared in lower concentrations. Quercetin-3-O-galactoside (**62**), quercetin3-O-glucoside (**67**), three isomers of quercetin hexuronide (**61, 64** and **66**), quercetin pentosides (**73** and **77**), and a quercetin rhamnoside (**81**) were observed as described earlier (Hokkanen et al., 2009; Ieri et al., 2013). The berry characteristic quercetin-3-O-(4"-(3-hydroxy-3-methylglutaryl))-α-rhamnoside (**100**) was identified in all the morphological parts of the plant. The structure of the latter compound was confirmed using NMR experiments by Ek et al. (2006) who found it in lingonberry fruit and leaves. 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 only detected by mass spectrometry in leaves of May, when it was

quantified in July and September (4th flavonol) and appeared in quantifiable amounts in stems from all seasons.

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

In fruits, compound **72** with a parent ion at *m/z* 493 and a major fragment at *m/z* 331 was assigned as 3'-O-methylmyricetin (laricitrin) hexoside based on literature (Lätti et al., 2010; Mikulic-Petkovsek et al., 2015). Only one kaempferol derivative was detected, namely kaempferol hexuronide (**79**) in leaves and fruits (Hokkanen et al., 2009; Ieri et al., 2013; Liu et al., 2014). 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 (Gobert et al., 2014).

3.2.4. Flavanols

In stems, flavanols were present from May to September (Table I-1) although they were more abundant in July and September (Table I-3). Epicatechin or catechin-based oligomeric flavanols encompass a large variety in stems 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. The presence of six B-type dimers indicates that catechin and epicatechin appear as both extension and terminal units and may be linked through both C4-C8 and C4-C6 linkages. Additionally, (-)-epigallocatechin (**15**) and (-)-epicatechin (**34**) were present in quantifiable amounts whereas (+)-gallocatechin (**1**) was only detected in trace amounts by mass spectrometry while (+)-catechin was absent. (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**). One compound with a parent ion at *m/z* 405, assigned as an (epi)catechin derivative (**5**) due to its major fragment ions at *m/z* 289 and 245, was also detected.

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

(Hokkanen et al., 2009). Two main fragmentation pathways were observed for cinchonains I with the first two isomers giving major fragment ions at *m/z* 289 and 245 and the others at *m/z* 341 and 217. None of them were in a quantifiable amount 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, when only one B-type dimer (**75**) was identified in fruits. No oligomers were quantitatively assessed in both these morphological parts. (-)Epicatechin (**34**) was only present in quantifiable amounts in leaves of bilberry from July and September while not in May. Furthermore, an (epi)catechin derivative (**13**) with parent ion at *m/z* 405 was also detected in leaves. Last, 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 from July and September (**59** and **60**) (inset in Figure A) and fruits from 2014 (**99**). In leaves from May, cinchonains I and II were either not found or not fragmented indicating a lower content compared to samples harvested in July and September. They thus appear to be specifically biosynthetized from spring to summer.

Table I-1. Phenolic compounds identified by UPLC/ESI-MSn 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[e]	S	S	S	S	S	S
2	1.7	240	389	227, 209, 183, 165, 139	Monotropein[e]	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)[h]**	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**	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, 575 MS^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)[e]	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)[d,g]	S	S	S	S[a]	S[a]	S[a]
17			341	179, 135	**Caffeic acid-4-O-β-D-hexoside (2)**		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}	

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.
18	3.8	295sh, 324	353	191	5-O-Caffeoylquinic-acid (std)[g,h]	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)[c]	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)[e]	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	Coumaroylquinic acid (1)[e,l,h]	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)	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,	B-type trimer (2)	L[a], S	L, S	L, S	S[a]	L[a], S	S

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.
				575, 451, 425, 407, 289, 287,							
34	6.85	278	289	245, 205, 125	(–)-Epicatechin (std)[e,i,j,l]	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[e,g,i]	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)[e]	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]
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		337	191, 163	Coumaroylquinic acid (2)	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)[g]	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

No.	t_R (min)	λ_{max} (nm)	$[M - H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.
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 hexoside[i]	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)[g]	L, S	L, S	L, S	L, S	L, S	L, S
62			463	301	Quercetin-3-O-galactoside (std)[l]	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,	*p*-Coumaroyl monotropein (1)[e,i,l]	L, S	L_a, S, F_{H_2O},	L[a], S[a]	L[a], S[a]	L[a], S, F_{H2O},	L[a], S

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.
				311, 267, 191, 163			F_{EtOH}			F_{EtOH}	
64	12.25	255, 352	477	301	Quercetin hexuronide (2)[i]	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[i]	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)**[i,j]	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[f]	–	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**[i]	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)**[i]	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[e,i,j]	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]

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)[e,i,j]	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)**[i,j,l]	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 malonyldihexose (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[f]	–	F_{H_2O}, F_{EtOH}	–	–	F_{H_2O}, F_{EtOH}	–
91	15.0		435	273	**Phloretin-2-O-hexoside**[k]	–	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 malonyldihexose (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)**[e,i]	L	–	–	L	–	–

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.
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[c,i,j,n]	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[l]	–	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[l]	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]Ek et al. (2006).
[d]Harris et al. (2007).
[e]Hokkanen et al. (2009).
[f]Lätti et al. (2010).
[g]Mane et al. (2011).
[h]Chanforan et al. (2012).
[i]Ieri et al. (2013).
[j]Liu et al. (2014).
[k]Gobert et al. (2014).
[l]Mikulic-Petkovsek et al. (2015).
[m]HMG = 3-hydroxy-3-methylglutaryl.

3.2.5. Anthocyanins

Fruit of bilberry are found to contained high levels of anthocyanins (Table I-3). Fifteen anthocyanins were identified in both aqueous and ethanolic fruit extracts (Table I-2). They 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. The five anthocyanidins were present as two hexosides (loss of 162 amu) and one pentoside (loss of 132 amu) whose structures were proposed based on previous work on lingonberry (Mane at al., 2011) and the studies on bilberry by, Može et al. (2011) and Mikulic-Petkovsek et al. (2015). In lingonberry, cyanidin-3-galactoside appears as the major anthocyanin (Mane et al, 2011), whereas delphinidin-3-O-galactoside and delphinidin-3-O-glucoside were predominant in fruit extracts as previously determined by Može et al. (2011) and Prencipe et al. (2014).

Table I-2. Anthocyanins identified by UPLC/ESI-MS^n in fruits extracts of bilberry

t_R (min)	λ_{max} (nm)	$[M-H]^+$ *(m/z)*	MS^2 fragments *(m/z)*	Proposed structure[a]	2013	2014
7.5	278, 522	465	303	Delphinidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.0	280, 522	465	303	Delphinidin-3-O-Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.4	278, 520	449	287	Cyanidin-3-O-Gal (std)	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.4		435	303	Delphinidin-3-O-Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.9	278, 518	449	287	Petunidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.1		479	317	Cyanidin-3-O-Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.4	278, 520	479	317	Petunidin-3-O- Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.6		419	287	Cyanidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.0	278, 524	449	317	Petunidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.0		463	301	Peonidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.5	278, 523	493	331	Malvidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.6	278, 523	463	301	Peonidin-3-O- Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
11.0	276, 526	493	331	Malvidin-3-O- Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
11.0		433	301	Peonidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
11.4	270, 528	463	331	Malvidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}

Gal: galactoside; Ara: arabinoside; Glc: glucoside; FH_2O: aqueous fruit extract; F_{EtOH}: ethanolic fruits extract; [std] compounds were identified by comparison with standards; [a] identified according to Može et al. (2011), Mane et al. (2011) and Mikulic-Petkovsek et al. (2015).

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

Relatively similar phenolic and anthocyanin profiles were found in bilberry leaves, stems, and fruits 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 I-1). The importance of major phenolic sub-groups (caffeic acid and coumaric acid derivatives, flavonol glycosides, flavanol monomers and flavanol oligomers) is reported in Table I-3. In addition, the individual contribution of the major phenolic compounds to the leaf and stem extracts is given in Table I-4.

In leaves, caffeic acid derivatives were the most representative group of phenolic compounds (Martz et al., 2010) whatever the period and the year of harvest, their level ranging between 67 and 79% of the Dry Extract weight (Table I-3). Their seasonal evolution differed between years 2013 and 2014. Chlorogenic acid (**18**) contributed for more than half of the Dry Extract weight and its relative content varied between 55% (May 2013) and 75% (July 2013) (Table I-4). Althouh leaves are exposed to light, the *cis* isomer represented less than 2% of the chlorogenic acid pool.

Flavonol glycosides were present in lesser contents in May compared to *p*-coumaric acid derivatives although this ranging became opposite 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 submitted to light (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-galactoside (**62**) from July to September in year 2013 and July 2014 (3-8%).

The second and third 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%).

p-Coumaric acid derivatives appeared at their highest levels in May while decreasing during the season. The other main contributor to this group is *p*-coumaroyl monotropein (**70**) ranking 4th in May 2013 and May 2014, although this ranking largely decreases in July and September in both 2013 and 2014.

Finally, flavanol monomers and oligomers, which were mainly composed of (-)-epicatechin and cinchonains II, respectively, became quantifiable in July and September although in trace amounts. There were opposite seasonal variations between flavanol groups and inter-annual effects for (-)-epicatechin in September.

Overall, there were seasonal and inter-annual variations for all the phenolic sub-groups studied.

In stems, flavanol oligomers were the major group, representing between 54 to 62% of the total phenolic content (w/w of DE) (Table I-3). 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 (both ≥14%). Ranking third is dimer B2 (**32**) for all the 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 dominated in September (7%). Finally, the next compounds highly present were variable: *p*-coumaroyl malonylhexoside (**96**) in May and July 2013 and September 2014, quercetin hexuronide (**64**) in May and July 2014, quercetin-3-O-galactoside (**62**) in May 2013 and (-)-epigallocatechin (**15**) in September 2014. As for leaves, inter-annual variations were observed for most groups in the stem extracts. This can be attributed to different weather conditions like air and soil temperatures, hours of sunshine, and level of precipitations (Martz, Jaakola, Julkunen-Tiitto, & Stark, 2010; Uleberg et al., 2012). The year effect was however higher for stems than for leaves, suggesting that abiotic stress clearly influences polyphenol biosynthesis. Genetic control is rather observed in the type of polyphenol predominantly synthetized as observed with flavanol oligomers in stems.

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 I-3). 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). Flavanol monomers were only constituted by a cinchonain I isomer (**99**). Flavanol oligomers were not identified using UPLC/MS. Last, the content in phenolic compounds may be underestimated in fruit extracts as no phenolic compound could be integrated between 4 and 12 min owing to anthocyanin elution and absorption in this area.

Table I-3. 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]	–	99.8 ± 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]	–	**96.4 ± 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 (a)***	**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 ***	**1.49 ± 0.07**	**0.94 ± 0.03 ***	**0.13 ± 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.

Table I-4. 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-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
	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 parantheses correspond to compound number in Table 1. [b]Mean for $n = 3$.

3.4. Characterization of flavan-3-ol oligomers

Thioacidolysis was used to reveal the total amount of flavanol oligomers and their intrinsic composition. As a matter of fact, oligomers only up to tetramers were detected by UPLC.

3.4.1. In freeze-dried extracts

In leaves, (-)-epicatechin was quantified in the control analysis of the thioacidolysis experiment although it was not quantified by UPLC in May 2013 and 2014. EC decreased from July to September with both methods although the levels are quite different.

Flavanol oligomers could appear as the second class of phenolic compounds when considering data from thioacidolysis (Table I-5) and UPLC (Table I-3), ranging between 13 and 32 mg/g DE in July and September 2013 and 2014. This is rather contradictory with data from UPLC (Table I-3) where no type-A or type B oligomers, but only cinchonain II (**59**) were quantified in leaves (1.1-1.4 mg/g DE for the same period). mDP ranging from 2.9 to 4.5 suggests the presence of small-size oligomers which should not have escaped UPLC analysis unless there is a diversity of contributors. Epicatechin was the only constituting unit of flavanol oligomers with 22 to 35% of terminal unit and the rest as extension units.

In stems, flavanol oligomers appeared as the first phenolic group as already observed in UPLC quantification. The range was between 48 and 70 mg/g DE after thioacidolysis and between 40 and 71 mg/g DE by UPLC. Moreover, flavanol monomers and oligomers evolved similarly along seasons in 2013 and 2014 independently of the methods. One should note that flavanol monomers are constituted by epicatechin and epigallocatechin in UPLC analysis (4-12 mg/g DE) while by epicatechin only in the thioacidolysis method (5-18 mg/g DE). There is thus more epicatechin titrated by the thioacidolysis method. Additionally, the oligomer contents appears underestimated after thioacidolysis in accordance with type-A oligomers being incompletely degraded during thioacidolysis as well as the lack of quantification of (epi)gallocatechin units contained in oligomers. mDP ranging between 2 and 3 are also slightly underestimated in view of the two A-type and B-type trimers (**45** and **46**) that were predominant by UPLC. Last,

catechin appeared as both terminal units (2-8%) and extension units (1-3%), while absent as a monomer.

In fruits, nearly equal amounts of epicatechin and catechin were detected in the control experiment of thioacidolysis (Table I-5). By contrast, no catechin or epicatechin were found by MS or UPLC while only cinchonain I (**99**) constituted the flavanol monomer class (Table I-1). Low amounts of flavanol oligomers (2-5 mg/g DE) were provided in the thioacidolysis method when, again, no oligomers were quantified by UPLC. mDP (2-3) remained low. Surprisingly, catechin appeared as a terminal unit only with a 9% rate when epicatechin was the sole extension unit. Finally, 55% aqueous ethanol was twice as efficient at extracting monomeric and oligomeric flavanols although this difference was not evidenced for the different phenolic classes evaluated by UPLC.

3.4.2. In freeze-dried fruits

Freeze-dried fruits were directly analyzed by thioacidolysis, mostly confirming the results described for extracts for mDP and relative composition in monomers. Catechin (1%) was however detected as an extension unit. It is noteworthy that contents in flavanol monomers and oligomers, mDP, and the composition of oligomers are similar between 2013 and 2014.

Table I-5. Flavan-3-ol composition and mDP in bilberry leaves, stems and fruits determined by HPLC following thioacidolysis.

Morphological part extracts	Period of vegetation	Flavanol monomers (mg/g DE)		Procyanidin characterization				Flavanol oligomers (mg/g DE)	mDP
				Terminal units (%)		Extension units (%)			
		CAT	*EC*	*CAT*	*EC*	*CAT*	*EC*		
Leaf extracts									
	May 2013	–	1.48 ± 0.12(A)	–	34.7 ± 4.5(A)	–	65.3 ± 16.6(A)	4.25 ± 0.99(A)	2.9 ± 0.5(A)
	May 2014	**–**	**4.74 ± 0.80(a)***	**–**	**34.5 ± 5.5**	**–**	**65.5 ± 2.2**	**2.11 ± 0.56(a)***	**3.0 ± 0.4(a)**
	July 2013	–	2.00 ± 0.58(A)	1.98 ± 0.03	27.9 ± 5.8(B)	–	70.1 ± 2.3(B)	32.4 ± 2.2(B)	3.4 ± 0.4(A)
	July 2014	**–**	**7.22 ± 1.46(b)***	**–**	**22.1 ± 0.3(b)**	**–**	**77.9 ± 1.2(b)**	**25.5 ± 1.4(b)***	**4.5 ± 0.1(b)***
	Sept. 2013	–	0.60 ± 0.10(B)	–	26.3 ± 2.3(C)	–	73.7 ± 0.8(C)	23.8 ± 0.6(C)	3.8 ± 0.2 (A)
	Sept. 2014	**–**	**4.83 ± 0.39(a,b)***	**–**	**28.0 ± 3.7(c)***	**–**	**72.0 ± 1.7(c)***	**12.7 ± 0.6(c)***	**3.6 ± 0.3(a)**
Stem extracts									
	May 2013	–	5.36 ± 1.15(A)	2.17 ± 0.11(A)	36.1 ± 1.8(A)	–	61.7 ± 3.0(A)	51.7 ± 2.1(A)	2.6 ± 0.0(A)
	May 2014	**–**	**9.65 ± 4.47(a)**	**–**	**32.6 ± 0.1(a)***	**1.61 ± 0.37(a)**	**65.8 ± 0.4(a)***	**60.0 ± 4.3(a)***	**3.1 ± 0.0 (a)***
	July 2013	–	8.87 ± 0.91(B)	3.10 ± 0.87(A)	37.5 ± 1.1(B)	–	59.4 ± 0.4(B)	63.6 ± 0.3(B)	2.5 ± 0.1(B)
	July 2014	**–**	**17.3 ± 3.03(a)***	**–**	**33.7 ± 0.6(b)***	**1.20 ± 0.17(a)**	**65.1 ± 0.9(a)***	**69.6 ± 2.3(b)***	**3.0 ± 0.0(b)***
	Sept. 2013	–	9.83 ± 0.96(B)	5.86 ± 1.06(B)	36.5 ± 1.0(B)	–	57.6 ± 1.9(B)	60.3 ± 0.3(C)	2.4 ± 0.0 (C)
	Sept. 2014	**–**	**17.9 ± 2.28(a)***	**7.90 ± 0.28**	**38.1 ± 0.9(c)**	**2.66 ± 2.60(a)**	**51.4 ± 2.0(a)***	**47.6 ± 1.0(c)***	**2.2 ± 0.1(c)***
Fruit extracts									
H_2O	July 2013	0.49 ± 0.02	0.49 ± 0.07	9.48 ± 2.61	34.6 ± 2.0	–	55.9 ± 23.5	2.21 ± 0.68	2.3 ± 0.6
	July 2014	**0.34 ± 0.02***	**0.39 ± 0.02**	**9.63 ± 0.62**	**37.6 ± 0.3***	**–**	**52.8 ± 0.9**	**2.45 ± 0.06**	**2.1 ± 0.0**
EtOH 55%	July 2013	0.94 ± 0.32	0.90 ± 0.24	8.99 ± 2.50	33.6 ± 4.5	–	57.4 ± 28.0	4.18 ± 1.50	2.3 ± 0.5
	July 2014	**0.21 ± 0.01***	**0.46 ± 0.06***	**5.75 ± 0.33**	**27.1 ± 1.0**	**–**	**67.2 ± 2.1**	**5.42 ± 0.25**	**3.0 ± 0.0***
Freeze-dried fruits[a]	July 2013	0.35 ± 0.03	0.78 ± 0.27	5.42 ± 0.59	38.1 ± 2.2	1.32 ± 0.15	55.2 ± 3.7	5.71 ± 0.40	2.3 ± 0.1
	July 2014	**0.33 ± 0.04**	**0.67 ± 0.08**	**6.72 ± 0.30***	**36.4 ± 0.6**	**1.49 ± 0.00**	**55.4 ± 2.7**	**5.71 ± 0.38**	**2.3 ± 0.1**

CAT: (+)-catechin. EC: (–)-epicatechin. Sept.: September. mDP: average degree of polymerization of monomeric and oligomeric flavan-3-ols. Values represented mean ± SD (n = 3). [a]Results are expressed in mg/g of Dry Matter. ¯ 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 ($p < 0.05$).

3.5. Antioxidant activity of bilberry extracts

The antioxidant activity of the various bilberry extracts was determined by two complementary methods. The Folin-Ciocalteu method, which measures the ability of a sample to reduce transition metal ions such as in the complex between sodium phosphomolybdate and phosphotungstate, gives access to the Total Phenolic Content (TPC). As to the DPPH (2,2-diphenyl-1-picrylhydrazyl) test, it relies on the ability of reducing molecules to transfer an electron or a hydrogen atom to the nitrogen-centered DPPH radical.

The TPC was reported in weight per Dry Matter (DM) for years 2013 and 2014 and in weight per Dry Extract (DE) in 2014 (Table I-3; Figure I-5A). The difference is due to the extraction yield of the DM (ca. 58, 52, and 85% for leaves, stems and fruits, respectively) (Annexe I-1). When expressed in mg of gallic acid per gram of DE (year 2014), the TPC values were in the same range or higher as those found by summing all the phenolic compounds quantified by UPLC (Table I-3). 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 (Figure I-6A and B). The removal of the May data markedly increased the correlation (solid line, 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 leaf samples are characterized by unexpectedly low TPC when compared to TPC of the samples from July and September. It is worth noting that *p*-coumaric acid derivatives contribute for 20% to the phenolic pool in May and only 7% in July and September. Besides, *p*-Coumaric acid derivatives remained at constant levels in stems (11-14%) and in fruits (4-5%) from May to September in agreement with the good correlations observed.

The DPPH scavenging activities of bilberry extracts showed contrasted seasonal variations in both 2013 and 2014 as already observed for TPC and the Sum of Phenolic Compounds (Figure I-5B). For example, 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 more modest in 2014.

It can also be noted that the DPPH values are slightly higher when the extract concentration is lower (5 rather than 10 mg dry extract/mL) (Figures I-5 B and C). This difference is supported by the fact that the reaction between the DPPH and the phenolic compounds or their oxidation products has not reach completion in 30 min. Last, the annual effect on the DPPH antioxidant activities is similar to that exhibited for the TPC values except for the September stem sample.

Additionally, the antioxidant activity in the DPPH test and the Sum of Phenolic Compounds (w/w of Dry Extract) were highly correlated with R^2 of 0.70 and 0.77 in 2013 and 2014 for all eight samples (Figure I-6C and D). The graph patterns are similar to the ones observed for Sum of Phenolic Compounds vs TPC (Fig. I-6 A and B). The difference in reactivity of the May leaf sample 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. 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, Everette et al. (2010) tested the reactivity of the Folin-Ciocalteu reagent regarding various biological substances. The relative reactivity of gallic acid (1.0), caffeic acid (0.96), chlorogenic acid (1.36), and rutin (1.53), which belong to caffeic acid derivatives and flavonol glycosides, was found higher than that of salicylic acid (0.26) and tyrosine (0.38), which are structurally related to *p*-coumaric acid. Tryptophan, ascorbic acid, as well as Cu(II), Fe(II), and Zn(II) complexes also react with the Folin-Ciocalteu reagent although more weakly than most phenolic compounds. These substances are generally present in plant aqueous extracts and they may thus contribute to the overall antioxidant activity.

On the other hand, both mono- and dihydroxyphenyl moieties are likely able to transfer an electron or a hydrogen atom towards a radical species like the DPPH species although examples in the literature are lacking to strengthen this point. Interestingly, catechin extension or terminal units in dimer B3 (Cat-(4-8)-Cat) and trimer C2 (Cat-(4-8)-Cat-(4-8)-Cat) are able to transfer an electron or a H-atom towards the DPPH radical although they appear slightly less reactive than monomeric catechin (Goupy et al., 2003).

Owing to the high correlation between the DPPH test and the Sum of Phenolic Compounds, the DPPH test appears as a valuable tool to assess both the relative content in phenolic compounds and the total antioxidant activity of bilberry extracts from all morphological parts.

The Total Phenolic Content determined by the Folin-Ciocalteu method strongly correlates with the DPPH radical scavenging activity with R^2 of 0.91 and 0.94 for samples from 2013 and 2014, respectively (Figures I-6E and F). Very similar reducing effects of the extracts are thus outlined. 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-centered radical as in the DPPH test.

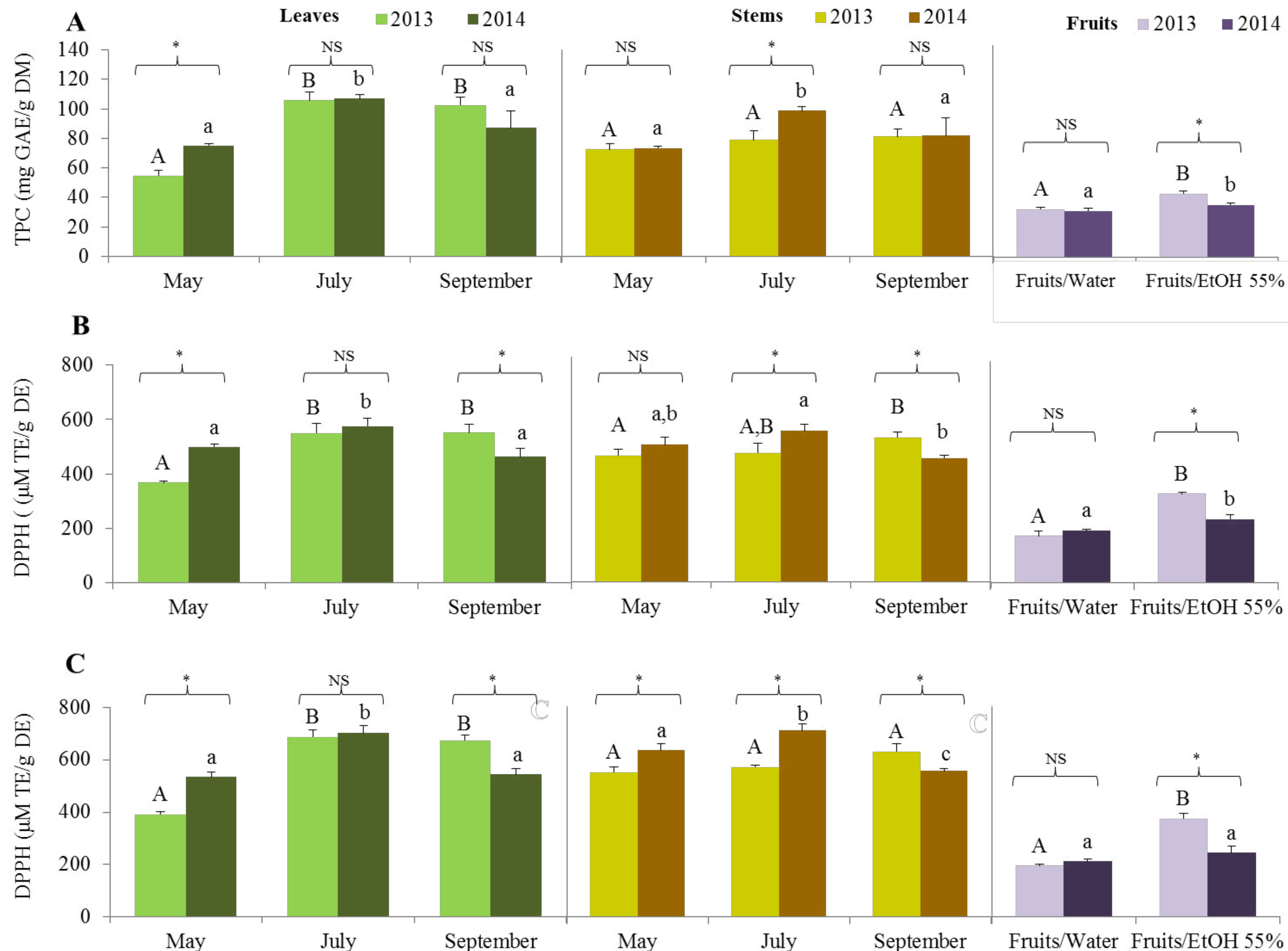


Figure I-5. Influence of different harvest periods on Total Phenolic Content (A) and DPPH radical scavenging activity (B: samples at 10 mg/mL; C: samples at 5 mg/mL) in leaf, stem and fruit extracts of bilberry (mean±SD, n = 3-4). Different letters indicate a significant difference between the periods 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.

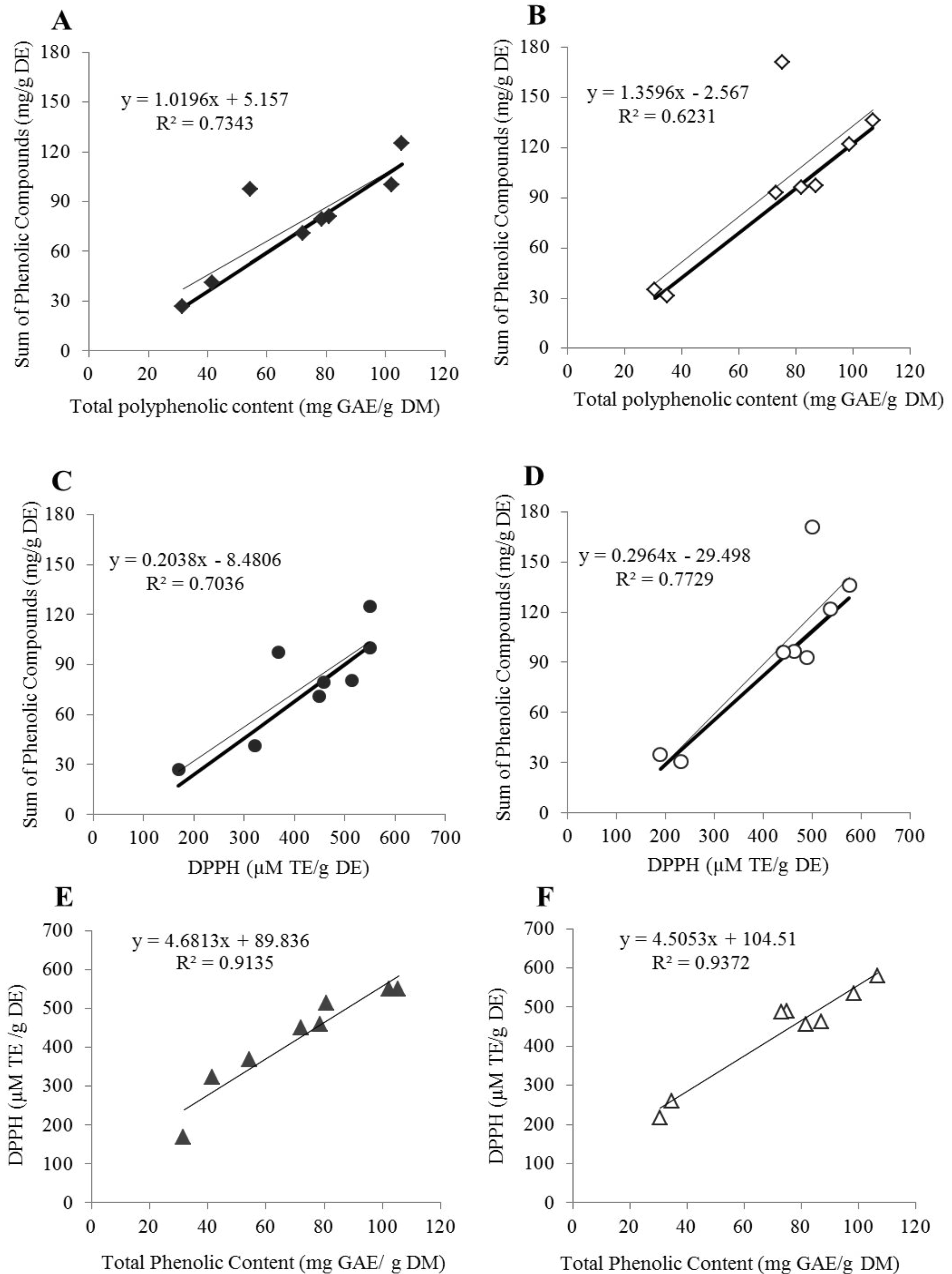


Figure I-6. Correlation between the Sum of Phenolic Compounds and the TPC (A: samples from 2013; B: samples from 2014), between the Sum of Phenolic Compounds and the DPPH radical scavenging activity (C: samples from 2013; D: samples from 2014) and between the TPC and the DPPH radical scavenging activity (E: samples from 2013; F: samples from 2014). The thin line is the linear regression for all samples. The solid line is the linear regression without leaf from May.

4. Conclusions

This study reports the most comprehensive qualitative analysis ever performed on bilberry phenolics in leaf, stem and fruit extracts. In particular, 45 phenolic compounds were either newly identified or named among which four hydroxybenzoic or propionic acid glycosides, eight caffeoylquinic acid derivatives, two caffeoyl hexosides, one caffeoylmalonyl glycoside, three *p*-coumaroyl(dihydro)monotropeins, fourteen *p*-coumaroylglycosides which could be further acetylated or malonylated, two ill-defined *p*-coumaroyl deivatives, two quercetin glycosides, phloretin-2-hexoside and various A-type and B-type flavanol oligomers up to the tetramers.

Quantitative analysis allowed to accurately determine the more important contributors of the following groups: caffeic acid derivatives, *p*-coumaric acid derivatives, flavonol glycosides, anthocyanins, and flavanol monomers and oligomers. These major contributors were markedly more affected by the season and year in leaves than in stems. In leaves, chlorogenic acid was the major compound for all seasons and both years and variability was observed starting at the second and third contributors which were principally but not exclusively a quercetin hexuronide and quercetin-3-galactoside. In stems, an A-type and a B type trimers were the first two contributors while dimer B2 was ranking third and chlorogenic acid fourth. The most important variations were however outlined between May and July with 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 various phenolic groups were generally different for years 2013 and 2014. The weather informations for the Borca region during the study periods showed that 2013 and 2014 were relatively different in terms of minimum and maximum soil and air temperatures as well as amounts of precipitation. April and May 2014 were colder and more rainy, June 2014 colder but less rainy, July and August 2014 similar in temperatures although July 2014 was more rainy, while September 2014 was warmer and less rainy. By comparison, the Sum of Phenolic Compounds was higher in May 2014 in leaves and from May to July for stems 2014. Polyphenol contents appear thus greater in leaves and stems with cold weather (Annexe I-5).

The phenolic content was highly correlated to the antioxidant activity in leaf, stem and fruit extracts of bilberry using the evaluation of the Total Polyphenol content and the antioxidant activity in the DPPH test. Besides, these two tests correlate very well with each other (R2 > 0.9) suggesting a similar reactivity of all the compounds in the plant extract towards transition metal ions constituting the Folin Ciocalteu reagent and the N-centered radical of the DPPH reagent. It was however outlined that the presence of *p*-coumaric derivatives, as found in large amounts in May leaves, decreased the antioxidant activity of the bilberry extract.

Results from this study indicate that all the morphological parts of bilberry are suitable for valorization 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 herbal supplements. The harvest period can be refined based on the desired phenolic structures and their potential health effect. The stability of the collected dry matter remains however to be assessed to determine its optimal shelf life.

ANNEXES

Annexe I-1. Extraction yield of the Dry Matter (DM) for bilberry leaves, stems and fruits from 2014 at three different periods of vegetation (*n* = 1).

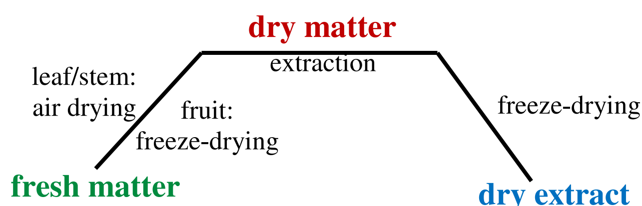
Morphological parts/ Period of vegetation	Extraction yield of the DM (%)
Leaves	
May	58
July	59
September	56
Stems	
May	49
July	52
September	53
Fruits with H_2O	73
Fruits with EtOH 55%	97

Annexe I-2. Residual moisture of grinded bilberry leaves, stems and fruits at three different periods of vegetation and for two different years.

Morphological parts	Period of vegetation	Residual moisture (%)*
Leaves	May 2013	8.2
	May 2014	8.6
	July 2013	9.5
	July 2014	7.9
	September 2013	9.3
	September 2014	7.9
Stems	May 2013	8.8
	May 2014	8.6
	July 2013	8.6
	July 2014	7.5
	September 2013	9.3
	September 2014	8.6
Fruits	July 2013	8.7
	July 2014	7.2

*Mean for *n* = 3.

Annexe I-3. Influence of the processing on the Total Phenolic Content in bilberry leaves, stems and fruits.

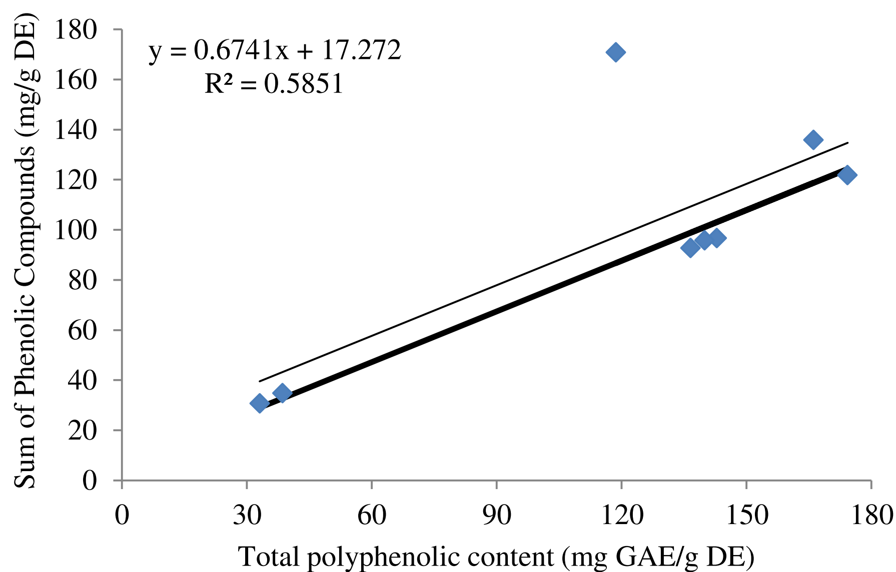


Processing steps for bilberry leaves, stems and fruits

Extract /Period of vegetation	Moisture content (%)*	Total Phenolic Content (mg GAE/g FM)	Total Phenolic Content (mg GAE/g DM)	Total Phenolic Content (mg GAE/g DE)
Leaves				
July 2014	38	60.6 ± 1.7(a)	106.9 ± 2.9 (b)	166.1 ± 4.4 (b)
September 2014	46	43.7 ± 5.9(b)	87.1 ± 11.7 (a)	142.9 ± 19.2 (a,b)
Stems				
July 2014	34	60.5 ± 1.0 (a)	98.7 ± 4.6 (b)	174.3 ± 2.8 (b)
September 2014	41	44.2 ± 5.9 (b)	81.8 ± 11.0 (a)	140.0 ± 18.8 (a)
Fruits with H_2O				
July 2014	86	4.09 ± 0.23	30.5 ± 1.7	38.6 ± 2.2
Fruits with EtOH 55%	86			
July 2014		4.66 ± 0.13	34.7 ± 1.0	33.1 ± 0.9

Values represented mean ± SD (n = 3). *Moisture content refers to the quantity of water contained in fresh samples. Total Phenolic Content is obtained by the Folin-Ciocalteu method. FM: Fresh Matter. DM: Dry Matter. DE: Dry Extract. Different letters indicate a significant difference between the two periods of vegetation at $p < 0.05$.

Annexe I-4. Correlation between the Sum of Phenolic Compounds and the Total Phenolic Content for samples from 2014 reported both in weight per Dry Extract



Thin line is the linear regression for all samples.
Solid line is the linear regression without leaf from May.

Annexe I-5. Weather informations for the Borca region (Romania) during the study period.

month	year	average temperature (°C)	maximum temperature (°C)	minimum temperature (°C)	average humidity (%)	average soil temperature (°C)	maximum soil temperature (°C)	minimum soil temperature (°C)	total precipitations (mm)
February	2013	-0.8	10.8	-8.7	84	-2	5	-15.3	24.7
	2014	**-0.3**	**16.2**	**-18.3**	**86**	**-1.3**	**10.2**	**-22**	**5.3**
March	2013	0.4	13.6	-10.6	75	-0.3	20.6	-12.9	31.0
	2014	**7**	**20.9**	**-1.2**	**69**	**6.9**	**34.5**	**-2.5**	**29.2**
April	2013	11	29.9	-0.7	66	12	46.4	-1.6	45.2
	2014	**9.4**	**21.8**	**0.1**	**76**	**10.9**	**37.5**	**-0.3**	**95.5**
May	2013	16.8	30.5	5.9	64	19.8	52.4	4.4	51.6
	2014	**14.7**	**28.3**	**2.3**	**71**	**17.9**	**49.7**	**0.6**	**139.4**
June	2013	18.9	31.5	8.3	76	21.6	53.0	6.7	233.4
	2014	**17.4**	**29.6**	**8.2**	**72**	**20.1**	**52.9**	**5.5**	**59.8**
July	2013	19.9	32.7	10.2	68	23.5	56.8	7.6	50.0
	2014	**20.1**	**30.6**	**10.1**	**-**	**22.4**	**48.7**	**9.8**	**240**
August	2013	19.8	31.2	9.1	68	23.6	54.5	8.3	51.6
	2014	**19.9**	**32.5**	**7.8**	**72**	**22.9**	**52.9**	**6.0**	**44.8**
September	2013	13.5	25.7	3.3	72	14.1	42.3	1.2	50.4
	2014	**15.2**	**26.8**	**0.3**	**71**	**17.9**	**43.6**	**-1.2**	**19.2**

Chapter II.

PHENOLIC PROFILE AND ANTIOXIDANT ACTIVITY OF LEAF, STEM AND FRUIT EXTRACTS OF LINGONBERRY (*VACCINIUM VITIS-IDAEA* L.) AT THREE VEGETATIVE STAGES

1. Background

Lingonberry (*Vaccinium vitis-idaea* L.), a wild shrub of the *Ericaceae* family, is known as a natural source of food, beverage and dietary supplements due to its richness in nutritional and bioactive compounds. Although lingonberry constituents have multiple biological activities, most of the research has focused on the phenolic compounds. Previous studies have shown that the most abundant polyphenols in lingonberry are proanthocyanidins in fruits and arbutins in leaves, but quercetin glycosides, derivatives of caffeic acid and anthocyanins are also detected (Kylli et al., 2011; Ieri et al., 2013; Liu et al., 2014). Health effects of lingonberry fruit extracts containing polyphenols (mainly type-A proanthocyanidins) have been shown in the prevention of urinary tract infections (Davidson et al., 2014). *In vitro*, these extracts may be antimicrobial against *Staphylococcus aureus* or inhibit hemagglutination of *Escherichia coli* (Kylli et al., 2011).

As shown by several studies, the content as well as the biological activities of phenolic compounds in plants are influenced by plant organs and growth conditions such as latitudinal, place of the growth site, harvest season, day length, light quality and temperature (Harris et al., 2006; Jaakola & Hohtola, 2010; Zhu et al. 2013).
For *Vaccinium angustifolium* Ait., Harris et al. (2006) have found that leaves contain high concentrations of chlorogenic acid and quercetin glycosides which are also present in the fruit and stem while procyanidin dimers were exclusively detected in the stem and root. The results obtained by Zhu et al. (2013) revealed that the blueberry leaves from different seasons have different contents of total polyphenols, total flavonoids, and proanthocyanidins as well as different antioxidant activities.

For these reasons, in the present investigation, the dynamic accumulation of phenolic compounds in various morphological parts of lingonberry was studied by comparing the total phenolic content, the phenolic composition and the antioxidant activity at three different periods of vegetation during two years. A method based on the thioacidolysis of the oligomeric procyanidins is used for analysis of the degree of polymerization and flavanol unit constitution.

No analysis of procyanidins from lingonberry leaves and stems has previously been reported. The correlation between contents in total polyphenols, quantified by the Folin-Ciocalteu method or by UPLC, and the antioxidant capacity in the DPPH test are also assessed.

2. Methodology

2.1. Materials

2.1.1. Lingonberry samples

Fruits, leaves and stems of lingonberry (*Vaccinium vitis-idaea* L.) were collected in May, July and September during the years 2013-2014 from natural mountain habitats of Borca village, Neamt, Romania (altitude: 1300 m to 1800 m; coordinate: 47° 11′ 34″ N and 25° 47′ 8″ E).

Fresh lingonberry fruits were frozen at - 24 °C, then lyophilized in a Christ Alpha 1-4 LSC (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 were dried at room temperature, in the shade, for 10 days. After drying, the leaves were manually separated from stems, ground (using the same mill as for fruits) and sieved through a standard sieve to a final particle size < 0.315 mm. Grinded samples were kept in a dessicator to prevent adsorption of moisture from the air until extraction. Before extraction, residual moisture (%) of grinded samples was determined using a RADWAG MAX 50/1 moisture analyzer; triplicate measurements were made for each sample and the mean values was used to estimate the Total Phenolic Content. Residual moisture between 7% and 9.5% were found for all plant materials, except for lingonberry fruits which showed a moisture content of 13.3% for the 2013 sample and 11.9% for the 2014 sample, respectively.

2.1.2. Chemicals and solvents

Standard phenolics, reagents and solvents are the same as the ones used in the bilberry study (Chapter I, Section 2.1.2).

2.2. Extraction of phenolic compounds

The extracts (Figure II-1) were prepared by the extraction method described for bilberry (Chapter 1, Section 2.2), using the same extraction conditions and equipment. The solutions of extracts obtained were freeze-dried and stored at 4 °C in a refrigerator before DPPH, thioacidolysis-HPLC and UPLC/MS analyses. For each sample, the extraction was realized in triplicate.

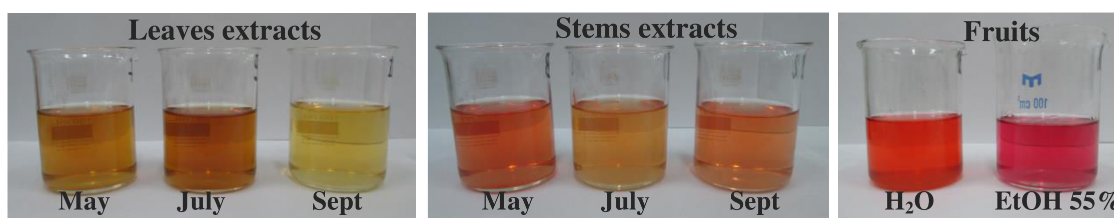


Figure II-1. Leaf, stem and fruit lingonberry extracts

2.3. Qualitative and quantitative analyses of phenolic compounds

Identification and quantification of phenolic compounds by UPLC/MS were achieved as in the bilberry study (Chapter I, Sections 2.3.1 and 2.3.2).

For UPLC/MS analyses, freshly prepared solutions of lyophilized lingonberry leaf and stem extracts (10 mg/mL) in water were directly used. For the lingonberry fruit extracts (20 mg/mL), purification was first conducted to eliminate sugars and organic acids that could interfere in the analysis of phenolic compounds. The volume of extract injected was 3 µL for analysis of phenolic compounds and 1 µL for analysis of anthocyanins. All samples were injected in triplicate after independent sample extractions.

For quantification of phenolic compounds, chlorogenic acid, (+)-catechin, (–)-epicatechin, (–)-epigallocatechin, procyanidin B2, procyanidin A2, procyanidin C1, quercetin-3-glucoside, quercetin-3-galactoside, quercetin-3-rhamnoside and cyanidin-3-galactoside, were used for 6 point-calibrations. Caffeic acid derivatives (4-O-caffeoylquinic acid, caffeoylarbutins) were quantified as chlorogenic acid (325 nm);

coumaric acid derivatives, ferulic acid, ferulic acid hexoside and cinnamic acid-hexoside as *p*-coumaric acid (330 nm); benzoic acid derivatives as (+)-catechin (280 nm); quercetin glycosides as quercetin-3-galactoside except for quercetin-3-glucoside and quercetin-3-rhamnoside (350 nm) which had their own standards; A-type dimers were expressed as procyanidin A2 (280 nm), B-type dimers as procyanidin B2 (280 nm), A-type and B-type trimers as procyanidin C1 (280 nm), cinchonain I as (–)-epicatechin and anthocyanins as cyanidin-3-galactoside (520 nm).

2.4. Analysis of procyanidins using thioacidolysis

Procyanidin analysis was achieved as in the bilberry study (Chapter I, Section 2.4).

2.4.1. Freeze-dried extracts

Solutions of freeze-dried extracts in dry methanol were prepared at the following concentrations:

- 15 mg/mL for leaves and stems of lingonberry extracts,
- 40 mg/mL for aqueous extracts of ethanolic extract of lingonberry fruits,
- 80 mg/mL for aqueous extracts of lingonberry fruits.

Solutions were analyzed with or without thioacidolysis.

Analyses were done in triplicate.

2.4.2. Freeze-dried fruits

For the analysis of procyanidins 50 mg of lyophilized lingonberry fruit were used. Analyses were done in triplicate.

2.5. Antioxidant activity by applying spectrophotometric methods

2.5.1. Total Phenolic Contents by the Folin Ciocalteu method

The Folin–Ciocalteu spectrophotometric method used previously in the bilberry study (Chapter I, Section 2.5.1) was applied for the determination of the Total Phenolic Content of the lingonberry extract solutions. The results were expressed as mg of gallic acid equivalents per gram of dry matter (mg GAE/g DM). Each extract was analyzed as triplicates of independent extract solutions.

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

The DPPH test based on a method developed by Goupy et al. (2003) and applied previously in the bilberry study (Chapter I, Section 2.5.2) was used for analysis of the DPPH radical scavenging activity. The results were expressed as micromoles of Trolox equivalents (TE) per gram of dry extract (µM TE/g DE). All determinations were carried out three to four times and independent extract solutions were utilized each time.

2.6. Statistical analyses

Results are expressed as the mean ± standard deviation (SD). Significant differences at a 95% confidence interval ($p < 0.05$) were assessed through the analysis of ANOVA with Tukey−Kramer honestly significant difference (HSD) post hoc test to identify differences among groups using the statistical XLStat software (version 2008.3.02, Addinsoft SARL, Paris, France).

3. Results and discussion

3.1. Phenolic profile and content of lingonberry extracts

Data from UPLC/MS analyses, mass fragmentation, retention times (t_R), and UV-VIS spectra, were used for the identification and quantification of the phenolic compounds in leaf, stem and fruit extracts of wild lingonberry at three different periods of vegetation and for two different years.

Available standards such as, *p*-coumaric acid, (+)-catechin, (–)-epicatechin, (-)-epigallocatechin, procyanidin B1, procyanidin B2, procyanidin A2, procyanidin C1, 5-O-caffeoylquinicacid, quercetin-3-O-galactoside, quercetin-3-O-glucoside and quercetin-3-O-rhamnoside, and previous literature data on phenolic compounds in lingonberry and in other plant materials also permitted the assessment of the identity of some phenolic structures. In all the morphological parts of lingonberry **127** phenolic compounds were tentatively identified (Table II-1), 75 being in leaf extracts, 94 in stem extracts, 56 in fruit extracts. Among which, **50** compounds have newly been detected in the different morphological parts of lingonberry. Chromatographic profiles of lingonberry leaves, stems and fruits are presented in Figures II-2 and II-3.

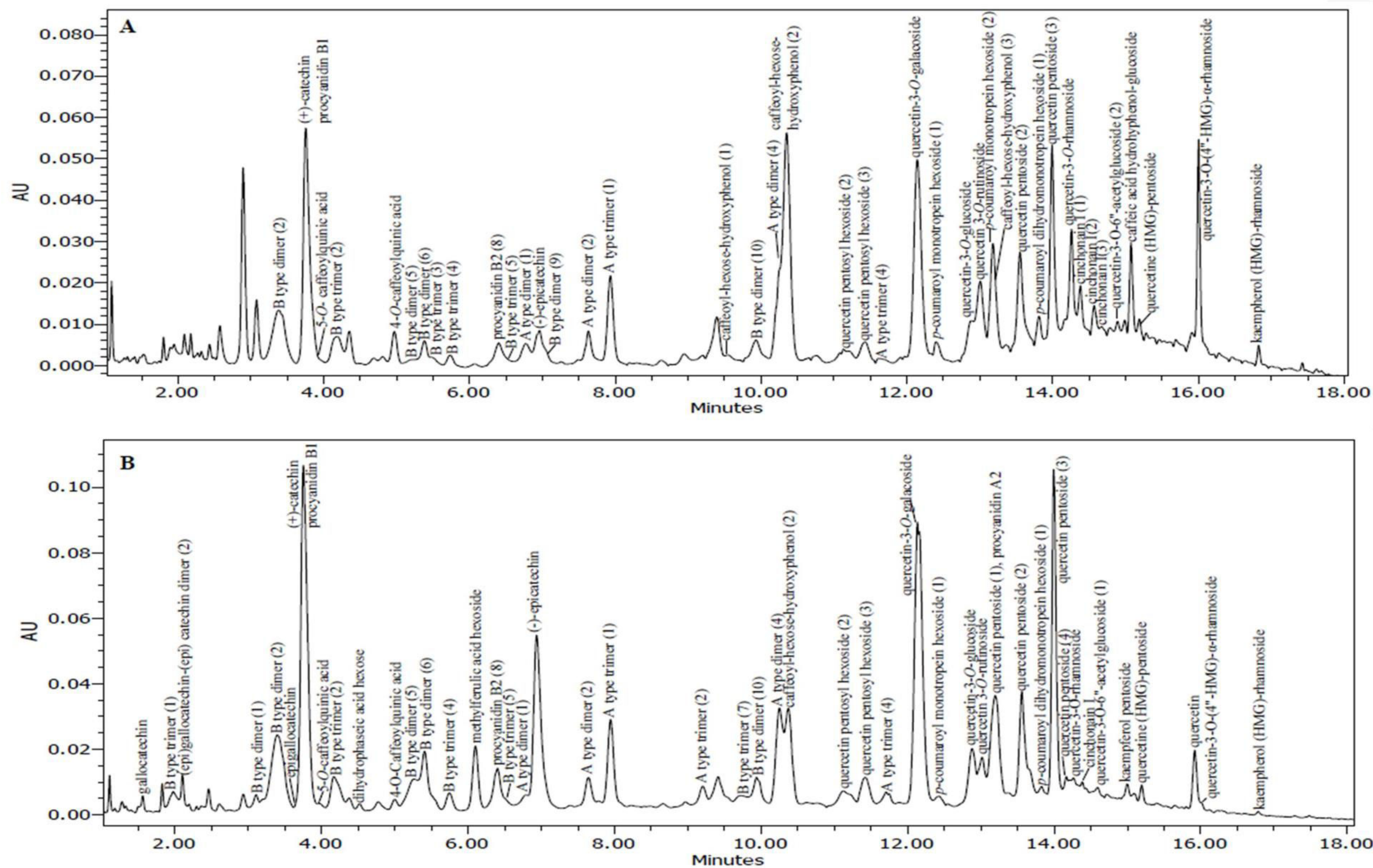


Figure II-2. Chromatographic phenolic profile of leaf (A) and stem (B) extracts of lingonberry at 280 nm

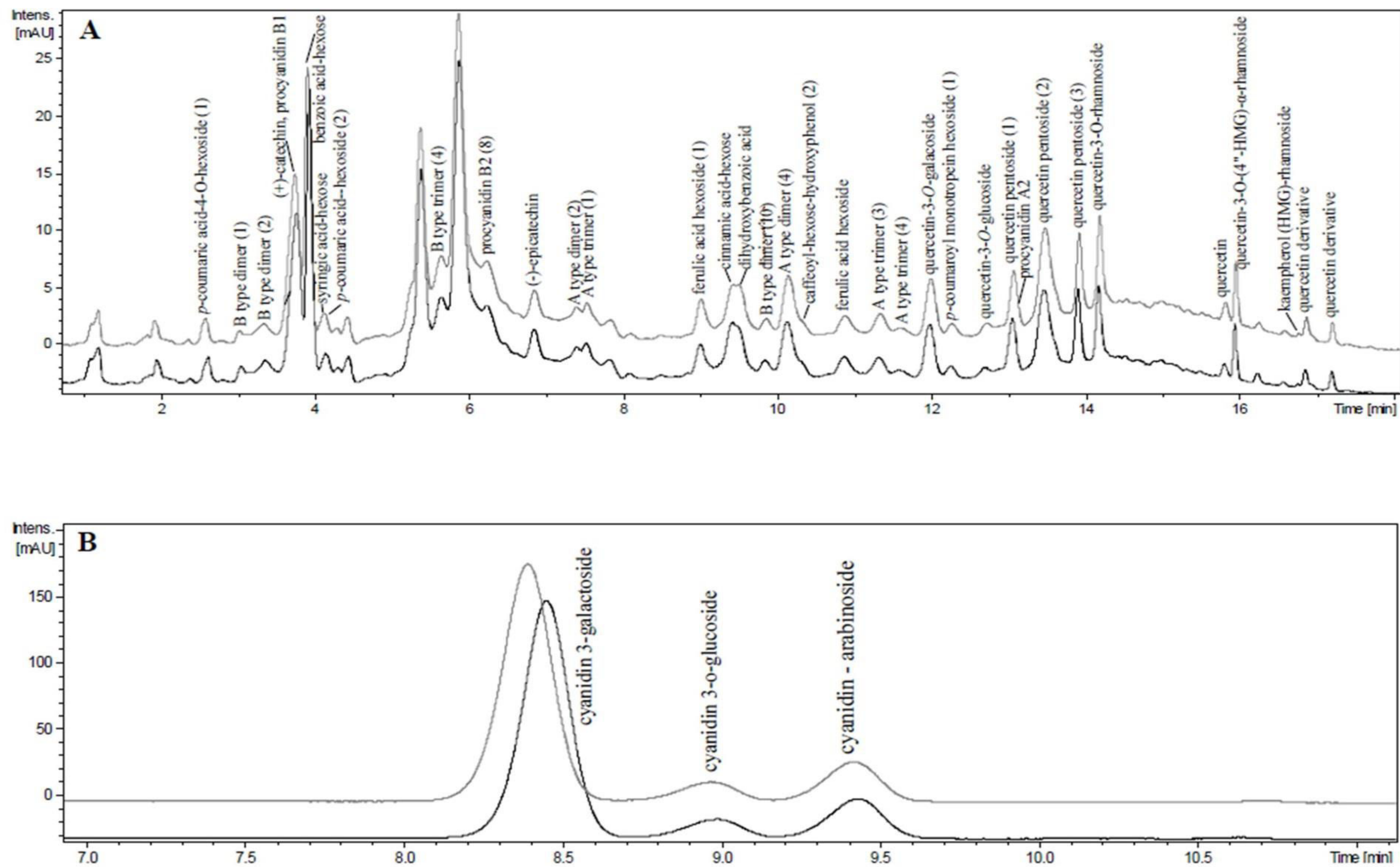


Figure II-3. Chromatographic profile at 280 nm (A) and at 520 nm (B) of aqueous (black) and ethanolic (grey) fruit extracts

3.1.1. Hydroxycinnamic acid derivatives

3.1.1.1. Caffeic acid and ferulic acid derivatives

In leaves, caffeic acid derivatives appear in considerable amounts from May to September (Table II.3), while in stems they are found in a lesser content. By contrast, caffeic acid derivatives are present in extremely low concentrations in fruits. Caffeic acid derivatives were found as unknown derivatives (*m/z* 315 at t_R 2.4 min, *m/z* 299 at t_R 3.4 min; *m/z* 431 at t_R 6.5 min;), 3-caffeoylquinic acid (t_R 2.4 min), 5-O-caffeoylquinic acid (chlorogenic acid, t_R 4.2 min), 4-O-caffeoylquinic acid (t_R 5.25 min), caffeoylquinic acid hexosides (t_R 1.4, 1.55 min), caffeoyl monotropein (t_R 11.35 min), caffeic acid-hydroxyphenol-hexose + 98 amu (t_R 1.4), caffeoylarbutins (caffeic acid-hydroxyphenol-hexose, t_R 9.5, 10.5, 13.15 min), acetylcaffeoylarbutin (t_R 15.0 min), and caffeic acid-hexose-hydroxyphenol containing one additional hexose or caffeic acid unit (t_R 10.2 min). The latter compound exhibited a parent ion at *m/z* 595 and fragment ions at *m/z* 433 (loss of hexose or caffeic acid) and *m/z* 323 (subsequent loss of hydroxyphenol) and it was detected in leaves and stems.

The two isomers of caffeoylquinic acid hexosides were identified according to their parent ion at *m/z* 515 (t_R 1.4, 1.55 min) and fragment ions at *m/z* 353 (loss of hexose) and 173 (quinic acid moiety - H_2O), and detected in stems only in trace amounts.Various caffeoylarbutins may result from the acylation by caffeic acid of different hydroxyl groups on the hexose moiety as well as the presence of glucose and galactose monosaccharides. Ek et al. (2006) found two caffeoylarbutins in lingonberry fruits. One was assessed as 2-O-caffeoylarbutin (glucose moiety) using NMR studies. Liu et al. (2014) found it also in lingonberry leaves after NMR identification.

Caffeoyl monotropein (t_R 11.35 min) with a parent ion at *m/z* 551 displayed, a fragmentation pattern in mass analogous to the one observed for bilberry leaves and fruits (Chapter I, Table I-1). Wheras several p-coumaroylmonotropeins were present in all the morphological parts, only one caffeoyl monotropein was identified, in leaves only.

Furthermore, only 4-O-caffeoylquinic acid, whose fragmentation pattern in mass is similar to that reported by Chanforan et al. (2012) in tomato, was observed in

quantifiable amount in all the morphological parts of lingonberry. In stems, caffeoyl arbutin (caffeic acid-hydroxyphenol-hexose at t_R 10.5 min) was the only other quantifiable contributor to the pool of caffeic acid derivatives. In leaves, caffeoylarbutin was quantified along with acetylcaffeoylarbutin (t_R 15.0 min).

Two ferulic acid hexosides (t_R 9.3 and 10.5 min) with a parent ion at *m/z* 355 and a typical fragment at m/z 193 were detected in fruits only, as in Mane et al. (2011) and Ek et al. (2006). Both of them could be quantified. Aditionally, a **newly identified** compound displaying a parent ion at *m/z* 369 and major fragments at *m/z* 207 (loss of hexose) and 193 (further demethylation) was assigned as **methylferulic acid hexoside (**t_R 6.3 min). It was only identified by mass spectrometry in leaves and stems and occurred in quantifiable amounts in stems.

Three sinapic acid hexoside (t_R 5.55, and 7.1 min) displaying a parent ion at *m/z* 385 and a fragment ion at *m/z* 223 (loss of hexose) were also newly identified in lingonberry stems and leaves.

3.1.1.2. Coumaric acid derivatives

Coumaric acid derivatives were present in all the morphological parts of lingonberry (Table II-1). They appear less abundant than caffeic acids derivatives in leaves while the reverse is true in stems and fruits. Coumaric acid was first found linked to hexosides through ester- or ether-type covalent bonds. Four compounds with a parent ion at *m/z* 325 and a major fragment ion at *m/z* 163 typical of *p*-coumaric acid were assigned as *p*-coumaric acid hexoside (t_R 2.6, 2.8, 4.6, and 5.1 min) while a compound with a parent ion at *m/z* 327 and a fragment ion at *m/z* 165 was assessed as dihydro-*p*-coumaric acid hexoside (t_R 4.3 min). *p*-Coumaric acid hexoside were identified in all the morphological parts when dihydro-*p*-coumaric acid hexoside only in leaves.

Next, three isomeric forms of coumaric acid-hexose-hydroxyphenol (t_R 12.95, 13.95, and 14.25 min) displayed a parent ion at *m/z* 417 and major fragments at *m/z* 307 (loss of hydroxyphenol) and *m/z* 145 coresponding to the coumaryl moiety (further loss of hexose), were identified in all the morphological parts of lingonberry as previously observed in lingonberry leaves and fruits and lingonberry leaves by Ek et al. (2006)

and Hokkanen et al. (2009), respectively. These compounds are also named *p*-coumaroylarbutins. It was proposed that the structural difference between these isomers could be attributed to the presence of glucose or galactose moieties or cis/trans isomerisation of the coumaroyl unit (Hokkanen et al., 2009).

Last, iridoid glycosides acylated by *p*-coumaric acid with parent ion at *m/z* 535 and *m/z* 537 were identified as *p*-coumaroyl monotropeins (t_R 12.55, and 13.15 min) and *p*-coumaroyl dihydromonotropein (t_R 13.7 min). The only difference beetwen these two isomers is a 2 amu (2H atoms) difference in the molecular weight and fragment ions. The three compounds were detected in all the morphological parts of lingonberry. *p*-Coumaroyl dihydromonotropein was **newly identified in lingonberry** as it was also in the different parts of bilberry (2 isomers).

3.1.2. Flavonol glycosides

Flavonol glycosides represent the second most abundant group of phenolic compounds after monomeric and oligomeric flavanols from May to September in lingonberry leaves, stems, and fruits although being relatively less predominant in the latters (Table II-1; Table II-3). Quercetin (t_R 15.75 min) was detected in stems and fruits with a fragmentation pattern similar to that reported by Mane et al. (2011). A variety of fourteen quercetin glycosides such as quercetin-3-O-galacoside (t_R 12.25 min), quercetin-3-O-glucoside (t_R 12.85 min), quercetin rutinoside (t_R 12.95 min), quercetin pentoside (t_R 13.15, 13.45, 13.8 and 13.95 min), quercetin-3-O-rhamnoside (t_R 14.05 min), quercetin-acetylhexosides (t_R 14.55 and 14.8 min), quercetin pentosylhexosides (t_R 9.3, 11.35, and 11.55 min), and quercetin-3-O-(4"-(3-hydroxy-3-methylglutaryl))-α-rhamnoside (t_R 15.95 min) were present in various morphological parts of lingonberry confirming previous studies (Ek et al., 2006; Hokkanen et al., 2009; Mane et al., 2011; Liu et al., 2014).

The compound found in leaves and stems with a parent ion at *m/z* 741 and fragment ions at *m/z* 300 and 271 was assigned as a quercetin-hexose-deoxyhexose-pentose (t_R 11.4 min). A structurally related compound, namely quercetin-3-O-(2"-O-β-apiofuranosyl-6"-O-α-rhamnopyranosyl-β-glucopyranoside) was identified earlier in tomato (Chanforan et al., 2012). A flavonol glycoside (t_R 15.1 min) was **newly**

identified in all the parts of the plant and its structure was attributed to quercetin-(3-hydroxy-3-methylglutaryl)-pentoside by analogy. Two quercetin derivatives (t_R 16.85 and 17.15 min) were also detected **for the first time** in lingonberry fruits only, based on the major fragment ion at m/z 301 typical for quercetin and the maximal absorption wavelength (λ_{max}) at 272 and 360 nm.

Additionaly, hexosides and pentosides of kaempferol were also identified in lingonberry: kaempferol hexoside (t_R 13.55 min) in leaves and **for the first time in fruits**, kaempferol rutinoside (t_R 14.05 min) in leaves and stems, kaempferol pentoside (t_R 14.9 min) in all the morphological parts of the plant, kaempferol-3-O-rhamnoside (t_R 15.1 min) in fruit (Ek et al., 2006; Hokkanen et al., 2009; Mane et al., 2011; Liu et al., 2014). Kaempferol glycosydes were less abundant compared to quercetin glycosides as it has been reported by Ek et al. (2006) and Hokkanen et al. (2009).

<u>*3.1.3. Flavanols*</u>

In stems, flavanols represented the most predominant sub-class of phenolic compounds whatever the period and the year of harvest being more abundant in July and September than in May (Tables II-1 and II-3; Figures II-2B). In total, eleven B-type dimers (t_R 3.25, 3.6, 4.0, 4.2, 5.4, 5.55, 5.65, 6.5, 7.2, 10.1 and 13.45 min), eight B-type trimers (t_R 2.2, 4.3, 5.65, 5.8, 6.65, 9.5, 9.9, and 13.7 min), five A-type dimers (t_R 6.9, 7.75, 9.9, 10.4 and 13.15 min) and four A-type trimers (t_R 8.0, 9.3, 11.55, and 11.9 min) were identified in large amounts. Their structures were assesed using mass fragmentation and retention times. Catechin (*m/z* 289; t_R 4.0 min) and epicatechin (*m/z* 289; t_R 7.1 min) were also identified in considerable amounts through running of available standards (Ek et al., 2006). Next, gallocatechin (t_R 1.75 min) was found in quantifiable amounts whereas (-)-epigallocatechin (t_R 3.9 min) was only detected in trace amounts. Their occurrence has never been reported in stems so far, only in leaves by Hokkanen et al., 2009. Additionaly, (epi)gallocatechin was detected in 3 mixed type-B dimers with (epi)catechin (*m/z* 593 at t_R 1.8, 2.4, and 3.15 min) and one type-A mixed dimer (*m/z* 591 at 9.6 min).

Finally, five cinchonain I (t_R 5.4, 11.9, 14.15, 14.25 and 14.4 min) were also detected based on their parent ion at *m/z* 451 and fragment ions at *m/z* 341, 217, 189, 177 (Hokkanen et al.,

2009). Only cinchonain I at t_R 14.25 min was quantified in stem extracts from May to September, the others being present below the limit of quantification.

In leaves, like in stems, flavanols are also largely distributed from May to September. Regarding the literature data on lingonberry leaves, three dimers of the B-type and two dimers of the A-type have been reported by Ek et al. (2006), one trimer of the A-type and two dimers the of A-type by Hokkanen et al. (2009, leaves), one trimer of the A-type and one tetramer of the B-type by Ieri et al. (2013, leaves and buds), and one dimer of the B-type, two trimers of the A-type and two dimers of the A-type by Liu et al. (2014, leaves). In this study, a great diversity of flavanol oligomers is detected in leaf extracts: nine B-type dimers (t_R 3.6, 4.0, 4.2, 5.4, 5.55, 5.65, 6.5, 7.2, and 10.1 min), five B-type trimers (t_R 2.2, 4.3, 5.65, 5.8, and 6.65 min), three A-type dimers (t_R 6.9, 7.75, and 10.4 min) and two A-type trimers (t_R 8.0 and 11.55 min). Flavanol monomers catechin (t_R 4.0 min) and epicatechin (t_R 7.1 min) were present in high amount whereas (-)-epigallocatechin (t_R 3.9 min) was only detected in trace amounts. Last three cinchonains I (t_R, 14.15, 14.25 and 14.4 min) and two additional cinchonain II (*m/z* 739; t_R 9.4 and 14.75 min) were identified in leaves of lingonberry. Among these, only two cinchonains I (t_R 14.25 and 14.4 min) could be quantified from May to September, the others being present below the limit of quantification.

In fruits, flavanols were assigned based on their mass fragmentation and on a previous work from the INRA-SQPOV Unit on lingonberry fruits (Mane et al., 2011). Seven B-type dimers (t_R 3.25, 3.6, 4.0, 6.5, 7.2, 10.1 and 13.45 min), two B-type trimers (t_R 5.8 and 9.5 min), three A-type dimers (t_R 7.75, 10.4 and 13.15 min) and four A-type trimers (t_R 8.0, 9.3, 11.55, and 11.9 min) were evidenced as well as (+)-catechin (t_R 4.0 min) and (-)-epicatechin (t_R 7.1 min).

When comparing with the results of Mane et al. (2012), three B-type dimers and trimers as well as two A-type dimers and trimers were aditionally detected in fruit extracts in this study. Cinchonains I (t_R 14.15, 14.25 and 14.4 min) were **newly identified in fruits of lingonberry** based on their major fragment ions at *m/z* 341 and 217 and common fragment ions at *m/z* 289, 231, 189, 177**.** None of them appeared in quantifiable amounts being below the limit of quantification.

3.1.4. Other compounds in lingonberry extracts

Different phenolic acids were characterized as hexosides of benzoic acid (*m/z* 283; t_R 4.3 min), dihydroxybenzoic acid (*m/z* 315 at t_R 1.6, 1.7, 2.2, 2.3 and 2.4 min) and hydroxymethoxybenzoic acid (*m/z* 329; t_R 2.05 min). The second compound was **newly identified in lingonberry stems and the third one in stems and leaves.** Dihydroxybenzoic and hydroxymethoxybenzoic acid hexosides were reported in bilberry stems (Chapter I, Table I-1). Only the benzoic acid hexoside was quantified in fruit extracts.

Further hexose acetylation was observed with dihydroxybenzoic acid acetylhexoside (t_R 6.8 min) and hydroxymethoxybenzoic acid acetylhexoside (t_R 7.75 min) which display parent ions at *m/z* 357 and 371, respectively. These two compounds were detected **for the first time in lingonberry, in leaves only**. The compound assessed as dihydroxybenzoic acid-hexose-pentose at t_R 2.8 min were also identified in stems only.

Last, hydroxymethoxyphenylacetic acid hexoside (t_R 4.95 min), dihydroxyphenylacetic acid hexoside (t_R 5.55 min) and hydroxymethoxyphenylpropionic acid hexoside (t_R 5.55 min) were **newly identified** in lingonberry leaves and stems. The presense of the compounds at t_R 5.55 min has already been observed in tomato products by Chanforan et al. (2012). None of these compounds could be quantified. In stems and fruits, a dihydroxybenzoic acid derivative displaying a parent ion at *m/z* 319 and a fragment ion at *m/z* 153 was detected at t_R 9.9 min in agreement with the earlier reporting by Mane et al. (2011). It was quantified only in fruits.

Furthermore, a dihydrochalcone (t_R 14.95 min), displaying a parent ion at *m/z* 435 and a major fragment ion at *m/z* 273 (loss of hexose), was assigned as phloretin hexoside. This **newly identified compound in lingonberry fruit** was largely present in apple (Gobert et al., 2014).

Last, monotropein (*m/z* 389; t_R 1.8 min), whose fragmentation pattern in mass is analogous to the one observed for bilberry stems (Chapter I, Table I-1), was also identified in stems of lingonberry only. The precursor of caffeoyl- and *p*-coumaroylmonotropeins was previously identified in lingonberry juice by Jensen et al. (2002).

Table II-1. Phenolic compounds identified by UPLC/ESI-MS in leaf, stem and fruit extracts of lingonberry.

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.	Literature
1	1.4	278	531	433, 323, 263, 221, 179, 161	**caffeoyl-hexose- hydroxyphenol + 98 amu**	L[a]	L	–	L	L	L	
2			515	479, 353, 341, 173	**caffeic acid-quinic acid-hexoside (1)**	S	S	–	S[a]	S[a]	S	
3	1.55		515	353, 173	**caffeic acid-quinic acid-hexoside (2)**	S	S	S	S[a]	S[a]	S	
4	1.6	314	315	153, 109	**dihydroxybenzoic acid hexoside (1)**	S	S	S	S[a]	S	S	Bilberry study
5	1.7	314	315	153	**dihydroxybenzoic acid hexoside (2)**	S	S	S	S	S	S	
6	1.75	270	305	261, 219, 179, 125	(+)-gallocatechin	S	S	S	S	S	S	Hokkanen et al. (2009)
7	1.8	275	389	227, 209, 183, 165, 139	**monotropein**	S	S	–	S	S	S	Jansen et al. (2002) Bilberry study
8			593	575, 549, 455, 409, 345	**(epi)gallocatechin-(epi)catechin dimer (1)**	S	–	–	S[a]	–	S[a]	
9	2.05	254, 286	329	167, 152, 123, 108	**hydroxymethoxybenzoic acid hexoside**	S	L,S	L	L, S[a]	L, S	L, S	Bilberry study
10	2.2	278	345	299, 161, 137	**unknown**	–	–	L[a]	L, S[a]	S[a]	L, S[a]	
11			865	739, 713, 695, 575, 543, 451, 363, 287	B-type trimer (1)	S	S	L, S	L[a], S	L[a], S	L[a], S	
12			315	153	**dihydroxybenzoic acid hexoside (3)**	S	S	–	S[a]	S	S	
13			453	409, 273, 247, 229, 193	**unknown**	–	–	S	S[a]	S[a]	S[a]	
14	2.3	278	315	153, 123	**dihydroxybenzoic acid hexoside (4)**	S	S	S	S	S	S	
15			593	575, 549, 467, 441, 423, 305, 287, 219	**(epi)gallocatechin-(epi)catechin dimer (2)**	S	S	S	S	S[a]	S	Bilberry study

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013			2014			Literature
						May	July	Sept.	May	July	Sept.	
16	2.4		315	153	**dihydroxybenzoic acid hexoside (5)**	S	S	S	S	S	S^a	
17			613	451, 289	**dihexoside of aglycone MW 290 (1) or cinchonain I hexoside**	L, S	L, S	L, S	L^a, S	–	L, S	
18			353	315, 191 > 179, 135	3-caffeoylquinic acid	–	–	L	L	L	–	
19			315	179, 135	**caffeic acid derivative**	–	L^a	–	L	L	L	
20	2.5	278	451	361, 331, 289	**hexoside of aglycone MW 290 (1)**	S	S	S	S	S	S	
21	2.6	286, 310sh	325	163, 119	*p*-coumaric acid-4-O-hexoside (1)		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		Bilberry study Mane et al. (2011)
22	2.65	278	451	289, 245, 161	**(epi)catechin derivative (1)**	S	L, S	L, S	L, S	L	L, S	Bilberry study
23	2.8	278	447	403, 315, 271, 153	**dihydroxybenzoic acid-hexose - pentose**	S	S	S	S	–	–	Bilberry study
24		284, 312sh	325	163, 119	*p*-coumaric acid-4-O-hexoside (2)	L^a	L^a	L^a	L	L	L	Bilberry study
25			593	575, 549, 467, 441, 423, 305, 287	**(epi)gallocatechin-(epi)catechin dimer (3)**	S	S	S	S	S	S	
26	3.25	276	577	559, 533, 493, 425, 393, 269, 241	B-type dimer (1)	S	S, F_{H_2O}, F_{EtOH}	S	S^a	S, F_{H_2O}, F_{EtOH}	S	
27			451	361, 331, 289, 245	**hexoside of aglycone MW 290 (2)**	S	S	–	S^a	S^a	S^a	
28	3.4	278	299	179, 161, 143, 119	**caffeic acid derivative**	L	L	L	L	L	L	
29	3.6	278	577	451, 425, 407, 289	B-type dimer (2)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	
30	3.9	271	305	261, 219, 179, 165, 137, 125	(–)-epigallocatechin (std)	L, S	L,S	S	L, S	L^a, S	L, S	Bilberry study Hokkanen et al. (2009)
31	4.0	278	289	245, 205, 179	(+)-catechin (std)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Mane et al. (2011)
32			577	451, 425, 407, 289, 245	procyanidin B1 (3) (std)	L, S	L, S, F_{H_2O}, F_{EtOH}	L,S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Mane et al. (2011)
33	4.2	320, 280sh	353	191	5-O-caffeoylquinic acid	L, S	L, S, F_{H_2O}, F_{EtOH}	L	L, S	L, S^a, F_{H_2O}, $F_{EtOH}{}^a$	L, S^a	

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013			2014			Literature
						May	July	Sept.	May	July	Sept.	
34			577	451, 425, 407, 289, 245	B-type dimer (4)	S	S	L, S	L[a], S	L[a], S	L, S	
35	4.3	278	865	847, 739, 713, 695, 619, 577, 543, 451, 407, 363, 287,	B-type trimer (2)	L, S	L, S	L, S	L, S	L, S	L, S	
36			305	287, 243, 195, 161 ,125	(epi)gallocatechin	S	S	S	S[a]	–	–	
37			451	421, 361, 331, 289, 245, 203	**hexoside of aglycone MW 290 (3)**	L,S	S	L, S	L[a], S[a]	L[a], S[a]	L, S[a]	
38			327	267, 203, 165, 123	**dihydro-*p*-coumaric acid hexoside**	–	–	L	L[a]	L[a]	L[a]	
39		274	283	121	**benzoic acid hexoside**		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		
40	4.6	284, 313	325	187, 163, 145, 119	*p*-coumaric acid hexoside (3)	L, S[a]	L, S[a], F_{H_2O}, F_{EtOH}	S[a]	L[a], S	L, S, F_{H_2O}, F_{EtOH}	L, S	Bilberry study
41			359	197	**syringic acid hexoside**		F_{H_2O}, F_{EtOH}			F_{H_2O}[a], F_{EtOH}		Chanforan et al. (2012) Bilberry study
42			613	451, 289	**dihexoside of aglycone MW 290 (2) or cinchonain I hexoside**	S	–	S	S[a]	–	S[a]	
43	4.95	275	343	223, 203, 181, 139, 124, 101	**hydroxymethoxyphenylacetic acid - hexose**	L	–	L	L	L[a]	L	
44	5.1	278, 306sh	325	187, 163, 145, 119	*p*-coumaric acid hexoside (4)		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		
45	5.25	300sh, 325	353	191< 179 < 173, 135	4-O-caffeoylquinic acid	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}[a]	L, S	Chanforan et al. (2012) Mane et al. (2011)
46	5.4	276	577	559, 451, 425, 407, 289, 245	B-type dimer (5)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Bilberry study
47			337	319, 173	4-*p*-coumaroyl quinic acid	L	L	L	L[a]	–	–	
48			451	289	(epi)catechin derivative (5) or cinchonain I (1)	S	S	S	S	–	S	Bilberry study
49	5.55	280	385	223	**sinapic acid hexoside (1)**	L, S	L, S	L, S	L, S	L, S	L, S	Bilberry study
50			577	559, 451, 425,	B-type dimer (6)	L, S	L, S	L, S	L, S	L, S	L, S	

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013			2014			Literature
						May	July	Sept.	May	July	Sept.	
				407, 289, 245								
51			329	269, 221, 209, <u>191</u>, 167	**dihydroxyphenylacetic acid - hexoside**	S	–	–	S[a]	S[a]	S[a]	Chanforan et al. (2012)
52			357	<u>195</u>, 177, 163, 136	**hydroxymethoxyphenylpropionic acid hexoside**	S	–	–	S	S	S[a]	Chanforan et al. (2012)
53	5.65	278	865	847, 737, 713, 695, 577, 575, 543, 451, 407, 363, 287, 245	B-type trimer (3)	L, S	L, S	L, S	L[a], S[a]	L[a], S[a]	L[a], S[a]	
54			577	559, 451, 425, 407, 289	B-type dimer (7)	–	L	L	L[a], S	L[a]	–	
55	5.8		865	847, 739, 713, 695, 577, 575, 543, 451, 363, 287	B-type trimer (4)	L, S	L, S, F_{H_2O}[a]	S	L[a], S	L[a], S	L[a], S	
56	6.3	300sh, 344	369	207, 193	**methylferulic acid hexoside (?)**	L, S	L, S	L, S	L, S	L, S	L, S	
57	6.5	275	577	559, 451, 425, 407, 289, 245	procyanidin B2 (8) (std)	L, S	L, S, F_{H_2O}[a], F_{EtOH}[a]	L, S	L[a], S	L[a], S, F_{H_2O}[a], F_{EtOH}[a]	L, S	
58			431	179, 161	**caffeic acid derivative**	–	L	–	–	–	L	
59	6.65	278	865	847, 739, 713, 695, 677, 619, 575, 451, 425, 407, 287	B-type trimer (5)	L, S	S	S	L[a], S[a]	L[a], S[a]	L[a], S[a]	
60	6.8	280	357	315, <u>153</u>	**dihydroxybenzoic acid - acetylhexose**	–	L	L	L	L	L	
61	6.9	276	575	559, 499, 451, 411, 331, <u>289</u>, 245, 205	A-type dimer (1)	L, S	L, S	L, S	L[a], S	L[a], S[a]	L[a], S	
62	7.1		385	<u>223</u>, 179	**sinapic acid hexoside (2)**	L	L	L	L, S	L, S	L	
63		278	289	245, 205, 179	(–)-epicatechin (std)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L[a], S	L[a], S, F_{H_2O}, F_{EtOH}	L, S	Mane et al. (2011)
64	7.2	278	577	559, 451, 425, 407, 289	B-type dimer (9)	L, S	L, F_{H_2O}[a], F_{EtOH}[a]	L	L[a], S[a]	L, S, F_{H_2O}[a], F_{EtOH}[a]	L, S	

No.	t_R (min)	λ_{max} (nm)	$[M - H]^-$ *(m/z)*	MS^2 fragments *(m/z)*	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.	Literature
65	7.75	276	575	451, 411, 289, 245	A-type dimer (2)	L, S	L, S, F_{H_2O}[a], F_{EtOH}	L, S	L[a], S	L[a], S, F_{H_2O}[a], F_{EtOH}[a]	L, S	
66			371	354, 209, 167, 152	**hydroxymethoxybenzoic acid acetylhexoside**	–	–	L	–	–	L	
67	8	278	863	711, 693, 573, 559, 531, 451, 411, 289, 285	A-type trimer (1)	L, S	L, S, F_{H_2O}[a], F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	
68	9.3		355	193	ferulic acid hexoside (1)		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		Mane et al. (2011)
69			595	463, 433, 301	**quercetin pentosylhexoside (1)**	S	S	S	S[s]	S[s]	S[s]	Bilberry study
70			863	711, 573, 531, 451, 411, 289	A-type trimer (2)	S	S, F_{H_2O}[a], F_{EtOH}[a]	S	S	S, F_{H_2O}[a], F_{EtOH}[a]	S	Bilberry study
71	9.4		739	721, 629, 587, 569, 435, 417, 339, 289	cinchonain II (1)	–	L	L	L[a]	L[a]	L[a]	Hokkanen et al. (2009)
72	9.5		433	387, 323, 179, 161, 133	caffeoylarbutin or caffeic acid-hydroxyphenol-hexose (1)	L	–	–	L	–	L	Ek et al. (2006) Mane et al. (2011) Hokkanen et al. (2009) Ieri et al. (2013) Liu et al. (2014)
73		280	865	847, 739, 713, 695, 661, 613, 577, 543, 451, 425, 339, 287	B-type trimer (6)	S	S, F_{H_2O}[a], F_{EtOH}[a]	S	S[a]	S[a], F_{H_2O}[a], F_{EtOH}[a]	S[a]	
74	9.5	280	309	147	cinnamic acid-hexose		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		
75	9.6	282	591	553, 465, 423, 305, 285, 245	A-type (epi)gallocatechin-(epi)catechin dimer	S	–	S	S	S	S	
76	9.9	278	865	847, 821, 739, 713, 695, 651, 619, 577, 543, 449,407, 287	B-type trimer (7)	S	S	S	S[a]	S[a]	S[a]	
77			575	557, 529, 499, 449, 411, 289	A-type dimer (3)	S	S	S	S[a]	S[a]	S[a]	

No.	t_R (min)	λ_{max} (nm)	[M – H]⁻ (*m/z*)	MS² fragments (*m/z*)	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.	Literature
78		276, 304sh	319	183, 165, 153, 109	dihydroxybenzoic acid derivative	S	S, F_{H_2O}, F_{EtOH}	S	S	S, F_{H_2O}, F_{EtOH}	S^a	Mane et al. (2011)
79	10.1	278	577	559, 451, 425, 407, 289	B-type dimer (10)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Bilberry study
80	10.2		595	433, 323	caffeic acid-hexose-hydroxyphenol – hexose or caffeic acid (dicaffeoylarbutin favored)	S	L, S	S	L, S	L, S	L, S	
81	10.4	278	575	557, 539, 449, 407, 327, 289, 245	A-type dimer (4)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	
82	10.5	298sh, 326	433	323, 161,	**caffeoylarbutin or caffeic acid-hydroxyphenol-hexose (2)**	L, S	S, F_{H_2O}, F_{EtOH}	L	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Ek et al. (2006) Mane et al. (2011) Hokkanen et al. (2009) Ieri et al. (2013) Liu et al. (2014)
83			355	193	ferulic acid hexoside (2)		F_{H_2O}, F_{EtOH}			$F_{H_2O}^a$, F_{EtOH}^a		Mane et al, 2011
84	11.35		551	507, 389, 345, 327, 283, 179	caffeoyl monotropein	L	–	–	L^a	L	L^a	Bilberry study
85			865	847, 821, 739, 713, 695, 651, 619, 577, 543, 449,407, 287	B-type trimer (7)	S	S	S	S^a	S^a	S^a	Bilberry study
86			595	445, 343, 300, 271	**quercetin pentosyl hexoside (2)**	L, S	L, S	L, S	L, S	L, S	L^a, S	Bilberry study
87	11.4		741	723, 609, 591, 475, 409, 343, 300, 271	**quercetin-hexose-deoxyhexose-pentose**	S	S	L, S	L^a	S^a	L^a, S^a	Chanforan et al. (2012)
88	11.55		595	445, 343, 300, 271, 179	**quercetin pentosyl hexoside (3)**	L, S	L, S	L, S	L, S	L, S	L, S	
89		278	863	737, 711, 693, 617, 575, 541, 449	A-type trimer (3)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	
90	11.9	278	863	845, 737, 711,	A-type trimer (4)	S	S, $F_{H_2O}^a$,	S	S	S, F_{H_2O},	S	

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.	Literature
				575, 559			F_{EtOH}[a]			F_{EtOH}		
91			451	341	cinchonain I (2)	S	S	S	S	S	S	Bilberry study
92	12.25	254, 354	463	301	quercetin-3-O-galactoside (std)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Mane et al. (2011) Harris et al. (2007) Ek et al. (2006)
93	12.55	278sh, 306	535	491, <u>371</u>, <u>329</u>, 311, 267, 191, <u>163</u>	*p*-coumaroyl monotropein (1)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Hokkanen et al. (2009) Bilberry study
94	12.85	254, 354	463	301	quercetin-3-O-glucoside (std)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Mane et al. (2011) Ek et al. (2006)
95	12.95	254, 352	609	343, <u>301</u>, 271	quercetin 3-O-rutinoside	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}[a], F_{EtOH}[a]	L, S	Ieri et al. (2013) Liu et al. (2014)
96			417	<u>307</u>, 145	coumaric acid-hexose –hydroxyphenol or coumaroylarbutin (1)	L, S[a]	L	L, S	L, S[a]	L[a], S	L, S	Ek et al. (2006) Hokkanen et al. (2009)
97	13.15		433	323, <u>301</u>, 179, 161	caffeic acid-hexose-hydroxyphenol or caffeoylarbutin (3)	L	L	L	L	L	L	Ek et al. (2006)
98			575	539, <u>449</u>, <u>423</u>, 327, 289	procyanidin A2 (6) (std)	S	S, F_{H_2O}, F_{EtOH}	S	S	S, F_{H_2O}, F_{EtOH}	S	
99			535	491, <u>371</u>, 329, 311, 267, 209, 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, S[a]	Bilberry study
100		254, 354	433	301	quercetin pentoside (1)	S	S, F_{H_2O}, F_{EtOH}	S	S	S, F_{H_2O}, F_{EtOH}	S	Mane et al. (2011) Ek et al. (2006) Bilberry study
101	13.45	254, 354	433	301	quercetin pentoside (2)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	
102			577	559, 451, 425, 407, 289	B-type dimer (11)	S	S, F_{H_2O}, F_{EtOH}	S	S	S, F_{H_2O}, F_{EtOH}	S	
103	13.55		447	327, <u>284</u>, 255, 151	**kaemferol hexoside**	–	L, **F_{H_2O}**, **F_{EtOH}**	L	–	L, **F_{H_2O}[a]**, **F_{EtOH}[a]**	L	Hokkanen et al. (2009)
104	13.7	280sh, 310	537	493, <u>373</u>, <u>331</u>, 313, 243, 193,	***p*-coumaroyl dihydromonotropein**	L, S	L, S, F_{H_2O}[a], F_{EtOH}[a]	L, S	L, S	L, S, F_{H_2O}[a], F_{EtOH}[a]	L, S	Bilberry study

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.	Literature
				163								
105			865	739, 713, 695, 650, 575, 449, 423, 287, 259	B-type trimer (8)	S[a]	S	S	S[a]	S[a]	S[a]	Bilberry study
106	13.8	256, 352	433	301	**quercetin pentoside (3)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Bilberry study
107	13.95		433	301	**quercetin pentoside (4)**	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Bilberry study
108			417	307, 145	**coumaric acid-hexose – hydroxyphenol or coumaroylarbutin (2)**	L	–	–	L[a]	L	L	
109	14.05	256, 354	447	301	quercetin-3-O-rhamnoside (std)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Ek et al. (2006)
110			593	285, 257	kaempferol rutinoside	L, S	L, S	L, S	L, S[a]	L, S[a]	L, S[a]	Hokkanen et al. (2009)
111	14.15	278	451	341, 299, 231, 217, 189, 177	cinchonain I (3)	L	S, F_{H_2O}, F_{EtOH}	–	L	L, F_{H_2O}, F_{EtOH}	L	Hokkanen et al. (2009)
112	14.25	278	451	341, 289, 231, 217, 189, 177	cinchonain I (4)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	
113			417	307, 145	coumaric acid-hexose-hydroxyphenol or coumaroylarbutin (3)	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S[a]	L, S, F_{H_2O}, F_{EtOH}	L, S	
114	14.4	278	451	343, 289, 231, 217, 189, 177	cinchonain I (5)	L	L, F_{H_2O}, F_{EtOH}	L	L	L, S, F_{H_2O}, F_{EtOH}	L, S	Hokkanen et al. (2009)
115	14.55		505	463, 301	**quercetin acetylhexoside (1)**	L, S	S	S	L, S	L, S	L, S	
116	14.75	282	739	722, 695, 629, 587, 569, 449, 339, 289	cinchonain II (2)	L	–	–	–	–	L[a]	
117	14.8	267	505	463, 343, 301, 271	**quercetin acetylhexoside (2)**	L, S	S, F_{H_2O}, F_{EtOH}	S	L, S	L, S,	L, S	
118	14.9		417	372, 285, 255	kaempferol pentoside	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Hokkanen et al. (2009) Ek et al. (2006) Liu et al. (2014)
119	14.95		435	273, 167	**phloretin hexoside**		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		Gobert et al. (2014) Bilberry study

No.	t_R (min)	λ_{max} (nm)	$[M-H]^-$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013			2014			Literature
						May	July	Sept.	May	July	Sept.	
120	15.0	282, 328	475	365, 203, 179, 161, 135	caffeic acid-acetic acid-hexose-hydroxyphenol or acetylcaffeoylarbutin	L, S	L, S	L	L, S	L, S	L, S	Ieri et al. (2012)
121	15.1	254, 356	577	515, 475, 433, 301	**quercetin (HMG)-pentoside**[b]	L, S	L, S, F_{H_2O}, F_{EtOH}[a]	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}[a]	L, S	
122		277	431	285, 255	kaempferol-3-O-rhamnoside		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		Mane et al. (2011)
123	15.9	254, 360	301	179, 151	quercetin	S	S, F_{H_2O}, F_{EtOH}	S	S	S, F_{H_2O}, F_{EtOH}	S	Hokkanen et al. (2009) Mane et al. (2011)
124	15.95	254, 346	591	529, 489, 447, 301	quercetin-3-O-(4"-HMG)-α-rhamnoside[b]	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	Hokkanen et al. (2009) Ek et al. (2006) Mane et al. (2011) Bilberry study
125	16.8		575	513, 473, 431, 285	kaemferol-3-O-(4”-HMG)- α rhamnoside[b]	L, S	L, S, F_{H_2O}, F_{EtOH}	L, S	L	L, F_{H_2O}, F_{EtOH}	L	Hokkanen et al. (2009) Ek et al. (2006) Mane et al. (2011)
126	16.85	272, 3560	567	301	**quercetin derivative**		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		
127	17.15		567	301	**quercetin derivative**		F_{H_2O}, F_{EtOH}			F_{H_2O}, F_{EtOH}		

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 standard; [a] not fragmented; [b]HMG = 3-hydroxy-3-methylglutaryl. Compounds in bold are newly described or identified.

3.1.5. Anthocyanins

The three major anthocyanins detected and quantified in both the aqueous and ethanolic fruit extracts (Table II-2) are: cyanidin-3-O-galactoside (t_R 8.4 min), cyanidin-3-O-glucoside (t_R 9.0 min), and cyanidin-3-O-arabinoside (t_R 9.4 min). Cyanidin-3-O-galactoside was confirmed to be the most abundant anthocyanin in lingonberry fruits as described previously by Mane et al. (2011) and Lee & Finn (2012).

Table II-2. Anthocyanins identified by UPLC/ESI-MS^n in fruits extracts of lingonberry

t_R (min)	λ_{max} (nm)	$[M – H]^+$ *(m/z)*	MS^2 fragments *(m/z)*	Proposed structure[a]	2013	2014
8.4	278, 516	449	287	cyanidin-3-*O*-galactoside (std)	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.0	278, 512	449	287	cyanidin-3-*O*-glucoside	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.4	280, 514	419	287	cyanidin-3-*O*-arabinoside	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}

F_{H_2O}: aqueous fruit extract; F_{EtOH}: ethanolic fruits extract; [std] compounds were identified by comparison with standard; [a] identified according to Mane et al. (2011).

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

Qualitative analyses revealed similar phenolic profiles for lingonberry leaves and stems harvested at the three different periods during the years 2013 and 2014. The quantitative results showed the predominant presence of flavanols monomers and oligomers followed by quercetin glycosides, then caffeic acid derivatives.

The Sum of Phenolic Compounds (UPLC method) was significantly affected by the factor year (2013 vs 2014) except for leaves from July and fruits (Table II-3). As matter of fact, more phenolic compounds are present in the stem and leaf extracts from year 2014. Similar annual variations are further reported for most of the sub-classes in stems and leaves. By contrast, the composition of the lingonberry fruit extract appears highy stable between years 2013 and 2014.

When considering the influence of the vegetation periods on leaves, one can see that the Sum of Phenolic Compounds increases slightly from May to September 2013 while markedly from May to September 2014. At the sub-class levels, this increase is true for caffeic acid derivatives and flavanol monomers in 2013 and caffeic acid derivatives, coumaric acid derivatives and flavanol monomers and oligomers in 2014. Only flavonol glycosides may be present in a lower content in leaves from 2014.

As far as lingonberry stems are concerned, the sum of phenolic acids increases more strongly from July to September in 2014 compared to 2013 as just observed for leaves. At the sub-class levels, this increase in content is true for caffeic acid derivatives and flavanol monomers and oligomers in 2013 and 2014. Only flavonol glycosides may tend to decrease in stems from years 2013 and 2014.

In leaves, flavanols oligomers were found to be the most representative sub-class of phenolic compounds with relative levels ranging between 36% (May 2014) to 48% (July 2013) of the total phenolic content (w/w of DE) (Table II-3). Flavonol glycosides appeared as the second most abundant group of phenolic compounds (Liu et al., 2014) followed by flavanol monomers. Caffeic acid derivatives were also found in significant quantifiable amounts from July to September. Caffeoylarbutin (caffeic acid-hexose-

hydroxyphenol, t_R 10.5 min) was found as the main compound of caffeic acid derivatives with relative levels ranging between 63% (May 2014) to 77% (September 2013).

As to the major contributor of the phenolic pool, three compounds could be outlined: catechin in May 2013 (12.3%), September 2013 (13.7%), July 2014 (10.6%) and September 2014 (13.3%) or an A-type trimer (t_R 7.75 min) in July 2013 (13.5%) or quercetin-3-O-galactoside (t_R 12.25 min) in May 2014 (11.8%) (Table II-4). Ranking second were the same A-type trimer (t_R 7.75 min) in May 2013 (11.6%), September 2013 (11.0%), catechin in July 2013 (10.9%), May 2014 (11.3%), quercetin-3-O-galactoside (t_R 12.25 min) in July 2014 (10.1%) or caffeoylarbutin (t_R 10.5 min) in September 2014 (11.4%). Ranking third were a B-type trimer (t_R 4.3 min) in May 2013 (8.8%) or caffeoylarbutin (t_R 10.5 min) in July 2013 (9.0%), July 2014 (9.3%) and September 2013 (8.5%) or an A-type trimer (t_R 7.75 min) in May 2014 (8.9%) and September 2014 (8.2%). In contrast, Liu et al. (2014) and Ieri et al. (2013) reported that arbutins (hexose-hydroxyphenol derivatives) as the most abundant phenolic compound in lingonberry leaves and lingonberry buds and leaves, respectively. The first group did not quantify flavanols while the second group largely underestimated flavanols by reporting only a couple of oligomeric flavanols. Liu et al. (2014) also observed flavonols as the second class of phenolic compounds whereas Ieri et al. (2013) found them in small amounts. Moreover, small variabilities in the content of phenolic compounds may also be due to the different origins of the plant material, the various methods of collection, extraction and storage adopted in each study (Ieri et al. 2013).

In stems, like in leaves, flavanol oligomers were the more abundant class of the of the total weight of phenolic compounds (47% in May 2014 to 50% in July 2013 and 2014 and September 2014) followed by flavonol glycosides (19% in September 2014 to 26% in May 2014) and flavanol monomers (18% in July 2014 and July 2014 to 26% in September 2013) (Table II-3). Both coumaric and caffeic acid derivatives were present in lower concentrations in stem extracts from May to September (Table II-3).

The two most abundant compounds in leaf extracts were catechin in July 2013 (13.9%), July 2014 (10.7%) and September 2014 (13.7%) as well as quercetin-3-O-galactoside (t_R 12.25 min) in May 2013 (10.9%), May 2014 (11.4%) and July 2014 (10.8%) (Table II-4). Ranking second were: catechin in May 2013 (10.5%), May 2014 (9.3%) and July 2014 (10.7%) or quercetin-3-O-galactoside (t_R 12.25 min) in July 2013 (9.1%) or

epicatechin (t_R 7.1 min) in September 2013 (11.0%) and 2014 (9.5%). Ranking third were: an A-type trimer (t_R 7.75 min) in May 2013 (8.3%) and July 2013 (8.6%) or quercetin-3-O-galactoside (t_R 12.25 min) in September 2013 (8.2%) and 2014 (7.8%) or epicatechin (t_R 7.1 min) in May 2013 (7.7%) or an A-type dimer (t_R 10.4 min) in July 2014 (6.9%).

In fruits, the Sum of Phenolic Compounds is 5- to 9-fold less compared to the one in leaves and stems and this could be attributed to the high concentration in sugar of the fruits**.** The monomeric and oligomeric flavanols (34 to 42%) were present as the major phenolic compounds in both fruit extracts (Kylli et al., 2011) while anthocyanins (25 to 29%), benzoic acid derivatives (18 to 28%) and flavanol glycosides (8 to 10%) constituted the second, thirds and fourth classes of phenolic compounds, respectively. Coumaric and caffeic acid derivatives were detected in small amounts.

Table II-3. Phenolic composition in lingonberry leaves, stems and fruits at three different periods of vegetation and for two different years.

Extract /Period of vegetation	Benzoic acid derivatives (mg/g DE)[a]	Caffeic acid derivatives (mg/g DE)	Coumaric acid derivatives (mg/g DE)	Flavonol glycosides (mg/g DE)	Flavanol monomers[b] (mg/g DE)	Flavanol oligomers[c] (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	–	5.64 ± 0.35(A)	1.41 ± 0.26(A)	14.5 ± 0.9(A)	9.64 ± 0.43(A)	24.2 ± 1.4(A)	–	55.3 ± 1.1(A)	–	96.8 ± 4.1(A)
May 2014	–	**6.48 ± 0.11(a)***	**1.70 ± 0.37(a)**	**21.5 ± 1.2(a)***	8.78 ± 0.28(a)*	22.0 ± 0.9(a)	–	**60.4 ± 1.7(a)***	**135.1 ± 1.3(a)**	**85.3 ± 0.8**(a)*
July 2013	–	8.03 ± 0.46(B)	1.32 ± 0.27(A)	14.5 ± 0.2(A)	9.35 ± 0.72(A)	31.3 ± 3.8(A)	–	64.5 ± 2.6(A,B)	–	93.1 ± 4.2(A)
July 2014	–	**9.30 ± 0.01(b)***	**2.78 ± 0.13(b)***	**21.2 ± 0.2(a)***	**8.64 ± 0.15(a)**	**24.3 ± 1.6(a)***	–	**66.2 ± 1.5 (b)**	**148.5 ± 8.6(a,b)**	**96.3 ± 5.6**(b)
September 2013	–	7.86 ± 0.18(B)	1.25 ± 0.18(A)	13.4 ± 1.1(A)	12.9 ± 0.3(B)	31.0 ± 5.1(A)	–	66.5 ± 6.1(B)	–	114.6 ± 21.1(A)
September 2014	–	**15.8 ± 0.1(c)***	**3.10 ± 0.18(b)***	**18.5 ± 0.1(b)***	**15.5 ± 0.4(b)***	**41.3 ± 1.6(b)***	–	**94.2 ± 1.7(c)***	**158.9 ± 6.0(b)**	**99.8 ± 3.7**(b)
Stems										
May 2013	–	4.42 ± 0.10(A)	1.59 ± 0.05(A)	22.4 ± 1.2(A)	17.7 ± 0.4(A)	43.7 ± 1.1(A)	–	89.8 ± 1.6(A)	–	80.5 ± 5.5(A)
May 2014	–	**5.77 ± 0.08(a)***	**1.95 ± 0.03(a)***	**27.6 ± 0.2(a)***	**19.9 ± 0.2(a)***	**49.9 ± 1.4(a)***	–	**105.1 ± 1.1(a)***	**131.7 ± 4.4 (a)**	**72.3 ± 2.4**(a,b)
July 2013	–	4.66 ± 0.05(B)	1.49 ± 0.07(A)	19.6 ± 2.4(A)	20.8 ± 1.1(B)	47.3 ± 1.2(B)	–	93.9 ± 2.5(A,B)	–	79.1 ± 4.7(A)
July 2014	–	**5.76 ± 0.04(a)***	**2.48 ± 0.05(b)***	**26.9 ± 0.3(b)***	**20.6 ± 0.3(b)**	**56.3 ± 2.6(b)***	–	**112.0 ± 3.1(b)***	**142.2 ± 6.4 (a,b)**	**70.5 ± 3.2**(b)
September 2013	–	5.03 ± 0.04(C)	1.28 ± 0.08(B)	19.5 ± 1.5(A)	25.4 ± 1.6(C)	47.3 ± 0.4(B)	–	98.4 ± 3.3(B)	–	82.3 ± 11.8(A)
September 2014	–	**6.00 ± 0.02(b)***	**1.99 ± 0.29(a)***	**25.3 ± 0.2(c)***	**32.2 ± 0.2(c)***	**65.0 ± 1.0(c)***	–	**130.5 ± 1.8(c)***	**147.7 ± 2.5 (b)**	**77.6 ± 1.3**(a)
Fruits with H_2O										
July 2013	2.00 ± 0.21	0.23 ± 0.07	0.12 ± 0.02	1.01 ± 0.04	1.72 ± 0.16	2.83 ± 0.78	2.97 ± 0.63	10.9 ± 1.3	–	13.5 ± 0.9
July 2014	**2.79 ± 0.17***	**0.26 ± 0.12**	**0.13 ± 0.02**	**0.99 ± 0.05**	**1.29 ± 0.07***	**2.83** ± 0.69	**3.44 ± 0.75**	**12.1 ± 1.0**	**13.1 ± 0.6**	**13.3 ± 0.6**
Fruits with EtOH 55%										
July 2013	2.09 ± 0.31	0.33 ± 0.12	0.14 ± 0.02	1.24 ± 0.18	1.68 ± 0.34	2.93 ± 0.69	3.50 ± 0.84	11.9 ± 1.9	–	17.2 ± 0.8
July 2014	**3.79 ± 0.95***	**0.25 ± 0.05**	**0.08 ± 0.01***	**1.25 ± 0.20**	**1.79 ± 0.34**	**3.11 ± 0.48**	**3.40 ± 0.49**	**13.7 ± 2.3**	**19.4 ± 0.3**	**15.4 ± 0.3***

Values represented mean ± SD (n = 3). Sum of Phenolic Compounds is obtained by 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]Benzoic acid derivatives contain benzoic acid hexoside (t_R 4.3 min) and dihydroxybenzoic acid derivative (t_R 9.9 min). [b]Flavanol monomers contain catechin, epicatechin and cinchonains I in leaves, gallocatechin, catechin, epicatechin and cinchonains I in stems, only catechin and epicatechin in fruits. [c]Flavanol oligomers contain B-type and A-type oligomers in all morphological parts.

Table II-4. Relative content of major phenolic compounds in lingonberry leaves and stems at three different periods of vegetation and for two different years.

Morphological part extracts	Major phenolic compounds	Relative content (%)[a]					
		2013			2014		
		May	*July*	*Sept.*	*May*	*July*	*Sept.*
Leaf extracts	B-type dimer (2) (3.6 min)	6.4	5.6	5.3	5.0	4.3	4.6
	(+)-Catechin (3.7 min)	12.3	10.9	13.7	11.3	10.6	13.3
	B-type trimer (2) (4.3 min)	8.8	7.7	7.0	8.7	7.2	7.0
	A-type trimer (1) (7.75 min)	11.6	13.5	11.0	8.9	7.2	8.2
	caffeoyl arbutin (10.5 min)	7.2	9.0	8.5	6.8	9.3	11.4
	A-type trimer (3) (11.3 min)	4.0	5.6	4.6	4.9	4.2	5.4
	Quercetin-3-O-galactoside (11.9 min)	8.1	6.7	5.6	11.8	10.1	4.2
Stem extracts	B-type dimer (2) (3.6 min)	5.0	6.5	5.1	4.2	4.6	4.9
	(+)-Catechin (3.7 min)	10.5	13.9	12.9	9.3	10.7	13.7
	B-type trimer (2) (4.3 min)	6.3	6.5	6.0	5.9	5.8	5.6
	(–)-Epicatechin (7.1 min)	7.7	7.0	11.0	8.0	5.9	9.5
	A-type trimer (1) (7.75 min)	8.3	8.6	7.7	7.0	5.9	6.7
	A type dimer (4) (10.4 min)	4.1	5.1	4.6	4.9	6.9	5.6
	Quercetin-3-O-galactoside (12.25 min)	10.9	9.1	8.2	11.4	10.8	7.8
	Quercetin pentoside (3) (13.8 min)	5.2	4.4	4.4	6.4	5.1	5.0

[a]Mean for $n = 3$.

3.3. Characterization of flavan-3-ol oligomers

In order to determine the total amount of flavanol oligomers and their composition as well as the average degree of polymerisation, HPLC analyses following thioacidolysis were carried out for lingonberry leaves, stems, and fruits. The results are given in Table II-5.

3.3.1. In freeze-dried extracts

In leaves, for all the harvest periods, the flavanol monomers were detected as (+)-catechin and (-)-epicatechin, the former being highly preponderant as already determined in the UPLC qualitative analysis (Figure II-2A). A good correlation for their levels between data from thioacidolysis (Table II-5) and UPLC (Table II-3) was found. Both (+)-catechin and (-)-epicatechin slightly increased from May to September although they were more abundant in September. Flavanol oligomers appeared from May to September as the most concentrated class of phenolic compounds which is in accordance with data from UPLC quantification. Moreover, their contents were greater after thioacidolysis suggesting that the degradation of B-type oligomers, which are predominantly found in lingonberry leaves (Table II-1), was complete during thioacidolysis.

The flavan-3-ol units, (+)-catechin and (-)-epicatechin were found both as extension and terminal units for all the period of vegetation. (+)-Catechin was relatively more abundant as terminal units (26-40%) than as extension units (18-23%) accounted for 43 to 60% of the constitutive units. By contrast, (–)-epicatechin was predominantly found as extension units (32-55%) than as terminal units (2-7%). The mDP ranging from 2.1 to 3.6 confirmed the large presence of dimers and trimers as detected by UPLC analysis (Table II-1). When flavanol monomers tend to increase from may to July for both years 2013 and 2014, contradictory seasonal variations are observed for flavanol oligomers. Annual variations are also observed for contents in flavanol monomers and oligomers which could be attributed to the environmental factors that are known to influence the biosynthesis of phenolic compounds in plants.

In stems, both (+)-catechin and (-)-epicatechin were found as flavanol monomers in accordance with both compounds being quantified by UPLC qualitative analysis (Figure II-2B). The catechin/epicatechin ratio ranges between 1 and 1.4 from May to September

for the two years and the highest flavanol monomer content was found for stems from September as also determined by UPLC. By contrast, Harris et al. (2007) determined for stems of blueberry (*Vaccinium angustifolium Ait.*) a ratio of 2:1 between epicatechin and catechin by HPLC quantification. This difference could be attributed to a gene difference between blueberry and lingonberry species or to the reaction of epimerization of (-)-epicatechin to (+)-catechin that occurs during thioacidolysis (Guyot et al., 1998). Epicatechin and catechin evolved similarly in 2013 and 2014 whatever the period of vegetation outlining no annual influence on their biosynthesis.

Flavanol oligomers represent the first phenolic group in agreement with data from UPLC quantification. For all the periods of vegetations, (+)-catechin and (-)-epicatechin were detected as both extension and terminal units. (+)-Catechin was relatively more abundant as terminal units (28-38%) than as extension units (18-21%) representing the main constitutive unit. (–)-Epicatechin was predominantly found as extension units (25-37%) rather than as terminal units (14-24%). Stems from all the periods of vegetation contain flavanol oligomers with lower mDP than in leaves, ranging from 1.8 to 2.3 being in agreement with the various B-type dimers and trimers that were quantified by UPLC.

In fruits, similar amounts of flavanol monomers were quantified in the control experiment of thioacidolysis (Table II-5) and by UPLC. By contrast and as expected, flavanol oligomers were evaluated in a larger content after thioacidolysis. These results are in agreement with those of Grace et al. (2014) and Jungfer et al. (2012) who detected lower amounts of flavanol monomers compared to flavanol oligomers.
(+)-Catechin and (-)-epicatechin appeared as extension as well as terminal units in both aqueous and ethanolic extracts (Table II-5). (+)-Catechin was relatively more abundant as terminal units (31-36%) than as extension units (8-9%) representing between 39 and 43% of all the constitutive units. (–)-Epicatechin was predominantly found as extension units (40-49%) than as terminal units (12-17%) representing the major constitutive unit. No significant differences were determined between the two types of fruit extracts (H_2O vs 55% aqueous EtOH) suggesting that water is a good solvent for the extraction of flavanol oligomers.

3.3.2. In freeze-dried fruits

The results related to the composition of flavanols in freeze-dried fruits are in good agreement with those described previously for fruit extracts. A remarkable similarity in the contents in flavanol monomers and oligomers, mDP, and the composition of oligomers was observed for fruits from 2013 and 2014.

Table II-5. Flavan-3-ol composition and mDP in lingonberry leaves, stems and fruits determined by HPLC following thioacidolysis.

Morphological parts	Period of vegetation	Flavanol monomers (mg/g DE)		Procyanidin characterization				Flavanol oligomers (mg/g DE)	mDP
				Terminal units (%)		*Extension units (%)*			
		CAT	*EC*	*CAT*	*EC*	*CAT*	*EC*		
Leaves extracts									
	May 2013	9.28 ± 0.63(A)	2.10 ± 0.15(A)	34.7 ± 1.9(A)	4.3 ± 0.49(A)	23.4 ± 1.3(A)	37.5 ± 3.4(A)	41.6 ± 1.80(A)	2.6
	May 2014	**7.57 ± 0.31(a)***	**2.62 ± 0.09(a)***	**31.2 ± 0.6(a)**	**3.1 ± 0.08(a)***	**20.1 ± 1.0(a)***	**45.7 ± 0.9(a)***	**40.2 ± 1.08(a)**	**2.9**
	July 2013	10.4 ± 0.70(A,B)	2.63 ± 0.28(B)	37.2 ± 2.0(A,B)	4.6 ± 0.08(A)	21.7 ± 0.8(B)	36.5 ± 1.5(A)	34.9 ± 2.74(B)	2.4
	July 2014	**7.53 ± 0.23(a)***	**3.39 ± 0.09(a)***	**26.0 ± 0.8(b)***	**1.9 ± 0.13(b)***	**17.5 ± 1.2(b)***	**54.7 ± 1.7(b)***	**50.3 ± 2.14(b)***	**3.6**
	September 2013	12.2 ± 1.47(B)	2.87 ± 0.02(B)	39.9 ± 1.4(B)	7.1 ± 0.15(B)	21.2 ± 1.1(B)	31.8 ± 2.0(B)	33.1 ± 2.53(B)	2.1
	September 2014	**12.3 ± 0.53(b)**	**3.72 ± 0.96(a)**	**37.8 ± 0.7(c)***	**4.6 ± 0.10(c)***	**20.5 ± 1.0(a)**	**37.1 ± 0.9(c)***	**41.9 ± 0.48(a)***	**2.4**
Stems extracts									
	May 2013	10.4 ± 0.77(A)	10.7 ± 0.69(A)	31.1 ± 1.1(A)	20.8 ± 1.8(A)	21.2 ± 2.1(A)	27.0 ± 2.0(A)	42.9 ± 1.64(A)	1.9
	May 2014	**11.3 ± 0.22(a)**	**11.9 ± 1.39(A,B)**	**28.4 ± 1.5(a)**	**18.7 ± 1.0(a)***	**19.7 ± 1.5(a)**	**33.2 ± 2.3(a)***	**42.1 ± 5.25(A)**	**2.1**
	July 2013	14.5 ± 0.77(B)	10.2 ± 0.56(A)	37.6 ± 0.7(B)	17.4 ± 0.3(B)	20.1 ± 0.9(A)	24.9 ± 0.5(B)	38.5 ± 2.34(A)	1.8
	July 2014	**13.5 ± 0.19(b)**	**10.6 ± 0.13(A)**	**30.9 ± 0.4(b)***	**13.6 ± 0.2(b)***	**18.4 ± 0.1(b)***	**37.2 ± 0.3(b)***	**50.5 ± 0.30(B)***	**2.3**
	September 2013	17.5 ± 1.90(B)	16.9 ± 2.00(B)	31.2 ± 0.5(A)	24.1 ± 0.3(C)	20.2 ± 0.1(A)	25.0 ± 0.2(B)	37.4 ± 3.72(A)	1.8
	September 2014	**18.6 ± 0.61(c)**	**14.9 ± 1.64(B)**	**34.8 ± 0.1(c)***	**20.6 ± 0.1(c)***	**20.0 ± 0.1(a)**	**25.0 ± 0.1(c)**	**48.7 ± 2.32(A,B)***	**1.8**
Fruits extracts									
H_2O	July 2013	0.71 ± 0.06	0.65 ± 0.15	33.7 ± 8.8	13.9 ± 3.0	8.6 ± 2.31	43.9 ± 11.8	2.97 ± 0.98	2.1
	July 2014	**0.77 ± 0.03**	**0.54 ± 0.02**	**32.8 ± 0.1**	**16.8 ± 0.3***	**8.6 ± 0.16***	**41.9 ± 0.0***	**4.95 ± 0.08***	**2.0**
EtOH 55%	July 2013	0.42 ± 0.16	0.64 ± 0.10	35.8 ± 5.6	16.4 ± 4.7	7.5 ± 0.65	40.3 ± 9.3	3.57 ± 0.30	2.0
	July 2014	**1.00 ± 0.05***	**0.61 ± 0.04**	**31.3 ± 0.7***	**12.2 ± 0.4***	**7.8 ± 0.09**	**48.7 ± 0.3***	**8.98 ± 0.16***	**2.3**
Freeze-dried fruits[a]	July 2013	1.00 ± 0.06	0.74 ± 0.10	24.0 ± 0.3	25.2 ± 0.8	15.8 ± 0.5	35.0 ± 2.5	4.21 ± 0.08	2.0
	July 2014	**0.94 ± 0.02**	**0.77 ± 0.05**	**23.7 ± 0.5**	**26.4 ± 1.9**	**16.9 ± 0.8**	**33.8 ± 2.5**	**4.24 ± 0.28**	**2.0**

CAT: (+)-catechin. EC: (–)-epicatechin. mDP: average degree of polymerization of monomeric and oligomeric flavan-3-ols. Values represented mean ± SD ($n = 3$). [a]Results are expressed in mg/g of Dry Matter. 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 ($p < 0.05$).

3.4. Antioxidant activity of lingonberry extracts

The antioxidant activity of leaf, stem and fruit extracts of lingonberry was carried out through the Folin-Ciocalteu method for the assessment of the Total Phenolic Content (TPC) and by using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test. The ability of lingonberry extracts to reduce transition metal ions and to scavenge DPPH was determined for samples collected in May, July and September during the years 2013 and 2014. The results obtained are presented in Figure II-5 and Table II-3.

The TPC of lingonberry extracts was expressed in weight per Dry Matter (DM) for the two years of study (2013 and 2014) and in weight per Dry Extract (DE) for year 2014 only (Table II-3; Figure II-5A). The difference between TPC contents is due to the extraction yields of the DM: ca. 58% for leaves, 48% for stems, and 79% for fruits.
When comparing the values expressed in weight per DE for year 2014 (Figure II-4), the TPC is ca. 2-fold higher than the Sum of the Phenolic Compounds quantified by UPLC (Table II-3) suggesting the possibility that other non-phenolic antioxidants such as vitamin C interfere with the response toward Folin-Ciocalteu reagent (Ma et al, 2007). One the other hand, a good correlation between these two results was found with R^2 of 0.73. These was not the same case when TPC (w/w of DM) were correlated to the Sum of Phenolic Compounds (w/w of DE) which resulted in a decrease of the correlation with R^2 of 0.56 and 0.52 in 2013 and 2014 for all the eight samples respectively (Figure II-6A and B). For leaves, the TPC (mg GAE/g DE) significantly increases from July to September 2014 as observed for the Sum of Phenolic Compounds. In the case of the TPC (mg GAE/g DM), the trends are similarly related to the one observed for the Sum of Phenolic Compounds although with annual variations. September remains the month where leaves diplay the highest content in phenolic compounds (Sum of Phenolic Compounds) or antioxidants (TPC).

For stems, the TPC (mg GAE/g DE) significantly increases from July to September 2014 as observed for the Sum of Phenolic Compounds. In the case of the TPC (mg GAE/g DM), the trends are less related to the one observed for the Sum of Phenolic

Compounds. Owing to less important seasonal variations for stems than for leaves, stems may be advantageously picked from July to September.

Last, there is no annual variation for fruits whatever the parameter followed (TPC and sum of phenolic acids), except for TPC contents (mg GAE/g DM) of fruit extract with 55% aqueous EtOH. Moreover, the total phenolic content in leaves and stems was higher than in fruits and this could be attributed to the high concentration in sugar of the latter. Similarly, a higher TPC in leaves than in fruits was previously observed in *Vaccinium myrtillus* L., which also belongs to the *Ericaceae* family as lingonberry (Vučić et al., 2013).

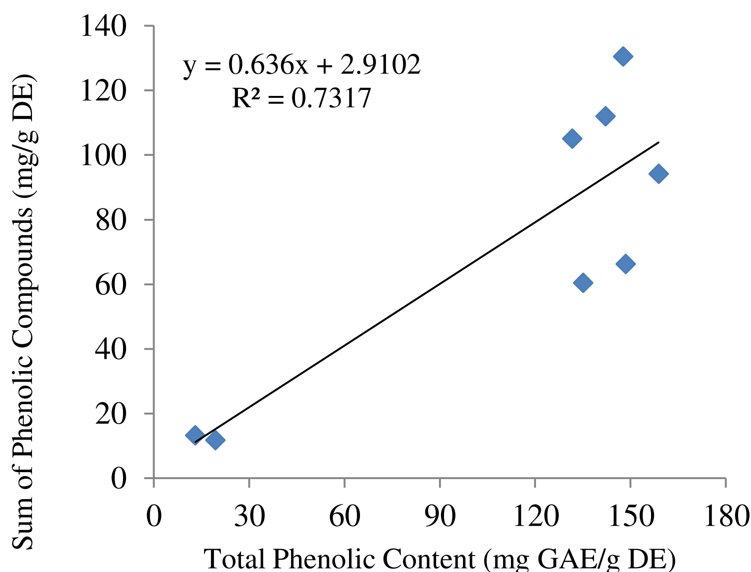


Figure II-4. Correlation between the Sum of Phenolic Compounds and the Total Phenolic Content for samples of dry extracts from 2014

The DPPH scavenging activity was assessed for two concentrations of leaf, stem and fruit extracts, at 10 mg DE/mL (Figure II-5B) and 5 mg DE/mL (Figure II-5C). For both concentrations tested, the DPPH activity is rather similar for leaves and stems of lingonberry in agreement with the similar TPC (mg/g DE) observed for 2014. The DPPH activity of fruits is lower, as also exhibited by TPC and this may be associated to the presence of sugars in their composition. Additionally, the use of ethanol-containing solvents slightly improved the extraction yields of the antioxidant compounds increasing the antioxidant activity by both TPC (ca. + 27%) and DPPH (ca. + 16%) tests

in 2013 whereas this effect was less clear in 2014. By contrast, the Sum of Phenolic Compounds was unaffected by the extraction solvent type. When comparing the DPPH test at the 10 or 5 mg/mL levels, one can note that the antioxidant activity of leaves and stems significantly, although slighlthy, increases from May to September 2014 as already observed for the Sum of Phenolic Compounds and TPC. This effect was not evidenced for year 2013 in agreement with leaf and stem TPC being unchanged during the seasons.

A good correlation was found between the DPPH radical scavenging activity and the Sum of Phenolic Compounds with R^2 of 0.83 and 0.85 for lingonberry extracts from 2013 and 2014, respectively (Figure II-6C and D). Based on these results, it is clear that phenolic compounds are largely responsible for the antioxidant activities of the lingonberry extracts. The relationships between the Total Phenolic Content determined by the Folin-Ciocalteu method and the DPPH radical scavenging activity were highly correlated with R^2 of 0.84 and 0.78 for samples from 2013 and 2014, respectively (Figure II-6E and F). The Folin-Ciocalteu method is based on the transition metal-reducing capacity of a molecule whenthe DPHH assay assesses the electron transfer capacity of a molecule towards a nitrogen-centered radical. The high similarity in correlation between the DPPH and TPC methods points to antioxidant compounds displaying both properties in the extracts. As a matter of fact, polyphenols containing the 1,2-*ortho*-dihydroxyphenyl moiety can act as antioxidants by both mechanisms. This moiety is indeed present in the large majority of phenolic compounds identified in the MS study. Caffeic acid derivatives, quercetin glycosides, monomeric and oligomeric flavanols as well as cyanidin glycosides are all contributing to the excellent antioxidant capacity of lingonberry extracts.

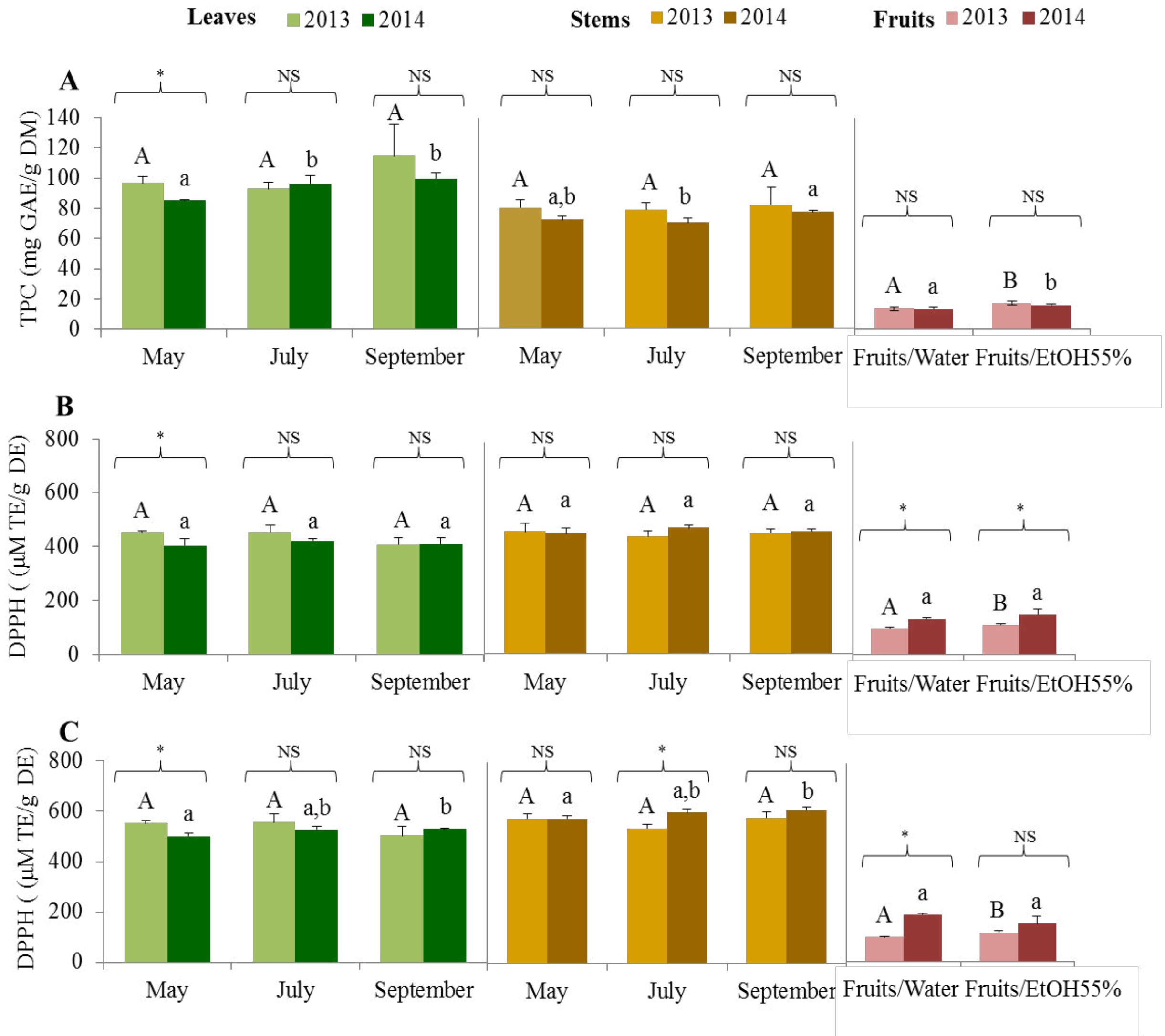


Figure II-5. Influence of different harvest periods on Total Phenolic Content (A) and DPPH radical scavenging activity (B: samples at 10 mg/mL; C: samples at 5 mg/mL) in leaf, stem and fruit extracts of lingonberry (mean±SD, *n* = 3-4). Different letters indicate a significant difference between three 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.

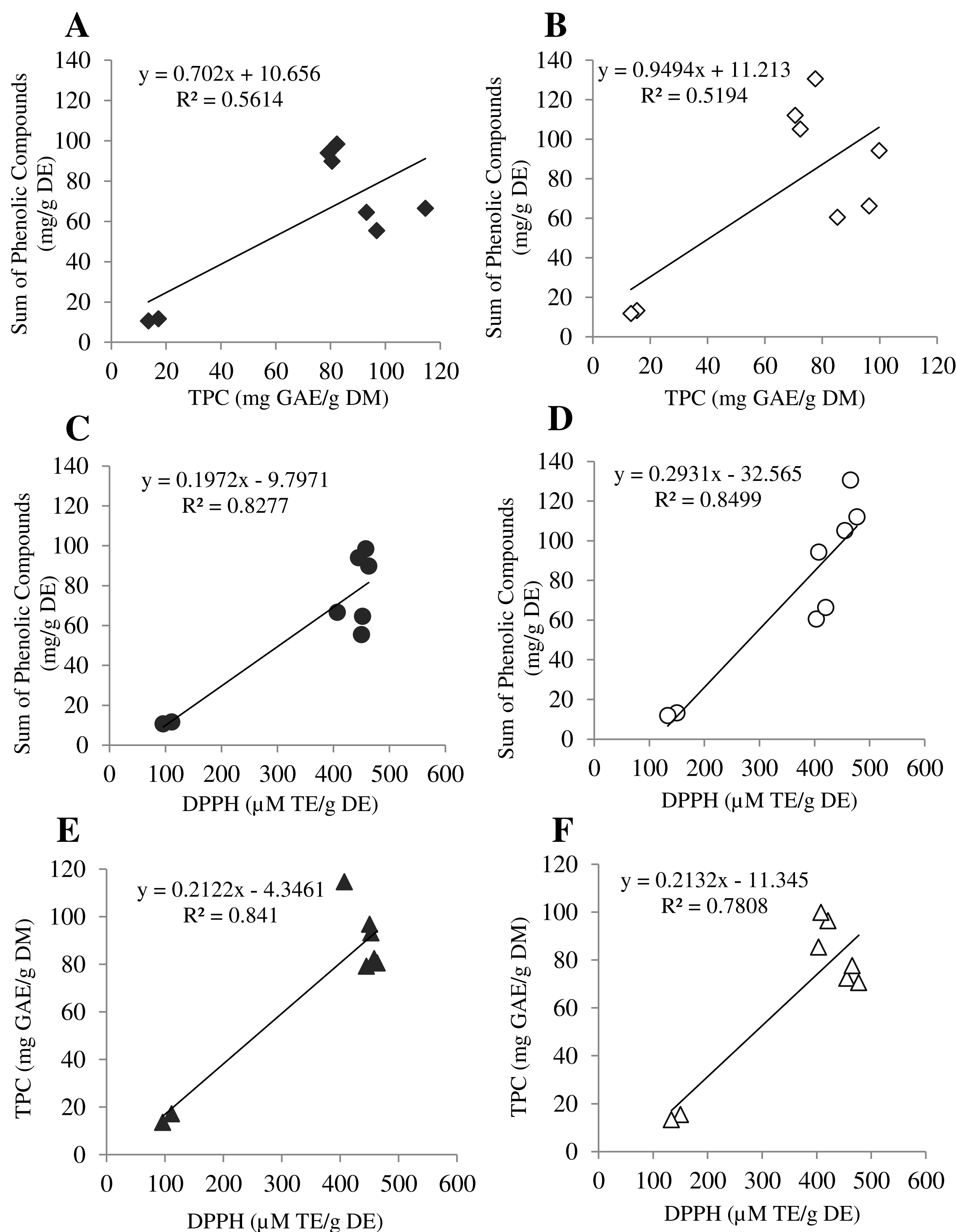


Figure II-6. Correlations between the Sum of Phenolic Compounds and the TPC (A: samples from 2013; B: samples from 2014), between the Sum of Phenolic Compounds and the DPPH radical scavenging activity (C: samples from 2013; D: samples from 2014) and between the TPC and the DPPH radical scavenging activity (E: samples from 2013; F: samples from 2014).

4. Conclusions

This study appears as the most complete qualitative and quantitative analysis of the phenolic compound of lingonberry leaves, stems and fruits never performed to date. Additionally, this study originally reports the content in individual phenolic compoundsat three different periods of vegetation. These data are thus extremely valuable for the field of ecology as well as for human health.

The influence of different harvest periods on the phenolic profile and content and antioxidant activity of leaf, stem and fruit extracts of lingonberry was evaluated through UPLC/MS analysis, the Folin-Ciocalteu method and DPPH (2,2-diphenyl-1-picrylhydrazyl) test. For lingonberry leaves and stems, procyanidins were characterized by thioacidolysis in order to determine the subunit composition and the average degree of polymerization (mDP). This determination was conducted for the first time.

Qualitative analysis revealed similar phenolic profiles for lingonberry leaves and stems harvested at the three different periods in 2013 and 2014. The structures of 127 phenolic compounds were assessed by UPLC/MS analyses. Fifty compounds detected in the lingonberry extracts were characterized for the first time. The results showed the predominant presence of monomers and oligomers of catechin and epicatechin and quercetin glycosides in all the morphological parts. Aditionally, the anthocyanins are quantified in significant amounts in fruits. Based on the results of thioacidolysis, lingonberry leaf, stem and fruit were found to contain (+)-catechin and (-)-epicatechin units as both extension and terminal units.

This study has also demonstrated a high antioxidant activity of leaf, stem and fruit extracts of lingonberry. Regarding the harvest period, the Total Phenolic Content (mg/g DE) and the Sum of Phenolic Compounds (UPLC) showed a slight but significant increase from May to September for both lingonberry stems and leaves. This increase was confirmed for the antioxidant activity by the DDPH test for both stems and leaves in 2014 and TPC (mg/g DM) for leaves in 2014.

Among the three periods of vegetation, leaves and stems can be collected in any one, May, July or September, as sustainable sources of natural antioxidants. If one is seeking for phenolic compounds with a significant antioxidant activity, July, or better, September, should be favored. Additionally, if flavanols are of interest, then September is the favoured month for picking.

Finally, the qualitative analysis of the extracts first helped understanding their high activity as antioxidant sources. In human health, this knowledge is of further interest since an extract enriched in a particular sub-class of phenolic compounds may be selected for a desired biological activity.

ANNEXES

Annexe I-1. Extraction yield of the Dry Matter (DM) for lingonberry leaves, stems and fruits from 2014.

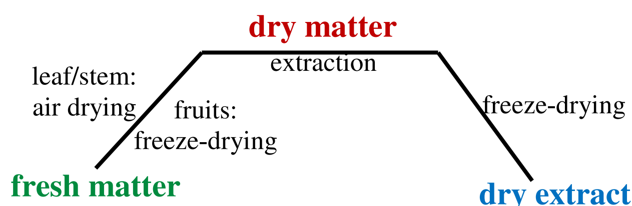
Morphological parts/ period of vegetation	Extraction yield of the DM (%)
Leaves	
May	57
July	60
September	58
Stems	
May	50
July	46
September	48
Fruits with H_2O	88
Fruits with EtOH 55%	70

Annexe I-2. Residual moisture of grinded lingonberry leaves, stems and fruits at three different periods of vegetation and for two different years.

Morphological parts	Period of vegetation	Residual moisture (%)*
Leaves	May 2013	8.7
	May 2014	9.4
	July 2013	8.9
	July 2014	8.1
	September 2013	8.7
	September 2014	8.4
Stems	May 2013	8.4
	May 2014	9.0
	July 2013	8.3
	July 2014	7.5
	September 2013	9.2
	September 2014	8.2
Fruits	July 2013	13.3
	July 2014	11.9

*Mean for $n = 3$.

Annexe I-3. Processing steps of lingonberry leaves, stems and fruits.



Annexe I-4. Influence of the processing on the Total Phenolic Content in lingonberry leaves, stems and fruits.

Extract /Period of vegetation	Moisture content (%)*	Total Phenolic Content (mg GAE/g FM)	Total Phenolic Content (mg GAE/g DM)	Total Phenolic Content (mg GAE/g DE)
Leaves				
July 2014	44	49.3 ± 2.9(a)	96.3 ± 5.6(a)	148.5 ± 8.6 (a)
September 2014	48	47.3 ± 1.8(a)	99.8 ± 3.7(a)	158.9 ± 6.0 (a)
Stems				
July 2014	33	43.4 ± 1.9(a)	70.5 ± 3.2(a)	142.2 ± 6.4 (a)
September 2014	30	49.7 ± 0.9(b)	77.6 ± 1.3(b)	147.7 ± 2.5 (a)
Fruits with H_2O				
July 2014	84	1.93 ± 0.1	13.3 ± 0.6	13.1 ± 0.6
Fruits with EtOH 55%				
July 2014	84	2.23 ± 0.0	15.4 ± 0.3	19.4 ± 0.3

Values represented mean ± SD (*n* = 3). *Moisture content refers to the quantity of water contained in fresh samples. Total Phenolic Content is obtained by the Folin-Ciocalteu method. FM: Fresh Matter. DM: Dry Matter. DE: Dry Extract. Different letters indicate a significant difference between the three periods of vegetation at $p < 0.05$.

Chapter III.

LIPID PROTECTION FROM OXIDATION BY BILBERRY AND LINGONBERRY PHENOLIC EXTRACTS: *IN VITRO* INVESTIGATION UNDER SIMULATED DIGESTION CONDITIONS

1. Background

Currently, there is a growing interest for the control of the oxidation of polyunsaturated lipids, phenomenon which is responsible for not only the deterioration of food quality, but also for damage to tissues. Indeed, dietary oxidized lipids play a key role in the earliest stage of atherosclerosis (Staprans et al. 1994). The formation of dietary lipid oxidation products (conjugated dienes, short-chain aldehydes and alcohols) can be generated *in vivo* and the gastric compartment has been proposed as a major site for diet-related oxidative stress (Ursini & Sevanian).

Recently, it was demonstrated that plant polyphenols provided as fruits and vegetables or an extract protect dietary lipids from oxidation during gastric digestion in minipigs (Gobert et al., 2014). Several approaches using *in vitro* models have been developed in order to study lipid oxidation and its inhibition by phenolic antioxidants: oxidation of oils and emulsions rich in polyunsaturated fatty acids (food models), oxidation in conditions mimicking those in the digestive tract (*in vitro* digestion models) and oxidation of cell membranes (physiological models).

Bilberry (*Vaccinium myrtillus L.*) and lingonberry (*Vaccinium vitis-idaea* L.) are two wild shrubs of the *Ericaceae* family whose fruits and aerial parts are consumed as dietary supplements for health benefits. These beneficial effects are attributed to the high content in polyphenols in bilberry and lingonberry (Kylli et al., 2011; Mane et al. 2011).

In the previous chapters of this study, it has been demonstrated that leaves, stems and fruits of wild bilberry and lingonberry are not only rich in phenolic compounds but also present a significant antioxidant activity highlighted through their capacity to reduce the DPPH radical and transition metal ions in the Folin-Ciocalteu test. Chlorogenic acid, flavonol glycosides and (epi)catechin monomers and oligomers were identified in significant amounts in leaves and stems of bilberry and lingonberry. At the same time, in previous research from the INRA-SQPOV group, abundant phenolic compounds in food such as quercetin and its glycosides (flavonols), catechin and its oligomers (tannins), but also phenolic acids (caffeic and chlorogenic acids)

have proved to be efficient antioxidants on lipid peroxidation (Goupy et al. 2007; Goupy et al. 2009; Lorrain et al. 2010; Lorrain et al. 2012).

Based on these premises, the aim of this study is to evaluate the *in vitro* antioxidant activity of fruits, leaves and stems of bilberry and lingonberry extracts and their phenolic compounds in lipid oxidation. Firstly, the investigation of lipid oxidation in an *in vitro* model of gastric digestion and its inhibition by extracts of bilberry and lingonberry was evaluated using two different emulsion systems as physico-chemical models of the gastric content. Oil-in-water emulsions were stabilized either by a protein (bovine serum albumin, BSA) or egg yolk phospholipids (PL), both emulsifiers mimicking dietary components. Secondly, the protective capacity against lipid oxidation of an extract of bilberry leaves was assessed in a complete static *in vitro* digestion model (oral, gastric and intestinal phase).

2. Methodology

2.1. Materials

2.1.1. Chemicals and solvents

Chemicals: Chlorogenic acid, (-)-epicatechin, sodium acetate trihydrate, disodium hydrogenophosphate, iron (II) sulphate heptahydrate, myoglobin (from horse heart, MW ca. 17600 g mol^{-1}, Lot No. SLBD8797V), bovine serum albumin (BSA) (A9647, MW ca. 66500 g mol^{-1}, Lot No. 051M1872V), L-α-phosphatidylcholine from dried egg yolk (≥50%, TLC, 61771, Lot No. 1203670), porcine pepsin (P6887, 2000 U/mg according to the COST protocol for enzyme activity determination, Lot No. SLBB9556V), pancreatin (from porcine pancreas, 8 × USP specifications, P7545, Lot No. 061M1822V), porcine bile extract (B8631, MW ca. 500 g mol^{-1}, Lot No. MKBQ8333V), calcium chloride dehydrate, acetic acid and NH_3 solution (28-30%) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France); procyanidin A2 and cyanidin-3-O-galactoside (ideain chloride) were from Extrasynthese (Genay, France) and citric acid from Merck (Darmstadt, Germany). Commercial L-α-phosphatidylcholine (PL) contained phosphatidylcholine (33%), phosphatidylethanolamine (13%), sphingomyelin (3%), lysophosphatidylcholine (2%) and phosphatidylinositol (2%) along with a neutral fraction containing triacylglycerols (47%) (analysis performed by the INRA-BIA unit in Nantes). Commercial sunflower oil (Lot No. A07611) was purchased from Auchan store and stored at –20 °C. According to the manufacturer (Auchan), the commercial sunflower oil contained 50 mg tocopherol/100 g of oil. Fatty acids were assayed in the sunflower oil and egg yolk phospholipids by GC/MS after slight modifications of the protocol from Berton et al. 2011: standard C19:0 (2.5 mg/mL) was solubilized in acetone/MeOH (2:1). The oil composition was: 1.4% C22:0, 5% C20:1, 0.5% C20:0, 0.2% C18:3n-3, 46% C18:2n-6, 28% C18:1n-9, 7% C18:0, and 10% C16:0. The PL composition was: 3% C20:4, 1% C20:1, 0.3% C18:3n-3, 9% C18:2n-6, 27% C18:1n-9, 24% C18:0, 1% C16:1 and 34% C16:0.

Solvents: HPLC-MS grade methanol and 2-propanol were purchased from Fisher Scientific (Illkirch Cedex, France). Sodium acetate (10 mM, pH 5.0) and citrate/phosphate buffer (8 mM : 4 mM, pH 3.0) were prepared with Millipore Q

water. Ultrapure water (resistivity 18.2 MΩ.cm^{-1} at 25 °C) was obtained with a Milli-Q water purification system (Millipore, Bedford, MA).

2.1.2. Antioxidants: bilberry and lingonberry samples and phenolic compounds

2.1.2.1. Bilberry and lingonberry extracts

Bilberry and lingonberry extracts prepared according to the protocols described in Chapter I, Section 2.2 were used. Solutions of freeze-dried extracts of the fruits, leaves and stems of bilberry and lingonberry collected in July 2013 were freshly prepared at the following concentrations:

- 10 mg/mL for leaves and stems of bilberry and lingonberry extracts,
- 20 mg/mL for aqueous and ethanolic extract of bilberry fruits,
- 40 mg/mL for aqueous and ethanolic extract of lingonberry fruits.

Solutions for aqueous extracts were prepared in Millipore Q water. Solutions for ethanolic extracts were prepared in 55% aqueous ethanol (v/v).

2.1.2.2. Freeze-dried fruits

Bilberry and lingonberry fruits lyophilized according to the protocol in Chapter I, Section 2.1.1 were used.

2.1.2.3. Phenolic compounds

Solutions of 10 mM chlorogenic acid, (-)-epicatechin, procyanidin A2 and cyanidin-3-O-galactoside were prepared in Millipore Q water. (-)-Epicatechin was subjected to ultrasound treatment for complete solubilization.

2.2. Lipid oxidation in gastric model emulsions

The preparation of emulsions and lipid oxidation conditions were carried out according to a procedure described by Katerina Asprogenidi (2015, PhD Thesis).

2.2.1. Preparation of emulsions

Dietary lipids in the course of gastric digestion were modeled by sunflower oil-in-water emulsions stabilized either by bovine serum albumin (BSA) or egg yolk phospholipids (PL). Sunflower oil was used for the preparation of emulsions based on its high content in n-6 polyunsaturated acids as found in the Western diet. In a 60 mL bottle containing 4 g of sunflower oil was added 36 mL of BSA or PL solutions prepared by solubilizing 160 mg of BSA or 100 mg of PL in a pH 5 or pH 3 (only for BSA model) buffer. The PL dispersion was best achieved using a rotor stator homogenizer (SilentCrusher M-01, Heidolph) at 24000 rpm for 2 min. The oil and aqueous phases were then homogenized at 24000 rpm for 2 min. The coarse emulsion was next sonicated in the ice for 8 periods of 30 s with a rest interval of 30 s and amplitude of 40% for both BSA and PL emulsions (Q700, QSonica, 20 kHz).

2.2.2. Preparation of lipid oxidation initiator solutions

Two forms of dietary iron, heme iron (metmyoglobin) and non-heme iron (Fe^{II}), were used as initiators of lipid oxidation. Metmyoglobin (44.1 mg) was dissolved in 10 mL of pH 5 buffer or Millipore Q water for pH 3 emulsions; the concentration of the initial solution is between 205-235 μM. The metmyoglobin concentration was standardized at 200 µM using ε=7700 M^{-1} cm^{-1} at 525 nm prior to use (Mikkelsen & Skibsted, 1995). The solution of Fe^{II} was prepared in Millipore Q water to a final concentration of 2 mM prior to use.

2.2.3. Lipid oxidation and its inhibition by phenolic extracts and fruits

2.2.3.1. Bilberry and lingonberry extracts and phenolic compounds

In a 50 mL round-bottom flask, a volume of 0.1 mL of the bilberry, lingonberry or phenolic compound solutions was added to 9 mL of the BSA or PL emulsion. Lipid

oxidation was initiated by adding 1 mL of a 200 µM solution of metmyoglobin or 2 mM solution of Fe^{II} leading to final concentrations of 20 µM or 200 µM, respectively. The system was protected by punched parafilm and placed in an oven at 37 °C under constant magnetic stirring at 280 rpm.

2.2.3.2. Freeze-dried fruits

In a 50 mL round-bottom flask, 20 mg of bilberry or lingonberry fruit powder were added to 9 mL of the PL emulsion. Lipid oxidation was initiated by adding 1 mL of a 200 µM solution of metmyoglobin. The system was protected by punched parafilm and placed in an oven at 37 °C under constant magnetic stirring at 280 rpm.

2.2.3.3. Determination of lipid-derived conjugated dienes

The accumulation of lipid-derived conjugated dienes was followed for 6 hours. Every hour, aliquots of 200 µL of emulsion were diluted by a factor 6 in 2-propanol followed by centrifugation at 10000 rpm for 5 min (4 °C). The concentration of conjugated dienes (CDs) was determined after dilution of the supernatant by a factor 11, 21 or 41 in 2-propanol and measurement of the absorbance at 234 nm (HP 8453 diode-array spectrometer; optical path length = 1 cm).

Absorbances were converted in concentrations by using $\varepsilon = 27000\ M^{-1}cm^{-1}$ as the molar absorption coefficient for conjugated linoleyl hydroperoxides (Pryor & Castle, 1984). Controls (experiments without antioxidant) were carried out for each emulsion systems and iron forms. CDs concentration vs time curves were plotted for all the experiments. The initial absorbance corresponding to CD levels between 0.4 and 0.6 mM was subtracted. The antioxidant activity was determined through the calculation of the area under the curves ((AUC for samples x 100) /control AUC) using a polyhedral method. The inhibition levels were calculated as the difference between 100 and relative AUC. The experiments were carried out three to six times.

2.3 Simulated static in vitro digestion: preliminary study on the inhibition of lipid oxidation by a bilberry leaf extract

The physico-chemical conditions for the oral, gastric and intestinal phases of digestion were simulated according to the standardized protocol for *in vitro* static digestion proposed by Minekus et al. (2014) (COST FA1005 Action INFOGEST) with slight modifications.

2.3.1. Bilberry leaf extract

For this study, the freeze-dried extract of bilberry leaves collected in July 2013 was used for the assessment of the antioxidant activity at the gastric and intestinal stages of digestion.

2.3.2. Preparation of o/w emulsions

To investigate the lipid protective capacity of the bilberry leaf extract, sunflower oil-in-water emulsions stabilized either by BSA or PL as models of dietary lipids were used. The BSA and PL emulsions were prepared in a sodium acetate buffer at pH 5 as described above, in the Section 2.2.1.

2.3.3. Preparation solutions of simulated digestion fluids

Solutions of simulated digestion fluids, Simulated Salivary Fluid (SSF, oral phase), Simulated Gastric Fluid (SGF, gastric phase) and Simulated Intestinal Fluid (SIF, intestinal phase) were made up of the corresponding electrolyte stock solutions (Tables III-1) and water. Preparation of stock solutions of simulated digestion fluids are given in Table III-1. All stock solutions of simulated digestion fluids and $CaCl_2(H_2O)_2$ solution were prepared in Millipore Q water. The stock solutions were made up to 250 mL with Millipore Q water to ensure the correct ionic composition in the simulated digestion fluids.

Table III-1. Preparation of stock solutions of simulated digestion fluids

Constituent	Molar mass (g·mol^{-1})	Stock conc.		SSF pH 7		SGF pH 5		SIF pH 6.5	
				vol. of stock[a]	conc. in SSF	vol. of stock[a]	conc. in SSF	vol. of stock[a]	conc. in SSF
		g/L	mol/L	mL	mmol/L	mL	mmol/L	mL	mmol/L
KCl	74.55	37.3	0.5	7.55	15.1	3.45	6.9	3.4	6.8
KH_2PO_4	136.09	68	0.5	1.85	3.7	0.45	0.9	0.4	0.8
$NaHCO_3$	84.01	84	1	3.4	13.6	6.25	25	21.25	85
NaCl	58.44	117	2	–	–	5.9	47.2	4.8	38.4
$MgCl_2(H_2O)_6$	203.30	30.5	0.15	0.25	0.15	0.2	0.1	0.55	0.33
NH_3	96.09	48.0	0.5	0.03	0.06	0.25	0.5	–	–
For pH adjustement of each simulated fluid									
NaOH	39.99	40.0	1	–	–	–	–	–	–
HCl -37%	36.46	218.8	6	–	–	–	–	–	–
$CaCl_2(H_2O)_2$		**g/L**	**mol/L**		**mmol/L**		**mmol/L**		**mmol/L**
	147.01	14.7	0.1		0.75 mM in final digestion mixture				0.49 mM in final digestion mixture

[a]The volumes of stock solutions are calculated for a final volume of 250 mL for each simulated fluid.

<u>*2.3.4. Experimental protocol of in vitro digestion*</u>

Porcine pepsin activity was determined according to the procedure in Minekus et al. (2014). It was found to be 2041 +/- 177 U/mg of powder. Pepsin level in the gastric phase is fixed at 2000 U/mL based on Minekus et al. (2014). Trypsin activity was determined according to the procedure in Minekus et al. (2014). It was found to be 5.6 U/mg of powder. Porcine pancreatin is added in the intestinal phase to reach a trypsin activity of 100 U/mL. Bile salt concentrations are estimated based on an average MW of 500 and 96% purity. A final level of 10 mM in bile salts should be brought in the intestinal phase. All enzymes are prepared on the day of use and kept in ice until their use.

2.3.4.1. Oral phase

In a 50 mL round-bottom flask, 5 mL of the BSA or PL emulsion were mixed with 36 mg of freeze-dried bilberry leaf extract made up in 1 mL of SSF solution and 45 μL of 0.1 M $CaCl_2$ (final concentration of 0.75 mM in the oral phase). $CaCl_2(H_2O)_2$ was added to the final mixture to avoid salt precipitation (Minekus et al., 2014). The pH was adjusted to 7 by addition of 1 M NaOH. The obtained mixture was protected by punched parafilm and incubated in an oven at 37 °C under constant magnetic stirring at 130 rpm for 5 min. A control was prepared without bilberry leaf extract.

2.3.4.2. Gastric phase

The gastric digestion phase was divided in two subsequent steps in order to simulate the early step of digestion (pH 5, 1 h) and the mid-course of digestion (pH 3, 1 h).
The 6 mL of the oral phase mixture were mixed with 3.8 mL of SGF, 1 mL of a 12 mg/mL pepsin solution made up in SGF solution and 1.2 mL of a 200 µM solution of metmyoglobin in SGF. The pH of the gastric phase mixture was adjusted to 5 by addition of 1 M HCl and incubation was continued in the oven at 37 °C under constant magnetic stirring at 280 rpm for 60 min. After this step, the pH was adjusted to 3 by addition of 1 M HCl and incubation was continued for another 60 min. During the gastric phase (2 h), aliquots of 200 µL of the gastric phase were sampled at t = 0, 30, 60, 90 and 120 min time intervals and diluted by a factor 6 in 2-propanol followed by centrifugation at 10000 rpm for 5 min (4 °C). The supernatant was used for the determination of CDs.

2.3.4.3. Intestinal phase

The 11 mL (12 mL - 1 mL sampling) of gastric phase mixture were mixed with 5 mL of a pancreatin solution (393 mg of pancreatin in the SIF solution and bringing 440 U of trypsin/mg), 6 mL of a 19 mg/mL bile solution in the SIF solution (final concentration of 10 mM in bile salts) and 66 μL of 0.1 M $CaCl_2(H_2O)_2$ (final concentration of 0.49 mM). The pH of the intestinal phase mixture was adjusted to 6.5 by addition of 1 M NaOH and then incubation was continued in the oven at 37 °C under constant magnetic stirring at 280 rpm for 120 min. During the intestinal phase (2h), aliquots of 200 µL of the intestinal phase were sampled at t = 0, 30, 60, 90 and 120 min time intervals and

diluted by a factor 6 in 2-propanol followed by centrifugation at 10000 rpm for 5 min (4 °C). The supernatant was used for the determination of CDs.

2.3.5. Determination of lipid-derived conjugated dienes

The concentration of CDs was determined after dilution of the supernatant by a factor 11 or 21 in 2-propanol and measurement of the absorbance at 234 nm (HP 8453 diode-array spectrometer; optical path length = 1 cm). Absorbances were converted in concentrations by using $\varepsilon = 27000\ M^{-1}cm^{-1}$ as the molar absorption coefficient for conjugated linoleyl hydroperoxides. The antioxidant activity was obtained after area integration of the CDs concentration vs time curve (Section 2.2.3). The experiments were carried out three times.

Overview of the simulated in vitro digestion phases

Oral Phase	**Gastric Phase**	**Intestinal Phase**
Mix: dietary lipids (BSA or PL emulsions) + antioxidant + $CaCl_2$ (0.75 mM) ⇩	Oral phase (1:1 dilution) + pepsin (2000 U/mL) + SGF + $MbFe^{III}$ (20 µM) ⇩	Gastric phase (1:1 dilution) + pancreatin (100 U/mL) + bile (10 mM) + $CaCl_2$ (0.49 mM) ⇩
Adjustment pH 7: 1 M NaOH ⇩	**Adjustment pH 5:** 1 M HCl ⇩	**Adjustment pH 6.5:** 1 M NaOH ⇩
Incubation: 5 min, 37 °C, 130 rpm ⇩	**Incubation:** ➔ **Sampling:** 1 h, 37 °C, 280 rpm; 0, 30, 60 min ⇩	**Incubation:** 2 h, 37 °C, 280 rpm ⇩
No sampling	**Adjustment pH 3:** 1 M HCl ⇩	**Sampling:** 0, 30, 60, 90, 120 min ⇩
	Incubation: ➔ **Sampling:** 1 h, 37 °C, 280 rpm; 90, 120 min ⇩	
	Determination of CDs	***Determination of CDs***

3. Results and Discussion

3.1. Inhibition of lipid oxidation by lingonberry and bilberry extracts in in vitro gastric digestion models

Inhibition of lipid oxidation by lingonberry and bilberry extracts was assessed in oil-in-water emulsions stabilized by BSA or egg yolk PL as models of the gastric content. In the gastric compartment, lipid oxidation occurs due to contact between dietary iron, dioxygen, and emulsified lipids. In previous works from the INRA-SQPOV group, BSA- or PL-stabilized oil-in-water emulsions by have been shown to be valuable systems to study the efficiency of dietary polyphenols to inhibit heme (metmyoglobin) and nonheme iron (Fe^{II})-induced lipid peroxidation (Lorrain et al., 2010; Lorrain et al., 2012). It was demonstrated that the rate of CD accumulation is influenced by several parameters: the emulsifier type (proteins vs. phospholipids), the iron forms (metmyoglobin vs. Fe^{II}/Fe^{III}), and by pH (Lorrain et al., 2012). In both BSA and PL emulsions, dietary polyphenols such as quercetin, rutin, and chlorogenic acid highly inhibited the metmyoglobin-initiated lipid oxidation in the early phase of digestion (pH 5.8).
In this study, all the experiments have been predominantly conducted at pH 5 corresponding to the pH in the early stage of gastric digestion in relation with the consumption of a standard Western diet containing beef meat and sunflower oil (Gobert et al., 2014). For comparison, a couple of experiments were also carried out at pH 3 (the mid-course of digestion).

3.1.1. Inhibition of lipid oxidation by bilberry extracts and various phenolic compounds

3.1.1.1. With $MbFe^{III}$ as the initiator

The lipid oxidation initiated by $MbFe^{III}$, the main form of dietary iron (red meat), was first investigated in both BSA and PL model emulsions in order to evaluate its inhibition by bilberry extracts and various phenolic compounds (Figure III-1; Table III-2). The inhibition levels were calculated for the first 4 h of the kinetics in order to better simulate the maximal duration of gastric digestion for a Western type meal. Bilberry extracts from leaves (0.1 mg DE/mL emulsion) and fruits extracted with 55% aqueous EtOH (0.2 mg DE/mL emulsion) proved to be efficient inhibitors of lipid oxidation in

the BSA model in a similar level of 73%. Bilberry stems were less inhibitory than leaves with an inhibition level of 49%. This is supported by lower Total Polyphenol Content and Sum of Phenolic Compounds (UPLC) in leaves than in stems (Table III-2). The high protection of lipids by the leaf extract could be attributed to the presence of more than 78% of caffeic acid derivatives in the extract, among which 75% are *cis* and *trans* chlorogenic acids (Chapter I, Tables I-3 and I-4). Standard phenolic compounds were thus tested and *trans* chlorogenic acid showed an 82% inhibitory effect at the concentration of 100 µM. If one calculates the concentration of chlorogenic acids when brought through the extract, one would find 214 µM. Thus, chlorogenic acid appears more antioxidant when added in the emulsion as a molecule.The aqueous fruit extracts was the least efficient antioxidant extract (30% inhibition) in agreement with its lower TPC and Sum of Phenolic Compounds.

On the other hand, the bilberry leaf extract and fruit extract with 55% aqueous EtOH were slightly less inhibitory in the PL emulsion than in the BSA emulsion with inhibition rates of 59% for the leaf extract and 51% for the fruit extract with 55% aqueous EtOH (Table III-2; Figure III-1C).

The stem and aqueous fruit extracts were as efficient in both digestion models. The difference in antioxidant capacity observed for the stem and leaf extracts in the PL and BSA models may be ascribed to their respective compositions. Indeed, the stem extract from July 2013 contains 70% w/w of monomeric and oligomeric flavanols when the leaf extract contains 75% of chlorogenic acids (Table I-3). Flavanols are more hydrophobic molecules than chlorogenic acids and their non-covalent interactions with the BSA protein or phospholipids are clearly different. Flavanols and chlorogenic acids may thus locate differently in these emulsified systems. Therefore, their ability to reduce the prooxidant ferrylmyoglobin form may be different.

The antioxidant capacity of selected molecules representing the major phenolic sub-classes in bilberry and lingonberry extracts was evaluated. Chlorogenic acid (5-CQA), (–)-epicatechin (EC), procyanidin A2 (PCA A2) and cyanidin-3-galactoside (Cy-3-Gal) strongly inhibited metmyoglobin-initiated lipid oxidation by 82 to 95% in the BSA emulsion at pH 5 (Table III-2; Figure III-1D). This result clearly supports an antioxidant role for phenolic compounds in bilberry extracts of the various morphological parts.

Table III-2. Inhibition levels of bilberry extracts and various phenolic compounds (100 µM) in the BSA and PL models with $MbFe^{III}$ as the initiator at pH 5 after 4 h of kinetics.

Extracts[a]/ phenolic compounds	% Inhibition		*TPC (mg/g DM)*	*Sum of Phenolic Compounds (mg/g DE)*
	BSA model	*PL model*		
chlorogenic acid	82.0 ± 8.5	–		
(–)-epicatechin	95.2 ± 0.4	–		
procyanidin A2	90.9 ± 2.5	–		
cyanidin-3-galactoside	91.1 ± 0.8	–		
leaf extract	73.2 ± 4.7	58.8 ± 3.9	105.7 ± 6.0	124.9 ± 14.4
stem extract	49.1 ± 10.3	55.3 ± 4.0	78.8 ± 9.3	79.3 ± 1.7
fruit extract with H_2O	29.7 ± 9.5	29.4 ± 3.5	31.8 ± 1.2	26.9 ±1.7
fruit extract with EtOH55%	73.1 ± 3.4	50.8 ± 3.7	41.9 ± 1.7	41.1 ± 11.1

[a] Stem and leaf extracts were at 0.1 mg DE/mL in the emulsion whereas fruit extracts were at 0.2 mg DE/mL. Values represented mean ± SD (*n* = 3-4).

Owing to their significant antioxidant activity, leaf and stem bilberry extracts were also tested in the BSA emulsion with $MbFe^{III}$ as the initiator at pH 3, in conditions corresponding to the mid-phase of gastric digestion (Figure III-1B). Preliminary works also suggest that at pH 4, quercetin, rutin, and chlorogenic acid are less efficient inhibitors of lipid peroxidation (Lorrain et al., 2012) than at pH 6. Our results confirm these findings, leaf and stem bilberry extracts having no effect on the inhibition of the formation of lipid-derived conjugated dienes in the BSA emulsion at pH 3. When pH is below 4, metmyoglobin releases its hematin co-factor. Lipid oxidation is thus initiated by the planar protoporphyrin IX. In the absence of the globin part, phenolic compounds no longer come into the proximity of the iron center and are thus unable to act as antioxidants.

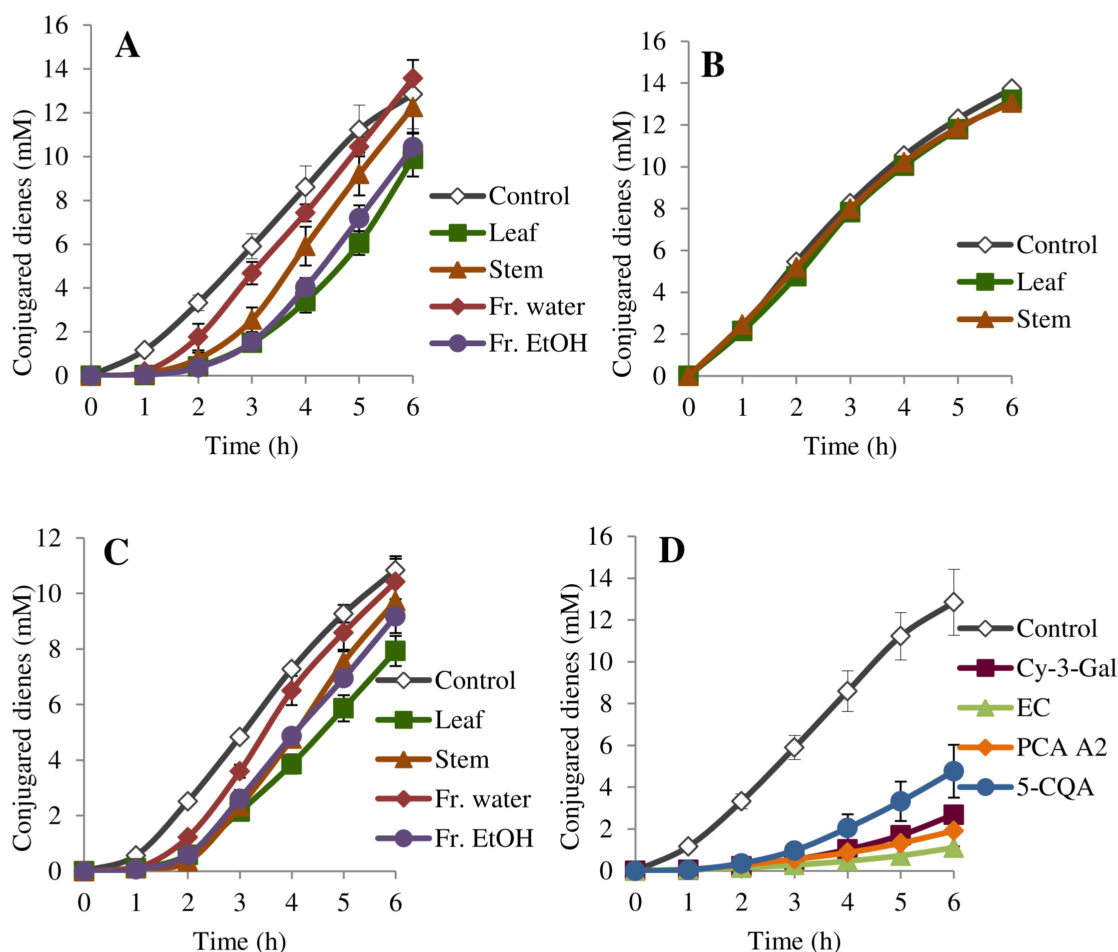


Figure III-1. Inhibition by bilberry extracts and by various polyphenols of the accumulation of conjugated dienes in the presence of metmyoglobin (20 µM) in BSA-stabilized emulsions at pH 5 (A, D) and pH 3 (B) and in PL-stabilized emulsions at pH 5 (C). Fr. water: fruit extract with H_2O. Fr. EtOH: fruit extract with 55% aqueous ethanol. Extract concentrations: leaf and stem extracts are at 0.1 mg DE/mL emulsion, both fruit extract are at 0.2 mg DE/mL emulsion. Polyphenol concentration = 100 µM. Bars represent the SDs for *n* = 3 (pH 5) and *n* = 2 (pH 3).

3.1.1.2. With Fe^{II} as the initiator

As leaf, stem and fruit with 55% aqueous EtOH extracts showed the most significant antioxidant activity with $MbFe^{III}$, their antioxidant capacity to inhibit of the Fe^{II}-initiated lipid oxidation was also evaluated in the BSA model at pH 5 and 3. First, the extent of lipid oxidation over the 6-hour long kinetics was 3-fold less with Fe^{II} than with metmyoglobin as the initiator. Additionally, a more linear pattern was exhibited by the Fe^{II}-initiated lipid oxidation. Leaf and stem extracts totally inhibited lipid oxidation when the ethanolic fruit extract was rather inactive (2% inhibition) in the early stage of gastric digestion at pH 5. When pH is decreased to pH 3, the Fe^{II}-initiated lipid

oxidation appeared very low with lipid-derived conjugated dienes amounting to 0.27 mM after 6 hours of kinetics (Figure III-2B). All the bilberry extracts displayed the same inhibitory effect in inhibiting lipid oxidation by 60-66% during the first four hours of the kinetics (Table III-3). Bilberry fruit anthocyanins were already found to show significant antioxidant activity toward Cu(II)-initiated lipid and protein oxidations in liposome systems (Viljanen, 2004). Anthocyanins are the major constituents of the bilberry fruit representing 84% in the 55% aqueous EtOH extracts. As reported by Lorrain et al. (2010, 2012), pH affects the prooxidant effect of initiators metmyoglobin and Fe^{II} in BSA and PL model emulsions.

Table III-3. Inhibition of lipid oxidation by bilberry extracts in the BSA model with Fe^{II} as the initiator at pH 5 and 3 after four hours of kinetics

Extracts[a]	% Inhibition	
	pH 5	*pH 3*
leaf	104.7 ± 16.8	66.4 ± 3.8
stem	101.5 ± 13.3	59.5 ± 11.9
fruit with EtOH55%	2.2 ± 7.9	61.9 ± 3.4

[a] Stem and leaf extracts were at 0.1 mg DE/mL in the emulsion whereas fruit extract was at 0.2 mg DE/mL. Values represented mean ± SD (n = 3).

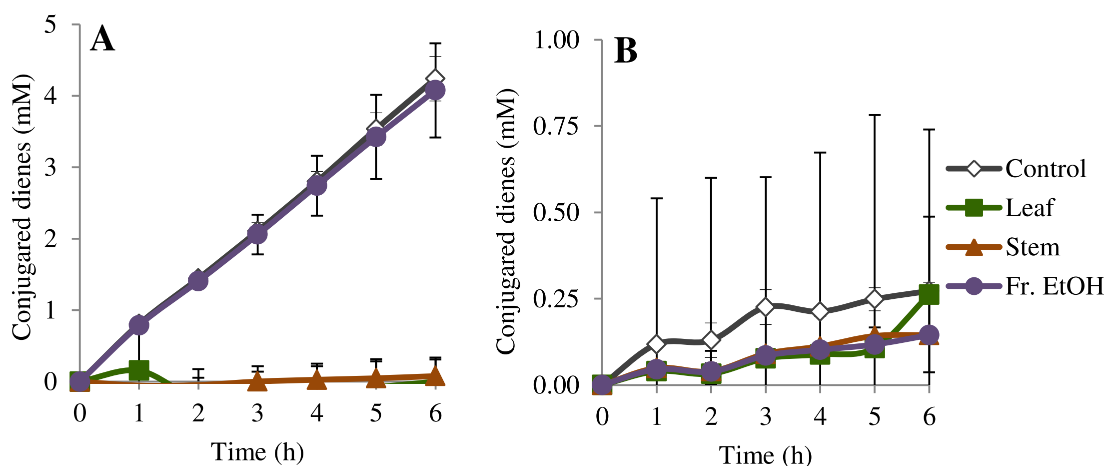


Figure III-2. Inhibition by bilberry extracts of the accumulation of conjugated dienes in the presence of Fe^{II} (200 µM) in BSA-stabilized emulsions at pH 5 (A) and pH 3 (B). Fr. EtOH: fruit extract with 55% aq. ethanol. Extract concentrations: leaf and stem extracts are at 0.1 mg DE/mL of emulsion; fruit extract are at 0.2 mg DE/mL. Bars represent the SDs for *n* = 3.

<u>3.1.2. Inhibition of lipid oxidation by lingonberry extracts</u>

Leaf and stem lingonberry extracts (0.1 mg DE/mL emulsion) were the most effective antioxidants toward the metmyoglobin-initiated lipid oxidation in both BSA (56% and 58%, respectively) and PL (59% and 42%, respectively) model emulsions (Figure III-3; Table III-4). Although 4-fold higher concentrations were used (0.4 mg DE/mL emulsion), the two aqueous and ethanolic fruit extracts were the least effective with inhibition rates of 8% and 12% in the BSA model, respectively. Surprisingly, both fruit extracts were more active in the PL model compared to the BSA model with similar inhibition rates of 21%. The weaker inhibition effect of fruit extracts appears to be due to the high content of sugar in fruit. In the earlier study on the phenolic composition of lingonberry extracts (Chapter II, Table II-3), the monomeric and oligomeric flavanols were present as the major phenolic compounds in leaves (63%), stems (73%), fruit extracted with H_2O (58%) and fruit extracted with 55% aqueous EtOH (54%). According to K. Asprogenidi (PhD thesis 2015), (–)-epicatechin and procyanidin oligomers (a trimer and an oligomeric fraction with an average degree of polymerization of 8) proved to be similarly inhibitory in the PL and BSA models at pH 5 (antioxidant activity expressed per (epi)catechin unit). Only quercetin proved to be more antioxidant in the PL model than in the BSA model. Consequently, these results suggest that monomeric and oligomeric flavanols are mainly responsible for the strong antioxidant activity of lingonberry extracts. Other contributors are quercetin derivatives and caffeic acid deivatives: all display the 1,2-dihydroxyphenyl moiety that is critical for the general antioxidant activity of phenolic compounds. A lingonberry extract titrated at 5% proanthocyanidins (flavanols) promoted an *in vivo* antioxidant protective effect in rats by decreasing the total oxidant status and favorably affected antioxidant defense enzymes (superoxide dismutase, glutathione reductase, and catalase) (Mane et al., 2011).

Table III-4. Inhibition of lipid oxidation by lingonberry extracts in the BSA and PL models with $MbFe^{III}$ as the initiator at pH 5 after four hours of kinetics

Extracts[a]/	% Inhibition		*TPC (mg/g DM)*	*Sum of Phenolic Compounds (mg/g DE)*
	BSA model	*PL model*		
leaf	56.2 ± 3.4	59.3 ± 2.2	93.1 ± 4.2	64.5 ± 2.6
stem	58.4 ± 0.6	41.8 ± 5.3	79.1 ± 4.7	93.1 ± 2.5
fruit with H_2O	8.1 ± 5.1	20.6 ± 3.9	13.5 ± 0.9	10.5 ±1.3
fruit with EtOH55%	12.0 ± 7.1	20.7 ± 9.3	17.2 ± 0.8	11.5 ± 1.8

[a] Stem and leaf extracts were at 0.1 mg DE/mL in the emulsion whereas fruit extract was at 0.4 mg DE/mL. Values represented mean ± SD (*n* = 3).

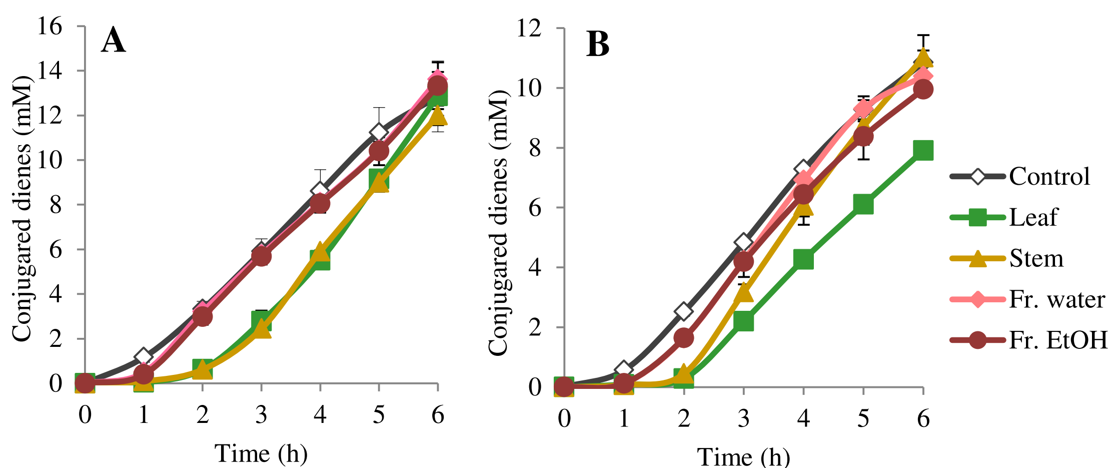


Figure III-3. Inhibition by lingonberry extracts of the accumulation of conjugated dienes in the presence of metmyoglobin (20 µM) at pH 5 in BSA-stabilized emulsion (A) and in PL-stabilized emulsion (B). Fr. water: fruit extract with H_2O. Fr. EtOH: fruit extract with 55% ethanol. Extract concentrations: leaf and stem extracts are at 0.1 mg DE/mL emulsion, both fruit extract are at 0.4 mg DE/mL emulsion. Bars represent the SDs for *n* = 3.

3.1.3 Inhibition of lipid oxidation: bilberry versus lingonberry extracts

The antioxidant capacity of the bilberry extracts was compared with that of the lingonberry extracts for both BSA and PL models, in the presence of metmyoglobin at pH 5 (Figure III-4). All the bilberry and lingonberry extracts inhibited almost totally the accumulation of the conjugated dienes during the first hour in BSA- and PL-stabilized emulsions. Lipid oxidation only resumes after 2 h for bilberry leaf, bilberry stem, bilberry fruit from 55% aqueous EtOH, lingonberry leaf, and lingonberry stem. These results are in agreement with the higher levels of phenolic compounds (TPC and Sum of Phenolic Compounds) in these extracts.

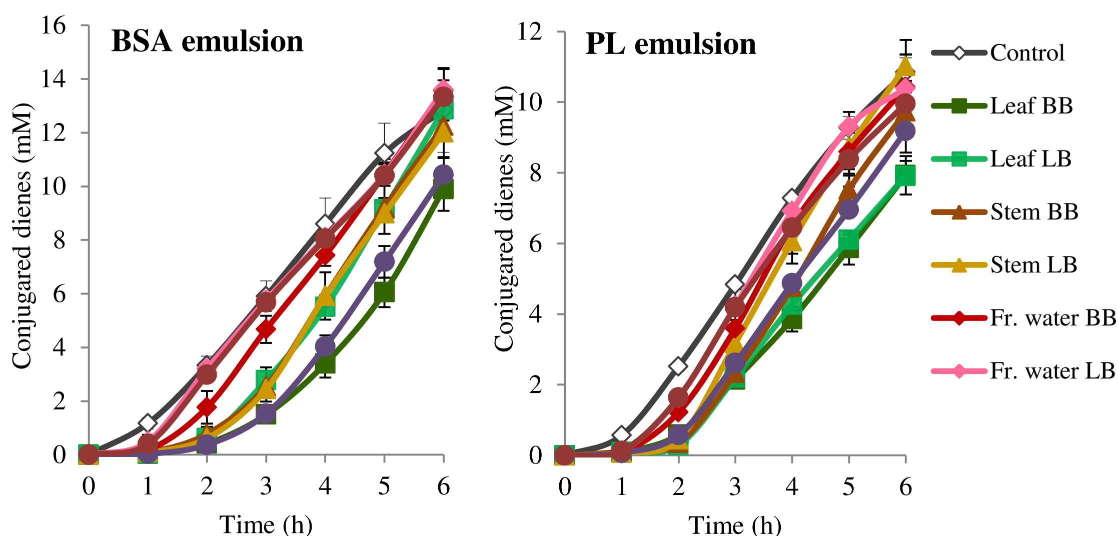


Figure III-4. Inhibition of the accumulation of conjugated dienes in the presence of metmyoglobin (20 µM) at pH 5 in BSA-stabilized emulsion and in PL-stabilized emulsion by bilberry extracts (BB) versus lingonberry extracts (LB).

In the BSA model, the inhibitory effect by bilberry and lingonberry extracts at 0.1 mg DE/mL decreased in the order (significantly at $p < 0.05$, 4 h of kinetics): bilberry leaf (73%) > lingonberry stem (58%) = lingonberry leaf (56%) > bilberry stem (49%).
When the PL model was considered, the capacity of bilberry and lingonberry extracts to inhibit the CDs accumulation ranked as follows (significantly at $p < 0.05$, 4 h of kinetics): bilberry leaf (59%) = lingonberry leaf (59%) = bilberry stem (55%) > lingonberry stem (42%). On the basis of these findings, bilberry and lingonberry leaf and stem extracts proved to be very similar inhibitors of the CD accumulation in the BSA and the PL models.

3.1.4. Inhibition of lipid oxidation by bilberry and lingonberry fruits

Lipid oxidation and its inhibition by bilberry and lingonberry fruits was evaluated in the PL model at pH 5 with initiator $MbFe^{III}$. The CD concentration vs time curves are presented in Figure III-5. Both bilberry and lingonberry fruits (2 mg DM/mL emulsion) highly inhibited the accumulation of lipid-derived conjugated dienes in the PL emulsion with inhibition levels reaching **94%** and **71%** during the first 4 h of the kinetics, respectively (Figure III-5).

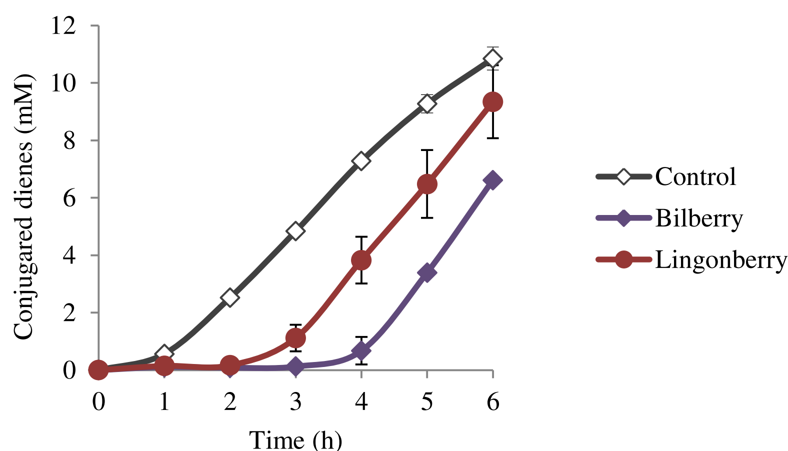


Figure III-5. Inhibition by bilberry and lingonberry fruits (2 mg DM/mL emulsion) of the accumulation of conjugated dienes in the presence of metmyoglobin (20 µM) at pH 5 in PL-stabilized emulsion. Bars represent the SDs for *n* = 3.

Their inhibitory capacity when comparing with the corresponding extracts (Section 3.1.1 and Section 3.1.2) increased significantly. However, the concentrations are higher for the fruits (2 mg DM/mL emulsion) than for the bilberry fruit extracts (0.2 mg DE/mL emulsion) and the lingonberry fruit extracts (0.4 mg DE/mL emulsion). Based on yield extracts of 87% and 68% for the aqueous extract and 55% aq. EtOH extract in 2014 (yields not measured in 2013), one can see that phenolic compounds are present at higher levels when brought by fruits supporting the stronger antioxidant effect observed for fruits.

Gobert et al. (2014) investigated the oxidation of dietary lipids and their inhibition by fruit & vegetables and the corresponding phenolic extract during the gastric digestion of a Western type diet ingested by minipigs. These authors showed less inhibition from the

phenolic extract compared to cubed fruit & vegetables on the CD accumulation. However, fruit & vegetables and the corresponding extract had a similar and markedly high inhibitory effect on the TBARS accumulation. To conclude, bilberry and lingonberry fruits as well as their extracts should be kept studying for their antioxidant properties.

3.2. Inhibition of lipid oxidation by bilberry leaf extract during digestion in an in vitro model of oro-gastro-intestinal digestion

In the present study, the metmyoglobin-initiated lipid oxidation in oil-in-water emulsions stabilized either by BSA or PL and its inhibition by an aqueous extract from bilberry leaf (6 mg/mL oral phase) was evaluated under simulated oral, gastric and intestinal conditions. During the gastric and intestinal phases of the *in vitro* digestion (2 h each), samples were collected every 30 minutes for the quantification of lipid-derived CD as primary markers of lipid oxidation. Why choosing a bilberry leaf extract for this study? The bilberry leaf extract from July 2013 was found to exhibit the best antioxidant activity toward lipid oxidation among the extracts as well as it shows the highest phenolic content and antioxidant capacity with the Folin method and DPPH test, respectively (Chapter I: Table I-3, Figure I-5).

Our results report the oxidizability of lipids and are expressed in µmoles of CD per gram of lipids. The successive dilutions during the digestion steps are taken into account. As expected from the previous results in Section III.3.1, lipid oxidation could be observed during the whole gastric step in both BSA and PL models (Figures III-6 and III-7). During the first hour at pH 5, metmyoglobin was likely the prooxidant form when for the next hour, at pH 3, hematin may have been released from $MbFe^{III}$. As observed earlier (Lorrain et al. 2010; K. Asprogenidi PhD thesis, 2015), lipid oxidation was more than 2-fold faster when the emulsifying agent was BSA rather than PL. The emulsifier type has a major influence on the rate of lipid digestion in the gastric step. In the intestinal phase, there were contrasted results. No oxidation of lipids could be evidenced in the BSA-stabilized emulsion when lipid oxidation kept proceeding although at a weaker rate for the PL-stabilized emulsion. In the intestinal step, emulsified lipids integrate mixed micelles owing to the addition of bile salts. Mixed micelles are very small structures containing free fatty acids, monoacyl- and diacyl glycerols as well as biliary salts, and with diameters lower than 10 nm. Thus, a difference in oxidizability was not expected for BSA and PL models.
In the presence of the bilberry leaf extract, lipid oxidation was almost totally inhibited in both the BSA and PL models during the whole gastric step. This is contrasting with results from *in vitro* models of gastric digestion which showed no inhibition by the leaf

extract at pH 3 (Figure III-1B). It may be due to the concentration of 3 mg/mL of bilberry leaf extracts in the gastric step that is 30-fold higher than that in the *in vitro* models of gastric digestion (0.1 mg/mL). With BSA, it may be outlined a very weak lipid oxidation between t0 and 120 min with the prooxidant form being likely hematin. Nevertheless, the leaf extract proved to be very efficient during gastric digestion.

During the inhibited intestinal digestion, the relative levels in CD increased from 162 to 226 µmol / g lipids over the two hours of study in the BSA model. By contrast, there was no noticeable oxidation of lipids in the PL model suggesting an antioxidant action of the leaf extract. In conclusion, the reactivity difference exhibited in the intestinal step by the BSA and PL models remain to be explained.

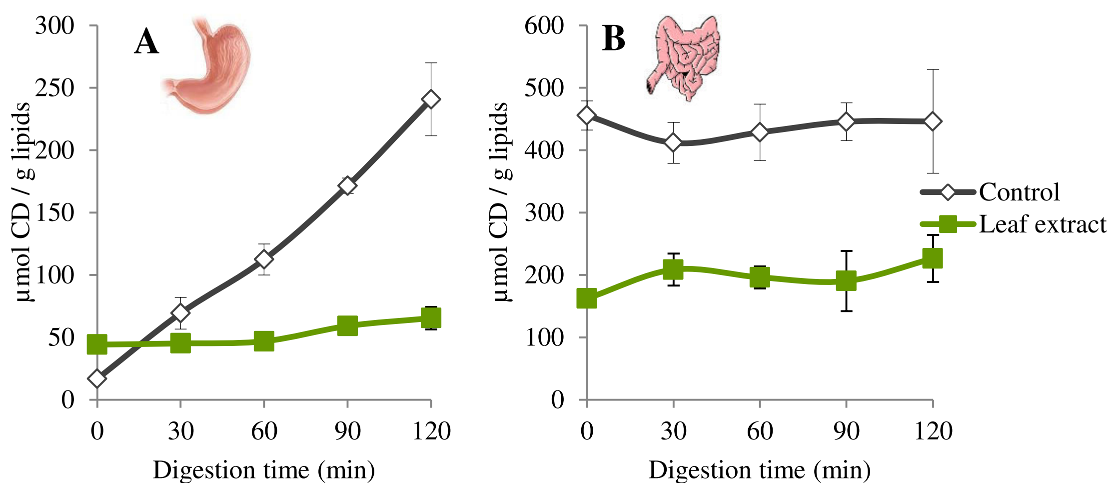


Figure III-6. Inhibition of the accumulation of conjugated dienes by a bilberry leaf extract in BSA-stabilized emulsions during the gastric (A) and intestinal (B) steps of digestion. Bars represent the SDs for *n* = 3.

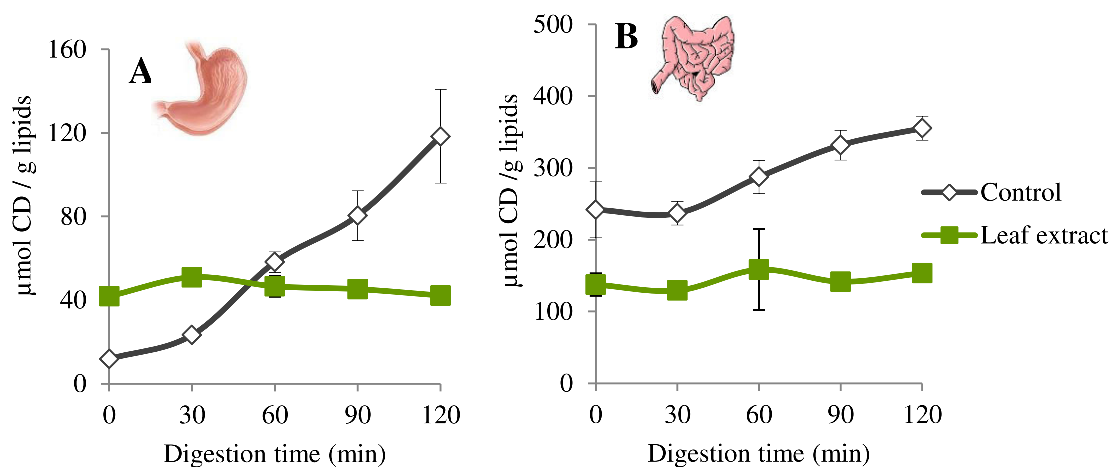


Figure III-7. Inhibition of the accumulation of conjugated dienes by a bilberry leaf extract in PL-stabilized emulsions during the gastric (A) and intestinal (B) steps of digestion. Bars represent the SDs for *n* = 3.

In the bilberry leaf extract, chlorogenic acid was found as the most abundant phenolic compound. The UV–Vis absorbance spectra between 250 and 450 nm for the isopropanol phase obtained at different times of the gastrointestinal digestion are displayed in Figure. III-8A and B. The first spectrum recorded for the BSA model at pH 5 with $MbFe^{III}$ as the initiator (GP – 0 min) showed a maximal absorption at 325 nm and a shoulder at ca. 295 nm (Figure III-8A) which are characteristic of chlorogenic acid (Figure III-8C). During gastric digestion, absorbance evolved to a relatively more intense band at 290 nm compared to that at 325 nm indicating the formation of degradation products from chlorogenic acid. These products likely no longer exhibit the conjugated carbon-carbon double bond located between the phenyl and the carboxyl groups of the caffeoyl unit. During the intestinal digestion, the absorption change wass more pronounced (Figure III-7B). Indeed, the spectral evolution between GP-120 and IP-0 may be assessed to the absorption of bile and pancreatin components, in particular at 270 nm. No further evolution was recorded upon intestinal digestion. Based on these results, it may be proposed that chlorogenic acid is an important contributor of the antioxidant activity of the bilberry leaf extract. New compounds are also formed upon its degradation.

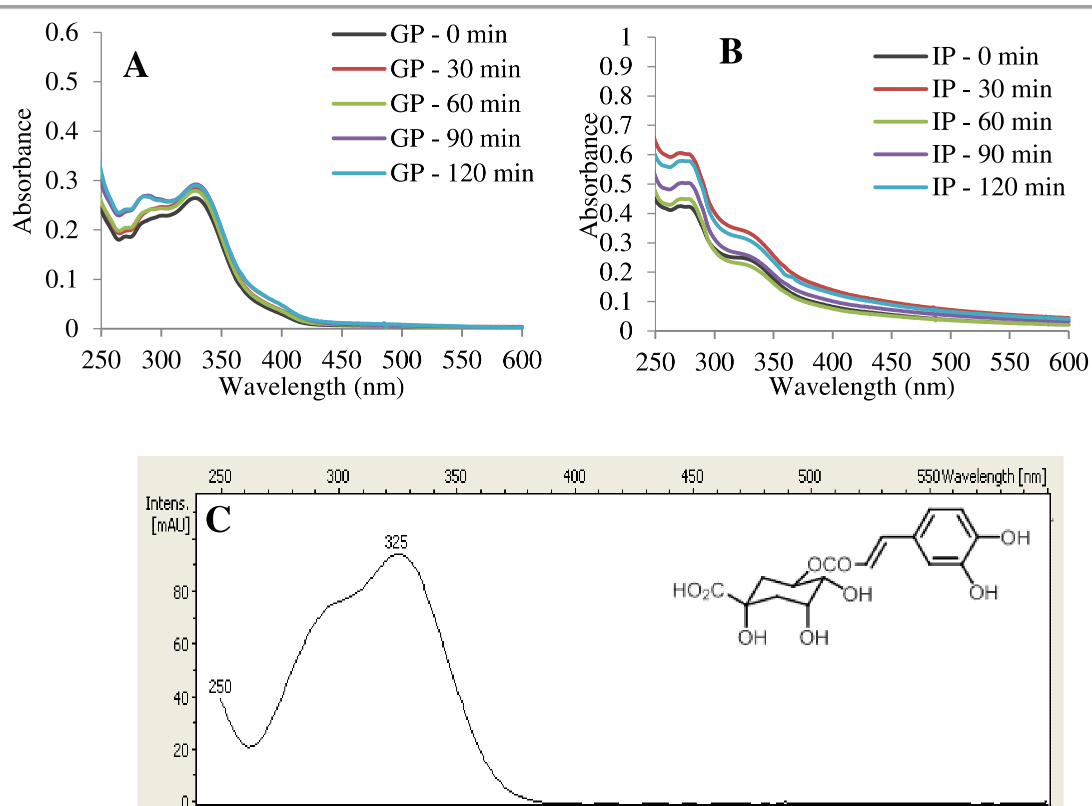


Figure III-8. UV–Vis spectra recorded for the bilberry leaf extract in the gastric (A) and intestinal (B) conditions in BSA model at pH 5 with MbFe^{III} as initiator and for chlorogenic acid (C). GP = Gastric Phase. IP = Intestinal Phase.

4. Conclusions

Leaf, stem and fruit extracts of bilberry and lingonberry can play a protective role towards polyunsaturated dietary lipids in the early step of gastric digestion (pH 5). In both protein-and phospholipid-emulsified systems, aqueous extracts from stems and leaves and the hydroethanolic fruit extract of bilberry proved to be the most efficient inhibitors of metmyoglobin-initiated lipid oxidation. They were rich in monomeric and oligomeric flavanols, caffeoyl derivatives and anthocyanins (fruits), respectively. Lingonberry extracts from leaves and stems showed a slightly weaker protective effect in both emulsion models when both lingonberry fruit extracts were the least effective ones. They were rich in monomers and oligomers of catechin and epicatechin and quercetin glycosides, respectively. Powdered fruits of lingonberry and bilberry exhibited interesting antioxidant capacities indicating that they could be used directly without extract preparation. In the second part of the digestion, at pH 3, extracts of bilberry and lingonberry had no inhibitory effect on lipid oxidation. Berry polyphenols were unable to reduce the prooxidant form of hematin as no interaction could develop between these species in the absence of the globin part. As observed in earlier works of the INRA-SQPOV team, lipid oxidation in oil-in-water emulsions and its inhibition by lingonberry and bilberry samples was obviously influenced by the emulsifier type, pH and the initiator form.

Next in this work, an oro-gastro-intestinal static model of digestion was implemented for the first time combining both our previous emulsion system for food modeling and the best physiological conditions ever described through the recommendations by the COST network InfoGest (Minekus et al., 2012). The obtained results gave us the first „image" of the real antioxidant activity of bilberry leaves in human digestion. The fast lipid oxidation in the gastric step (BSA and PL emulsion systems) and the slower lipid oxidation in the intestinal step (PL system) were totally inhibited by a bilberry leaf extract at the level of 3 mg/mL in the gastric step. Given the high content of phenolic compounds in the bilberry leaves, the bioaccessibility of the phenolic compounds should be further evaluated in the gastrointestinal digestion to give an insight into the protective mechanisms of phenolic compounds.

ANNEXES

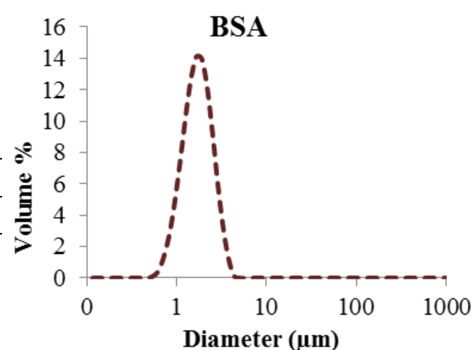
Annexe III-1. Characterization of the BSA and PL model emulsions by the laser granulometry

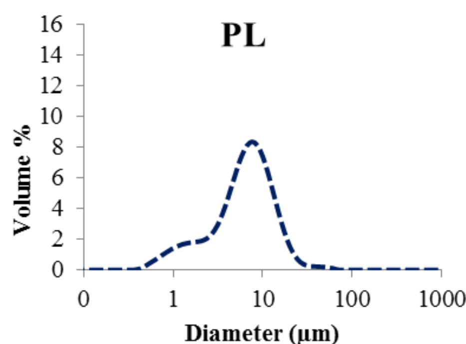
The size distribution of the lipid droplets was determined before the addition of metmyoglobin in the BSA model and after the addition of the metmyoglobin in the PL model. As demonstrated by Lorrain (2008) in the BSA model, the addition of metmyoglobin did not change the size of lipid droplets. The experiments were performed with a laser particle size analyzer (Mastersizer 2000, Malvern Instruments, Orsay, France). Three emulsion droplets were dispersed in distilled water and analyzed three times each. Calculations were performed using the Mie theory with the following refractive indexes: 1.475 for oil, 1.333 for the dispersant (water), and an absorbance value of 0.01 for the emulsion particle. The mean diameter was reported as the surface-weighted diameter $d_{3,2}$ while the mode was assessed as the most frequent diameter for this distribution.

The size distribution of the lipid droplets for the BSA and PL emulsion models at pH 5:

Characteristics of size of lipid droplets	Emulsion system	
	BSA[a]	**PL**[b]
d(3,2) (μm)		
t0	1.60 ± 0.06	6.06 ± 0.08
1h	–	7.81 ± 0.32
2h	–	8.85 ± 0.33
5h	–	7.03 ± 2.96
Mode (μm)		
t0	1.92 ± 0.10	12.5 ± 6.51
1h	–	27.6 ± 0.07
2h	–	33.1 ± 0.07
5h	–	23.1 ± 0.71

[a]Mean ± SD for n = 3 granulomery analyses of 1 emulsion.
[b]Mean ± SD for n = 2 independent emulsions with 3 granulomery analyses each. –: not determined.





Size distribution for the emulsion particules stabilized by BSA and PL

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis aimed to assess the seasonal variations of phenolic compounds in leaves, stems, and fruits of bilberry and lingonberry by comparing the total phenolic content, the phenolic composition and the antioxidant activity at different periods of vegetation (May, July and September) during two years (2013 and 2014). In addition, the evaluation of *in vitro* antioxidant activity of fruits, leaves and stems of bilberry and lingonberry extracts and their phenolic compounds in lipid oxidation under simulated digestion conditions was investigated.

This research reports the most complete qualitative and quantitative analysis ever conducted on phenolics from bilberry and lingonberry leaves, stems and fruits. The characterisation of procyanidins in leaves and stems from bilberry and lingonberry by thioacidolysis in order to determine the subunit composition and the average degree of polymerization (mDP) was conducted for the first time. In particular, this study originally demonstrates the protective capacity against lipid oxidation of bilberry and lingonberry extracts in an *in vitro* model of gastric digestion using two different emulsion systems as physico-chemical models of the gastric content as well as in a complete static *in vitro* digestion model.

In the first study of this thesis, "*Phenolic constituents in bilberry (Vaccinium myrtillus L.): accumulation in leaves, stems and fruits at different harvest periods and antioxidant activity",* structures were newly detected or named for 45 compounds among which several *p*-coumaroyldi- and triacetyl glycosides, caffeoyl- and *p*-coumaroylmalonyl glycosides, quercetin glycosides, and various A-type and B-type flavanol oligomers up to the tetramers. The more important groups in bilberry extracts were in the following order: caffeoyl derivatives, *p*-coumaroyl derivatives, flavon glycosides, anthocyanins, and flavanol monmers and oligomers. In general, these major contributors did not show any seasonal variations. Thioacidolysis revealed low degrees of polymerization (2-3) and (-)-epicatechin as the main flavan-3-ol 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 those of May leaves. The latter were

relatively richer 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 maximum in July for bilberry stems. The intra-annual variations for the various phenolic groups were mostly different for years 2013 and 2014.

In the second study of this research, "*Phenolic profile and antioxidant activity of leaf, stem and fruit extracts of lingonberry (Vaccinium vitis-idaea L.) at three vegetative stages",* qualitative and quantitative analyses by UPLC/MS showed the predominant presence of monomers and oligomers of catechin and epicatechin and quercetin glycosides in all the morphological parts. The structures of fifty phenolic compounds detected in the lingonberry extracts were characterized for the first time. Proanthocyanidins contain (+)-catechin and (-)-epicatechin as both extension and terminal units. Regarding the harvest period, both lingonberry stems and leaves showed a slight but significant increase of the Total Phenolic Content and the Sum of Phenolic Compounds (UPLC) from May to September. A similar trend was confirmed for the antioxidant activity by the DDPH test for both stems and leaves in 2014 and TPC for leaves in 2014. Among the three periods of vegetation, leaves and stems can be collected in any one, May, July or September, as sustainable sources of natural antioxidants but July, or better, September, should be favored.

In the third study of this thesis, "*Lipid protection from oxidation by bilberry and lingonberry phenolic extracts",* aqueous extracts from stems and leaves and ethanolic fruits extract of bilberry proved to be the most efficient inhibitors of metmyoglobin-initiated lipid oxidation in the early phase of digestion at pH 5. Lingonberry extracts from leaves and stems were also effective antioxidants toward the metmyoglobin-initiated lipid oxidation in both BSA and PL model emulsions. Extracts of bilberry and lingonberry showed very low inhibitory effect on the lipid oxidation in the midcourse of digestion at pH 3. Both powdered fruits of bilberry and lingonberry highly inhibited the accumulation of lipid-derived conjugated dienes indicating that they can be used directly without extract preparation. Last in this study, the fast lipid oxidation in the gastric step (BSA and PL systems) and the slower lipid oxidation in the intestinal step (PL

system) were totally inhibited by the bilberry leaf extract in an oro-gastro-intestinal static model of digestion.

Results from this study indicate that all the morphological parts of bilberry are suitable for valorization as sources of natural phenolic compounds al well as to be valuable feedstocks for the production of herbal supplements. In addition, leaf, stem and fruit extracts of bilberry and lingonberry can play a protective role towards polyunsaturated dietary lipids. The harvest period can be selected based on the desired phenolic structures and for a desired biological activity. However the stability of the collected dry matter remains to be assessed to determine its optimal shelf life.

This research gives us a first „image” of the potential antioxidant activity of bilberry leaves and more complex analyses requires to understand its real effect on lipid oxidation during digestion. Given the high content of phenolic compounds and the significant antioxidant activity of bilberry leaves, which were found to correlate strongly, the evaluation of the phenolic compounds bioaccessibility during the gastrointestinal digestion may be taken into account.

References of thesis

1. Aaby, K., Grimmer, S., & Holtung L. (2013). Extraction of phenolic compounds frombilberry (*Vaccinium myrtillus* L.) press residue: effects on phenolic composition and cell proliferation. *LWT - Food Science and Technology, 54*, 257-264.

2. Akerström, A., Jaakola, L., Bång, U., & Jäderlund, A. (2010). effects of latitude-related factors and geographical origin on anthocyanidin concentrations in fruits of *Vaccinium myrtillus* L. (bilberries). *Journal of Agricultural and Food Chemistry, 58*, 11939–11945.

3. Alhosin, M., León-González, A. J., Dandache, I., Lelay, A., Rashid, S. K., Kevers, C., Pincemail, J., Fornecker, L. M., Mauvieux, L., Herbrecht, R., & Schini-Kerth, V. B. (2015). Bilberry extract (Antho 50) selectively induces redox-sensitive caspase 3-related apoptosis in chronic lymphocytic leukemia cells by targeting the Bcl-2/Bad pathway. *Scientific Reports, 5*(8996), 1-10.

4. Andersen O.M. (1985). Chromatografic separation of anthocyanins in Cowberry (Lingonberry) *Vaccinium vites-idaea* L. *Journal of Food Science*, 50, 1230–1231.

5. Andersen O.M., & Markham K.R. (2006). Flavonoids: Chemistry, Biochemistry and Applications. *CRC Press*, ISBN 978-0-8493-2021-7.

6. Apetrei C.L., Tuchilus C, Aprotosoaie A.C., Adrian Oprea A., Malterud K.E., & Miron A. (2011). Chemical, antioxidant and antimicrobial investigations of *Pinus cembra* L. bark and needles. *Molecules*, *16*, 7773-7788.

7. Argyri, K., Komaitis, M., & Kapsokefalou, M. (2006). Iron decreases the antioxidant capacity of red wine under conditions of in vitro digestion. *Food Chemistry, 96,* 281–289.

8. Asprogenidi, K., (2015). Protection of lipids from oxidation by dietary polyphenols in gastric-like conditions. *PhD thesis*, Université d'Avignon et des Pays du Vaucluse.

9. Atawodi S.E. (2010). Polyphenol composition and *in vitro* antioxidant potential of nigerian *Canarium schweinfurthii* engl. Oil. *Advances in Biological Research*, 4(6): 314-322.

10. Balas A., & Popa V.I. (2007a). On characterization of some bioactive compounds extracted from *Picea abies* bark. *Roumanian Biotechnological Letters*, *12*(3), 3209-3215.

11. Balas A., & Popa V.I. (2007b). The influence of natural aromatic compounds on the development of *Lycopersicon esculentum* plantlets. *Bio/Resources, 2*(3), 363-370.

12. Baroffio C., Carron C-A., & Carlen C. (2012). Culture de canneberge et d'airelle en Suisse: utopie ou réalité?, *Revue suisse Viticulture*, Arboriculture, Horticulture, *44* (5), 280–286.

13. Benzie I. F. F., & Wachtel-Galor S. (editors). (2011). Boca Raton (FL):CRC Press, Available from: http://www.ncbi.nlm.nih.gov/books/NBK92770/.

14. Berton-Carabin, C. C., Ropers, M-H., & Genot, C. (2014). Lipid oxidation in oil-in-water emulsions: involvement of the interfacial layer. *Comprehensive Reviewsin Food Scienceand Food Safety, 13*, 945-977.

15. Bimakr M., Rahman A.R., Taip F.S., Chuan L.T., Ganjloo A., Selamat J., & Hamid A. (2009). Supercritical carbon dioxide (SC-CO_2) extraction of bioactive flavonoid compounds from spearmint (*Mentha Spicata L.*) leaves. *European Journal of Scientific Research,* ISSN 1450-216X, *33*(4), 679-690.

16. Blamey M., & Grey-Wilson C. (2003). La flore d'Europe occidentale, Plus de 2400 plantes décrites et illustrées en couleur. *Editor Flammarion*.

17. Bodarlau R., Teaca, C. A., & Popa, V. I. (2002). Utilization of vegetation for *in situ* bioremediation of contaminated soils. *Environmental Engineering and Management Journal, 1*(1), 67-78.

18. Bojor O. (2003). Ghidul plantelor medicinale și aromatice de la A la Z. *Editura Fiat Lux*, București, ISBN 973-9250-68-8.

19. Brescia J. P. (2012). Determination of antioxidant potential using an oxygen radical absorbance capacity (ORAC) assay with synergy™ H4. *Applications Scientist, BioTek Instruments*, Inc., Winooski, VT.

20. Bujor. O-B., Talmaciu, I. A., Volf, I., & Popa I. V. (2015). Biorefining to recover aromatic compounds with biological properties. *Tappi Journal*, *14(3)*, 187-193.

21. Bunea, A., Rugina, D., Pintea, A., Andrei, S., Bunea, C., Pop, R., & Bele, C. (2012). Carotenoid and fatty acid profiles of bilberries and cultivated blueberries from Romania. *Chemical Papers*, *66*(10), 935–939.

22. Burdulis, D., Ivanauskas, L., Dirsė, V., Kazlauskas, S., & Ražukas, A. (2007). Study of diversity of anthocyanin composition in bilberry *(Vaccinium myrtillus* L.) fruits. *Medicina Kaunas*, *43*(12), 971-977.

23. Burdulis, D., Sarkinas, A., Jasutiene, I., Stackeviciene, E., Nikolajevas, L., & Janulis, V. (2009). Comparative study of anthocyanin composition, antimicrobial and antioxidant activity in bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) fruits. *Acta Poloniae Pharmaceutica - Drug Research*, *66*(4), 399-408.

24. Castillo, F., Hernández, D., Gallegos, G., Rodríguez, R., & Aguilar, C. N. (2012). Antifungal properties of bioactive compounds from plants, Fungicides for plant and animal diseases. *Dr. Dharumadurai Dhanasekaran (Ed.)*, ISBN: 978-953-307-804-5.

25. Casas Cardoso, L.C., Mantell, Serrano C., Torrez Quintero, E., Pereyra López, C., Medrano Antezana, R., & Martínez de la Ossa, E.J. (2013). High pressure extraction of antioxidants from *Solanum stenotomun* peel. *Molecules*, *18*, 3137-3151.

26. Canter, P. H., & Ernst, E. (2004). Anthocyanosides of *Vaccinium myrtillus* (Bilberry) for night vision-a systematic review of placebo-controlled trials. *Survey of ophthalmology, 49*(1), 38-50.

27. Chanforan, C., Loonis. M., Mora, N., Caris-Veyrat, C., & Dufour, C. (2012). The impact of industrial processing on health-beneficial tomato microconstituents. *Food Chemistry*, *134*, 1786–1795.

28. Chemat, F., Zill-e-Huma, & Khan, M. K. (2011). Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry, 18*, 813–835.

29. Chemat, F., Vian, M. A., & Cravotto, G. (2012). Green extraction of natural products: concept and principles. *International Journal of Molecular Sciences*, *13*, 8615-8627.

30. Chen, H., & Zuo, Y. (2007). Identification of flavonol glycosides in American cranberry fruit. *Food Chemistry, 101*, 1357–1364.

31. Cheynier, V. (2005). Polyphenols in foods are more complex than often thought. *American Society for Clinical Nutrition,* 81, 223S–229S.

32. Cheynier V. (2012). Phenolic compounds: from plants to foods, *Phytochemistry Reviews*, 11, 153–177.

33. Chu, W., Cheung, S. C. M., Lau, R. A. W., & Benzie, I. F. F., (2011). Chapter 4. Bilberry (*Vaccinium myrtillus* L.) in herbal medicine: biomolecular and clinical aspects (2nd edition).

34. Clifford, M. N., Knight, S., & Kuhnert, N. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. *Journal of Agricultural and Food Chemistry*. *53*, 3821-3832.

35. Craft, D. B., Kerrihard, L. A., Amarowicz, R., & Pegg, B. R. (2012). Phenol-based antioxidants and the *in vitro* methods used for their assessment. *Comprehensive Reviews in Food Science and Food Safety, 11*(2), 148-173.

36. Cunha, R. W., Andrade de Silva, M. A., Veneziani, R. C. S., Ambrósio, S. R., & Bastos J. K. (2012). Lignans: chemical and biological properties, phytochemicals - a blobal perspective of their role in nutrition and health. *Dr Venketeshwer Rao (Ed.)*, ISBN: 978-953-51-0296-0.

37. Dai, J., & Mumper, & J. R. (2010). Plant Phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules, 15*, 7313-7352.

38. Danaila, M., Popa, V. I., & Volf, I. (2007). Influence of polyphenols on the biosynthesis of carotenoids by yeast. *Proceedings of the 8th ILI Forum*, Rome, Italy, 10-12, 105-108.

39. Danaila, M. (2009). Contributions to the study of influence on natural products with aromatic structure on microorganisms development. *PhD Thesis*, Gheorghe Asachi Technical University of Iasi.

40. Dangles, O. (2012). Antioxidant activity of plant phenols: chemical mechanisms and biological significance. *Current Organic Chemistry*, *16*(2), 1-23.

41. Davidson, E., Zimmermann, F. B., Jungfer, E., & Chrubasik-Hausmann, S. (2014). Prevention of urinary tract infections with *Vaccinium* products. *Phytotherapy Research, 28*, 465-70.

42. Del Rio, D., Borges, G., & Crozier, A. (2010). Berry flavonoids and phenolics: bioavailability and evidence of protective effects. *British Journal of Nutrition, 104*, S67–S90.

43. Denev P., Ciz M., Ambrozova G., Lojek A., Yanakieva I., & Kratchanova M. (2010). Solid-phase extraction of berries' anthocyanins and evaluation of their antioxidative properties. *Food Chemistry*, 123, 1055–1061.

44. Dent, M., Dragović–Uzelac, V., Penić, M., Brnčić, M., Bosiljkov, T., & Levaj, B. (2013). The effect of extraction solvents, temperature and time on the composition

and mass rraction of polyphenols in dalmatian wild sage (*Salvia officinalis* L.) extracts. *Food Technology and Biotechnology*, *51*(1), 84–91.

45. Díaz-García, M. C., Obón, J. M., Castellar, M. R., Collado, J., & Alacid, M. (2013). Quantification by UHPLC of total individual polyphenols in fruit juices. *Food Chemistry, 138*, 938–949.
46. Duarte, C. A. R, & Duarte, M. M. C. (Eds.) (2009). Current trends of supercritical fluid technology in pharmaceutical, nutraceutical and rood processing industries, *Bentham Science Publishers*, Bussum, The Netherlands, ISBN: 978-1-60805-046-8.
47. Dulf, V. F., Andrei, S., Bunea, A., & Socaciu, C. (2012). Fatty acid and phytosterol contents of some Romanian wild and cultivated berry pomaces. *Chemical Papers, 66* (10), 925–934.
48. Ek, S., Kartimo, H., Mattila, S., & Tolonen, A. (2006). Characterization of phenolic compounds from lingonberry (*Vaccinium vitis-idaea*). *Journal of Agricultural and Food Chemistry*, *54*, 9834-9842.
49. El Gharras H. (2009). Polyphenols: food sources, properties and applications - a review. *International Journal of Food Science and Technology, 44,* 2512–2518.
50. Erdogan, E., Ates, B., Durmaz, G., Yilmaz, I., & Seckin, T. (2011). Pressurized liquid extraction of phenolic compounds from Anatolia propolis and their radical scavenging capacities. *Food and Chemical Toxicology, 49,* 1592–1597.
51. Erlund, I., Koli, R., Alfthan, G., Marniemi, J., Puukka, P., Mustonen, P., Mattila, P., & Jula, A. (2008). Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *The American Journal of Clinical Nutrition, 87*, 323–31.
52. Escribano-Bailon, M. T., & Santos-Buelga, C. (2003). Polyphenols extraction from eoods. *in*: Methods in polyphenol analysis (eds. C. Santos-Buelga, G. Williamson). *Royal Society of Chemistry*, Cambridge, United Kingdom, pp. 1–16.
53. Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). A thorogh study of reactivity of various compounds classes towards the Folin-Ciocalteu reagent. *Journal of Agricultural and Food Chemistry*, *58(14)*, 8139–8144.
54. Faria A, Oliveira J, Neves P, Gameiro P, Santos-Buelga C, de Freitas V, & Mateus N. (2005). Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts, *Journal of Agricultural and Food Chemistry*, *53*, 6896-6902.
55. Ferrazzano, F. G., Amato, I., Ingenito, A., Zarrelli, A., Gabriele, Pinto, G., & Pollio, A. (2011). Plant polyphenols and their anti-cariogenic properties: a review. *Molecules, 16,* 1486-1507.
56. Ferruzzi, M. G., Böhm, V., Courtney, P. D., & Schwartz, S. J. (2002). Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays. *Journal of Food Science, 67*, 2589–2595.
57. Fischer E. (2000). Dicționarul plantelor medicinale – Ghid practic pentru cultivarea, recoltarea și utilizarea palntelor medicinale și aromatice. Editor Gemma Pres, București, ISBN: 973-9398-03-0.

58. Frank, J., Budek, A., Lundh, T., Parker, R. S., Swanson, J. E., Lourenço C. F., Gago, B, Laranjinha, J., Vessby, B., & Kamal-Eldin, A. (2006). Dietary flavonoids with a catechol structure increase α-tocopherol in rats and protect the vitamin from oxidation in vitro. *Journal of Lipid Research, 47*, 2718-2725.

59. Frostegård, J. (2013). Immunity, atherosclerosis and cardiovascular disease. *BMC Medicine*, 11(117).

60. Fulcrand, H., Mané, C., Preys, S., Mazerolles, G., Bouchut, C., Mazauric, J-P, Souquet, J-M, Meudec, E, Li, Y., Cole, R. B., & Cheynier, V. (2008). Direct mass spectrometry approaches to characterize polyphenol composition of complex samples, *Phytochemistry, 69*, 3131–3138.

61. Fursova A. Z., Gesarevich, O. G., Gonchar, A. M., Trofimova, N. A., & Kolosova, N. G. (2005). Dietary supplementation with bilberry extract prevents macular degeneration and cataracts in senescent accelerated OXYS rats. *Advances in Gerontology*, *16*, 176-179.

62. Garcia-Salas, P., Morales-Soto, A., Segura-Carretero, A., & Fernández-Gutiérrez, A. (2010). Phenolic-compound-extraction systems for fruit and vegetable samples, *Molecules, 15*, 8813-8826.

63. Gharras, E. H. (2009). Polyphenols: food sources, properties and applications. *International Journal of Food Science and Technology, 44*, 2512–2518.

64. Ghiselli, A., Serafini, M., Natella F., & Scaccinic, C. (2000). Total antioxidant capacity as a tool to assess redox status: critical view and experimental data. *Free Radical Biology & Medicine, 29*(11), 1106–1114.

65. Giovinazzo, G., Ingrosso, I., Paradiso, A., De Gara, L., & Santino, A. (2012). Resveratrol biosynthesis: plant metabolic engineering for nutritional improvement of food. *Plant Foods for Human Nutrition, 67,* 191–199.

66. Gobert, M., Rémond. D., Loonis, M., Buffière, C., Santé-Lhoutellier, V., & Dufour, C. (2014). Fruits, vegetables and their polyphenols protect dietary lipids from oxidation during gastric digestion. *Food & Function*, *5*, 2166-2174.

67. Goupy, P., Dufour, C., Loonis, M., & Dangles, O. (2003). Quantitative Kinetic Analysis of Hydrogen Transfer Reactions from Dietary Polyphenols to the DPPH Radical, *Journal of Agricultural and Food Chemistry, 51*(3), 615-622.

68. Goupy, P., Vulcain, E., Caris-Veyrat, C., & Dangles, O. (2007). Dietary antioxidants as inhibitors of the heme-induced peroxidation of linoleic acid: mechanism of action and synergism, *Free Radical Biology & Medecine*, 43, 933-946.

69. Goupy, P., Bautista-Ortin, A. B., Fulcrand, H., & Dangles, O. (2009). Antioxidant activity of wine pigments derived from anthocyanins: hydrogen transfer reactions to the DPPH radical and inhibition of the heme-induced peroxidation of linoleic acid. *Journal of Agricultural and Food Chemistry, 57*, 13, 5762-5770.

70. Grace, M. H., Esposito, D., Dunlap, K. L., & Lila, M. A. (2014). Comparative analysis of phenolic content and profile, antioxidant capacity and anti-inflammatory bioactivity in wild Alaskan and commercial *Vaccinium* berries. *Journal of Agricultural and Food Chemistry*, 62(18): 4007-4017.

71. Guyot, S., Marnet, N., Laraba, D., Sanoner, P., Drilleau, J.-F. (1998). Reversed-phase HPLC following thiolysis for quantitative estimation and characterization

of the four main classes of phenolic compounds in different tissue zones of a french cider apple variety (*Malus domestica* Var. Kermerrien). *Journal of Agricultural and Food Chemistry, 46*, 1698-1705.

72. Guyot, S., Marnet, N., & Drilleau, J-F. (2001). Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *Journal of Agricultural and Food Chemistry, 49,* 14-20.

73. Hainal, A. R., Ignat, I., Volf, I., & Popa, V. I. (2010). Study of the influence of aqueous extracts from *Asclepias syriaca* on the development of species of *Rhodotorula* sp. *Lucrări ştiinţifice, Seria Horticultură*, Editura "Ion Ionescu de la Brad" Iaşi, *53*(1), 603-608.

74. Hainal, A. C., Ignat, I., Volf, I., & Popa, I. V. (2011). Transformation of polyphenols from biomass by some yeast species, *Cellulose Chemistry and Technology, 45* (3-4), 211-219.

75. Hainal, A. C., Diaconescu, R., Volf, I. & Popa, V. I. (2012a). Studies concerning some possibilities of obtaining carotenoid pigments by cultivation of *Rhodotorula* spp. in the presence of *Asclepias syriaca* extracts. *Romanian Biotechnological Letters*, *17*(*2*), 7084-7092.

76. Hainal, A. C, Capraru, A. M., Volf, I., & Popa, V. I. (2012b). Lignin as a carbon source for the cultivation of some *Rhodotorula* species. *Cellulose Chemistry and Technology, 46 (1-2),* 87-96.

77. Han, R-M., Zhang, J-P., & Skibsted, L. H. (2012). Reaction dynamics of flavonoids and carotenoids as antioxidants. *Molecules, 17*, 2140-2160.

78. Harris, C. S., Burt, A. J., Saleem, A., Le P. M., Martineau, L. C., Haaddad, P. S., Bennet, S. A. L., & Arnason J. T. (2007). A single HPLC-PAD-APCI/MS method for the quantitative comparison of phenolic compounds found in leaf, stem, root and fruit extracts of *Vaccinium angustifolium*, *Phytochemical Analysis, 18*, 161–169.

79. Hokkanen, J., Mattila, S., Jaakola, L., Pirttilä, A. M., & Tolonen. A. (2009). Identification of phenolic compounds from lingonberry (*Vaccinium vitis-idaea* L.), bilberry (*Vaccinium myrtillus* L.) and hybrid bilberry (*Vaccinium x intermedium* Ruthe L.) leaves, *Journal of Agricultural and Food Chemistry, 57*, 9437–9447.

80. Ienaşcu, M. C. I., Balcu, I., Segneanu, E. A., Căta, A., & Damian, D. (2009). Anthocyanins profile of *Vaccinium myrtillus* alcoholic extracts revealed by electrospray ionization/mass spectrometry. *Ovidius University Annals of Chemistry*, *20*(1), 76-79.

81. Ieri, F., Martini, S., Innocenti, M., & Mulinacci, N. (2013). Phenolic distribution in liquid preparations of *Vaccinium myrtillus* L. and *Vaccinium vitis idaea* L., *Phytochemical Analysis, 24*, 467–475.

82. Ignat, I., Stingu, A., Volf, I., & Popa, V. I. (2009). Natural bioactive compounds as plant growth regulators. *Lucrări Ştiinţifice – seria Agronomie, 52.*

83. Ignat, I., Neagu, V., Bunia, I., Paduraru, C., Volf, I., & Popa, V. I. (2011a). A comparative study concerning the adsorption ofgallic acid onto polymeric adsorbents with amine functional groups. *Cellulose Chemistry and Technology*, *45* (3-4), 251-256.

84. Ignat, I., Volf, I., & Popa, V. I. (2011b). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry, 126,* 1821-1835.

85. Ignat, I., Stingu, A., Volf, I., & Popa, V. I. (2011c). Characterization of grape seeds aqueous extract and possible application in biological system. *Cellulose Chemistry and Technology*, *45*, 205–209.

86. Ignat, I., Radu, D. G., Volf, I., Pag, A. I., & Popa V. I. (2013). Antioxidant and antibacterial activities of some natural polyphenols, *Cellulose Chemistry and Technology*, *47 (5-6),* 387-399.

87. Isaak, C. K., Petkau, J. C., O, K., Debnath, S. C., & Siow Y. L. (2015). Manitoba lingonberry (*Vaccinium vitis-idaea*) bioactivities in ischemia-reperfusion injury. *Journal of Agricultural and Food Chemistry, 63,* 5660-5669.

88. Jaakola, L., & Hohtola, A. (2010). Effect of latitude on flavonoid biosynthesis in plants. *Plant, Cell and Environment,* 33, 1239-1247.

89. Jaakola, L., Uleberg, E., & Martinussen, I. (2013). Bilberry – wild superberry from Europe. The 8th Circumpolar Agricultural Conference & Arctic Inaugural Food Summit (University of Alaska Fairbanks). URL http://www.uaf.edu/files/cac/Presentations/Jaakola_-CAC_Alaska.pdf. Accessed 06.07.15.

90. Jakesevic, M., Aaby, K., A Borge, G-I. A., Jeppsson, B., Ahrné, S., & Molin, G. (2011). Antioxidative protection of dietary bilberry, chokeberry and *Lactobacillus plantarum* HEAL19 in mice subjected to intestinal oxidative stress by ischemia-reperfusion. *BMC Complementary and Alternative Medicine*, *11*(8).

91. Jensen, H. D., Krogfelt, K. A., Cornett, C., Hansen, S. H., & Christensen, S. B. (2002). Hydrophilic carboxylic acids and iridoid glycosides in the juice of american and european cranberries (*Vaccinium macrocarpon* and *V. oxycoccos*), lingonberries (*V. vitis-idaea*), and blueberries (*V. myrtillus*). *Journal of Agricultural and Food Chemistry*, 50, 6871–6874.

92. Jovančević, M., Balijagić, J., Menković, N., Šavikin, K., Zdunić, G., Janković, T., & Dekić-Ivanković M. (2011). Analysis of phenolic compounds in wild populations of bilberry (*Vaccinium myrtillus* L.) from Montenegro. *Journal of Medicinal Plants Research*, *5(6)*, 910-914.

93. Jungfer, E., Zimmermann, F. B., Ruttkat, A., & Galensa R. (2012). Comparing procyanidins in selected *Vaccinium* species by UHPLC-MS with regard to authenticity and health effects. *Journal of Agricultural and Food Chemistry*, *60*, 9688−9696.

94. Juadjur A., Mohn C., Schantz M., Baum M., Winterhalter P., & Richling E. (2015). Fractionation of an anthocyanin-rich bilberry extract and in vitro antioxidative activity testing. *Food Chemistry*, *167*, 418–424.

95. Kähkönen, P. M., Hopia, I. A., Vuorela, J. H., Rauha, J-P, Pihlaja, K., Kujala, S. T., & Heinonen, M. (1999). Antioxidant activity of plant extracts containing phenolic compounds. *Journal of Agricultural and Food Chemistry*, *47,* 3954-3962.

96. Kähkönen, P. M., Heinämäki, J., Ollilainen, V., & Heinonen, M. (2003). Berry anthocyanins: isolation, identification and antioxidant activities. *Journal of the Science of Food and Agriculture, 83*, 1403–1411.

97. Katyal, T., Sharma, M., Sidhu, K., Behera, D., & Das Budhiraja, R. (2009). Beneficial Effects of antioxidants on oxidative stress and diabetes-induced experimental nephropathy. *Pharmacologyonline, 1*, 252-263.

98. Karlsen, A., Paur, I., Bøhn, S. K., Sakhi, A. K., Borge, G. I., Serafini, M., Erlund, I., Laake, P., Tonstad, S., & Blomhoff, R. (2010). Bilberry juice modulates plasma concentration of NF-jB related inflammatory markers in subjects at increased risk of CVD. *European Journal of Nutrition*, *49*, 345–355.

99. Kaufmann, B., & Christen, P. (2002). Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction, *Phytochemical Analysis, 13*, 105–113.

100. Kelsey, A. N., Wilkins, M. H., & Linseman, A. D. (2010). Nutraceutical antioxidants as novel neuroprotective agents, *Molecules, 15*, 7792-7814.

101. Khoddami, A., Wilkes, A. M., & Roberts, H. T. (2013). Techniques for analysis of plant phenolic compounds, *Molecules, 18*, 2328-237.

102. Kivimäki, A. S., Siltari, A., Ehlers, P. I., Korpela, R., & Vapaatalo, H. (2014). Lingonberry juice negates the effects of a high salt diet on vascular function and low-grade inflammation. *Journal of Functional Foods, 7,* 238 –245.

103. Kylli, P., Nohynek, L., Puupponen-Pimiä, R., Westerlund-Wikström, B., Leppänen, T., Welling, J., Moilanen, E., Heinonen, M. (2011). Lingonberry (*Vaccinium vitis-idaea*) and european cranberry (*Vaccinium microcarpon*) proanthocyanidins: isolation, identification, and bioactivities. *Journal of Agricultural and Food Chemistry*, *59*, 3373–3384.

104. Kim, D. W., Curtis-Long, M. J., Yuk, H. J., Wang, Y., Song, Y. H., Jeong, S. H., & Park, K. H. (2014). Quantitative analysis of phenolic metabolites from different parts of *Angelica keiskei* by HPLC–ESI MS/MS and their xanthine oxidase inhibition. *Food Chemistry.* 153, 20–27.

105. Lashmanova, A. K., Kuzivanova, A. O., & Dymova, V. O. (2012). Northern berries as a source of carotenoids, *Acta Biochimica Polonica*, 59(1), 133–134.

106. Lätti, A. K., Riihinen, K. R., Kainulainen, P. S. (2008). Analysis of anthocyanin variation in wild populations of bilberry (*Vaccinium myrtillus* L.) in Finland, *Journal of Agricultural and Food Chemistry*, *56*, 190–196.

107. Lätti, A. K., Jaakola, L., Riihinen, K. R., & Kainulainen, P. S. (2010). Anthocyanin and flavonol variation in bog bilberries (*Vaccinium uliginosum* L.) in Finland. *Journal of Agricultural and Food Chemistry*, *58*, 427–433.

108. Le Bourvellec, C., Bouzerzour, K., Ginies, C., Regis, S., Plé, Y., & Renard, M. G. C. C. (2011). Phenolic and polysaccharidic composition of applesauce is close to that of apple flesh. *Journal of Food Composition and Analysis, 24*, 537–547.

109. Lee, J., Rennaker, C., Wrolstad, R. E. (2008). Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods, *Food Chemistry 110*, 782–786.

110. Lee, J., & Finn, C. E. (2012). Lingonberry (*Vaccinium vitis-idaea* L.) grown in the pacific northwest of north America: anthocyanin and free amino acid composition, *Journal of Functional Foods, 4*, 213 –218.

111. Lehtonen, H. M., Suomela, J. P., Tahvonen, R., Vaarno, J., Venojärvi, M ., Viikari, J., & Kallio, H. (2010). Berry meals and risk factors associated with metabolic syndrome. *European Journal of Clinical Nutrition*, 1-8.

112. Leopoldini, M., Marino, T., Russo, N., & Toscano, T. (2004). Antioxidant properties of phenolic compounds: H-atom versus electron transfer mechanism. *The Journal of Physical Chemistry A, 108*, 4916-4922.

113. Linderborg, K. M., Järvinen, R., Lehtonen, H-M., Viitanen, M., & Kallio, H. P. T. (2012). The fiber and/or polyphenols present in lingonberries null the glycemic effect of the sugars present in the berries when consumed together with added glucose in healthy human volunteers. *Nutrition Research, 32*, 471-478.

114. Liu, P., Lindstedt, A., Markkinen, N., Sinkkonen, J., Suomela, J-P., & Yang, B. (2014). Characterization of metabolite profiles of leaves of bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.). *Journal of Agricultural and Food Chemistry, 62*, 12015−12026.

115. Lorrain, B., Dangles, O., Genot, C., & Dufour, C. (2010). Chemical modeling of heme-induced lipid oxidation in gastric conditions and inhibition by dietary polyphenols. *Journal of Agricultural and Food Chemistry, 68* (1), 676-683.

116. Lorrain, B., Dangles, O., Loonis, M., Armand, M., & Dufour, C. (2012). Dietary iron-initiated lipid oxidation and its inhibition by polyphenols in gastric conditions. *Journal of Agricultural and Food Chemistry, 60*, 9074-9081.

117. Lorrain, B., Ky, I., Pechamat, L., & Teissedre, P. L. (2013). Evolution of analysis of polyhenols from grapes, wines, and extracts. *Molecules, 18*, 1076-1100.

118. Ma, Y-T., & Cheung, P. C. K. (2007). Spectrophotometric determination of phenolic compounds by enzymatic and chemical methods - a comparison of structure-activity relationship. *Journal of Agricultural and Food Chemistry*, *55*, 4222-4228.

119. Macías-Sáncheza, M. D., Mantell, C., Rodrígueza, M., Martínez de la Ossaa, E., Lubiánb, L. M., & Montero, O. (2009). Comparison of supercritical fluid and ultrasound-assisted extraction of carotenoids and chlorophyll a from *Dunaliella salina. Talanta, 77,* 948–952.

120. Mahugo Santana, C., Sosa Ferrera, Z., Torres Padrón, M. E., & Santana Rodríguez, J. J. (2009). Methodologies for the extraction of phenolic compounds from environmental samples: new approaches. *Molecules, 14*, 298-320.

121. Mandal, V., Mohan, Y., & Hemalatha, S. (2007). Microwave assisted extraction-an innovative and promising extraction tool for medicinal plant research, *Pharmacognosy Reviews*, *1*, 7–18.

122. Mane, C., Loonis, M., Juhel, C., Dufour, C., & Malien-Aubert C. (2011). Food grade lingonberry extract: polyphenolic composition and *in vivo* protective effect against oxidative stress. *Journal of Agricultural and Food Chemistry, 59*, 3330–3339.

123. Manzoor, M., Anwar, F., Saari, N., & Ashraf, M. (2012). Variations of antioxidant characteristics and mineral contents in pulp and peel of different apple (*Malus domestica* Borkh.) cultivars from Pakistan, *Molecules, 17*, 390-407.

124. Martinussen, I., Rohloff, J., Uleberg, E., Junttila, O., Hohtola, A., Jaakola, L., & Häggman, H. (2009). Climatic effects on the production and quality of bilberries (*Vaccinium myrtillus*). *Latvian Journal of Agronomy*, *12*, 71-75.

125. Martz, F., Jaakola, L., Julkunen-Tiitto, R., & Stark S. (2010). Phenolic composition and antioxidant capacity of bilberry (*Vaccinium myrtillus*) leaves in Northern Europe following foliar development and along environmental gradients, *Journal of Chemical Ecology, 36*, 1017–1028.

126. Mauray, A., Felgines, C., Morand, C., Mazur, A., Scalbert, A., & Milenkovic, D. (2010). Nutrigenomic analysis of the protective effects of bilberry anthocyanin-rich extract in apo E-deficient mice. *Genes Nutrition*, *5,* 343–353.

127. Mauray, A., Felgines, C., Morand, C., Mazur, A., Scalbert, A., & Milenkovic, D. (2012). Bilberry anthocyanin-rich extract alters expression of genes related to atherosclerosis development in aorta of apo E-deficient mice. Nutrition, *Metabolism & Cardiovascular Diseases*, *22*, 72-80.

128. Maurya, D. K., & Devasagayam T. P. A. (2011). Role of radioprotectors in the inhibition of DNA damage and modulation of DNA repair after exposure to gamma-radiation. *Selected Topics in DNA Repair*, Prof. Clark Chen (Ed.), ISBN: 978-953-307-606-5, InTech.

129. McDougall, G. J., Ross, H. A., Ikeji, M., & Steward, D. (2008). Berry extracts exert different antiproliferative effects against cervical and colon cancer cells grown *in vitro*. *Journal of Agricultural and Food Chemistry, 56*, 3016–3023.

130. Mckenna, D. J., Jones, K., Hughes, K., Tyler, & V. M. (2002). Botanical medicines: the desk reference for major herbal supplements. Second edition, *Haworth Herbal Press*, ISBN 0-7890-1265-0.

131. Miguel, M. G. (2011). Anthocyanins: antioxidant and/or anti-inflammatory activities. *Journal of Applied Pharmaceutical Science*, *1*(6), 07-15.

132. Mikkelsen, A., & Skibsted, L. H. (1995). Acid-catalyzed reduction of ferrylmyoglobin - product distribution and kinetics of auto-reduction and reduction by NADH. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung*, 200(3), 171-177.

133. Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F., & Veberic, R. (2015). A comparison of fruit quality parameters of wild bilberry (*Vaccinium myrtillus* L.) growing at different locations. *Journal of the Science of Food and Agriculture*, *95*, 776–785.

134. Minekus et al. (2014). A standardised static in vitro digestion method suitable for food – an international consensus. *Food & Function, 5*, 1113–1124.

135. Moreno-Montoro, M., Olalla-Herrera, M., Gimenez-Martinez, R., Navarro-Alarcon, M., & Rufia´n-Henares, J. A. (2015). Phenolic compounds and antioxidant activity of Spanish commercial grape juices. *Journal of Food Composition and Analysis*, *38*, 19–26.

136. Morrison, M. C., Liang, W., Mulder, P., Verschuren, L., Pieterman, E., Toet, K., Heeringa, P., Wielinga, P. Y., Kooistra, T., Kleemann, R. (2015). Mirtoselect, an anthocyanin-rich bilberry extract, attenuates non-alcoholic steatohepatitis and associated fibrosis in ApoE⁄3Leiden mice. *Journal of Hepatology, 62*, 1180–1186.

137. Moure, A., Cruz, J. M., Franco. D., Domínguez J. M., Sineiro, J., Domínguez, H., Núñez, M. J., & Parajó, J. C. (2001). Natural antioxidants from residual sources. *Food Chemistry, 72*(2), 145-171.
138. Može, Š., Polak, T., Gašperlin, L., Koron, D., Vanzo, A., Poklar Ulrih, N., & Abram, V. (2011). Phenolics in slovenian bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.). *Journal of Agricultural and Food Chemistry, 59(13)*, 6998-7004.
139. Müller, D, Schantz, M, & Richling E. (2012). High performance liquid chromatography analysis of anthocyanins in bilberries (*Vaccinium myrtillus* L.), blueberries (*Vaccinium corymbosum* L.), and corresponding juices. *Journal of Food Science, 77*(4), C340-5.
140. Mykkänen, O. T., Huotari, A., Herzig, K-H., Dunlop, T. W., Mykkänen, H., Kirjavainen, P. V. (2014). Wild blueberries (*Vaccinium myrtillus*) alleviate inflammation and hypertension associated with developing obesity in mice fed with a high-fat diet. *PLoS ONE, 9*(12), 1-21.
141. Ndhlala, R. A., Moyo, M., Van Staden, J. (2010). Natural antioxidants: fascinating or mythical biomolecules?. *Molecules, 15*, 6905-6930.
142. Nestby, R., Percival, D., Martinussen, I., Opstad, N., & Rohloff, J. (2011). The European Blueberry (*Vaccinium Myrtillus* L.) and the potential for cultivation. A review, *The European Journal of Plant Science and Biotechnology*, 5-16.
143. Ni, Q., Xu, G., Lu, G., Gao, Q., Zhou, C., & Zhang, Y. (2012). Investigation of the stability and antioxidant properties of anthocyanins-based purple potato colorants after processing. *African Journal of Biotechnology, 11*(14), 3379-3387.
144. Novotny, J. A., Baer, D. J., Khoo, C., Gebauer, S. K., & Charron C. S. (2015). Cranberry juice consumption lowers markers of cardiometabolic risk, including blood pressure and circulating C-reactive protein, triglyceride, and glucose concentrations in adults. *The Journal of Nutrition*, *145,* 1185-93.
145. Oancea, S., Stoia, M., & Coman D. (2012). Effects of extraction conditions on bioactive anthocyanin content of *Vaccinium corymbosum* in the perspective of food applications. *Procedia Engineering*, *42*, 489 – 495.
146. Oancea, S., Moiseenco, F., & Traldi, P. (2013). Total phenolics and anthocyanin profiles of Romanian wild and cultivated blueberries by direct infusion ESI-IT-MS/MS. *Romanian Biotechnological Letters, 18*(3), 8350-8360.
147. Ogawa, K., Kuse, Y., Tsuruma, K., Kobayashi, S, Shimazawa, M., & Hara, H. (2014). Protective effects of bilberry and lingonberry extracts against blue light-emitting diode light-induced retinal photoreceptor cell damage *in vitro*. *BMC Complementary and Alternative Medicine, 14*(120), 1-11.
148. Olthof, M. R., Hollman, C. H. P., & Katan, B. M. (2001). Chlorogenic acid and caffeic acid are absorbed in humans. *Journal of nutrition*, *131*, 66-71.
149. Paes, J., Dotta, R., Barbero, G. F., & Martinez, J. (2014). Extraction of phenolic compounds and anthocyanins from blueberry (*Vaccinium myrtillus* L.) residues using supercritical CO_2 and pressurized liquids. *The Journal of Supercritical Fluids*, *95*, 8–16.

150. Pandey, B. K., & Rizvi, I. S. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity, 2*(5), 270-278.

151. Patel, M. J. (2008). A review of potential health benefits of flavonoid, *Lethbridge Undergraduate Research Journal, 3*(2).

152. Penhallegon, R. (2006). Lingonberry production guide for the Pacific Northwest. *U.S. Department of Agriculture*, Oregon State University, PNW (Pacific Northwest) 583-E.

153. Peterson, J. J., Beecher, R. G., Bhagwat, A. S., Dwyer, T. J., Gebhardt, E. S., Haytowitz, B. D., & Holden, M. J. (2006). Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature. *Journal of Food Composition and Analysis, 19*, S74–S80.

154. Peterson, J., Dwyer, J., Adlercreutz, H., Scalbert, A., Jacques, P., & McCullough, L.M., Dietary lignans: physiology and potential for cardiovascular disease risk reduction. *Nutrition Reviews, 68*(10), 571–603.

155. Piberger, H., Oehme, A., Hofmann, C., Dreiseitel, A., Sand, P. G., Obermeier, F., Schoelmerich, J., Schreier, P., Krammer, G., & Rogler, G. (2011). Bilberries and their anthocyanins ameliorate experimental colitis. *Molecular Nutrition & Food Research*, 55, 1724–1729.

156. Pieroni, L. G., de Rezende, F. M., Ximenes, V. F., & Dokkedal A. L. (2011). Antioxidant activity and total phenols from the methanolic extract of *Miconia albicans* (Sw.) triana leaves, *Molecules, 16*, 9439-9450.

157. Plazonić, A., Bucar, F., Maleš, Ž., Mornar, A., Nigović, B., & Kujundžić, N. (2009). Identification and quantification of flavonoids and phenolic acids in burr parsley (*Caucalis platycarpos* L.), using High-Performance Liquid Chromatography with Diode Array Detection and Electrospray Ionization Mass Spectrometry. *Molecules, 14*, 2466-2490.

158. Popa, M. I., Aelenei, N., Popa, V. I., & Andrei, D. (2000). Study of the interactions between polyphenolic compounds and chitosan. *Reactive & Functional Polymers, 45*, 35–43.

159. Popa, V. I., Agache, C., Beleca, C., & Popa, M. (2002). *Polyphenols from spruce bark as plant growth regulators*. *Crop Resources, 24*, 398-406.

160. Popa, V. I., Dumitru, M., Volf, I., & Anghel, N. (2008). Lignin and polyphenols as allelochemicals. *Industrial Crops and Products, 27*(2), 144-149.

161. Prencipe, F. P., Bruni, R., Guerrini, A., Rossi, D., Benvenuti, S., & Pellati, F. (2014). Metabolite profiling of polyphenols in *Vaccinium* berries and determination of their chemopreventive properties. *Journal of Pharmaceutical and Biomedical Analysis*, *89*, 257-267.

162. Prior, L. R., Wu, X., & Schaich, K. (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry,* 53, 4290-4302.

163. Pryor, W. A., & Castle, L. (1984). Chemical methods for the detection of lipid hydroperoxydes. In *oxygen radicals in biological systems*, Packer, L., Ed., Academic Press: Orlando, 293–295.

164. Pyka, A., Bober, K., & Stolarczyk, A. (2007). Densitometric determination of arbutin in cowberry leaves (*Vaccinium vitis idaeae*), *Acta Poloniae Pharmaceutica - Drug Research, 63*(5), 395-400.

165. Pyrzynska, K., & Biesaga, M. (2009). Analysis of phenolic acids and flavonoids in honey. *Trends in Analytical Chemistry, 28*(7).

166. Radulović, N., Blagojević, P., & Palić, R. (2010). Comparative study of the leaf volatiles of *Arctostaphylos uva-ursi* (L.) spreng. and *Vaccinium vitis-idaea* L. (*Ericaceae*). *Molecules, 15*, 6168-6185.

167. Rainha, N., Lima, E., Baptista, J., & Rodrigues, C. (2011). Antioxidant properties, total phenolic, total carotenoid and chlorophyll content of anatomical parts of *Hypericum foliosum. Journal of Medicinal Plants Research, 5*(10), 1930-1940.

168. Reis, F. S., Rai, K. D., & Abu-Ghannam, N. (2012). Water at room temperature as a solvent for the extraction of apple pomace phenolic compounds. *Food Chemistry, 135*, 1991–1998.

169. Riihinen, K. R., Ou, Z. M., Gödecke, T., Lankin, D. C., Pauli, G. F., & Wuc, C. D. (2014). The antibiofilm activity of lingonberry flavonoids against oral pathogens is a case connected to residual complexity. *Fitoterapia, 97*, 78–86.

170. Rimando, M. A., & Suh, N. (2008). Biological/chemopreventive activity of stilbenes and their effect on colon cancer, *Planta Medica, 74*, 1635–1643.

171. Routray, W., & Orsat, V. (2011). Blueberries and their anthocyanins: factors affecting biosynthesis and properties. *Comprehensive Reviews in Food Science and Food Safety, 10*, 303-320.

172. Rouanet, J. M., Décordé, K., Del Rio, D., Auger, C., Borges, G., Cristol, J.-P., Lean, M. E. J., & Crozier, A. (2010). Berry juices, teas, antioxidants and the prevention of atherosclerosis in hamsters. *Food Chemistry*, *118*(2), 266-271.

173. Rupasinghe, H. P. V., Kathirvel, P., & Huber, M. G. (2011). Ultrasonication-assisted solvent extraction of quercetin glycosides from 'Idared' Apple Peels. *Molecules, 16*, 9783-9791.

174. Seeram, N. (2008). Berry fruits: compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. *Journal of Agricultural and Food Chemistry,* 56(3), 627-629.

175. Shilpi, A., Shivhare, U. S., & Basu, S. (2013). Supercritical CO_2 extraction of compounds with antioxidant activity from fruits and vegetables waste - a review. *Focusing on Modern Food Industry, 2*(1).

176. Silva, J. O. C., Costa, R. M. R., Teixeira, F. M., & Barbosa, W. L. R. (2011). Processing and quality control of herbal drugs and their derivatives, quality control of herbal medicines and related areas. Prof. Yukihiro Shoyama (Ed.), ISBN: 978-953-307-682-9, InTech.

177. Song, J., Li, Y., Ge, J., Duan, Y., Sze, S. C-W., Tong, Y., Shaw, P-C., Ng, T-B., Tsui, K.C., Zhuo, Y., & Zhang, K.I. (2010). Protective effect of bilberry (*Vaccinium myrtillus* L.) extracts on cultured human corneal limbal epithelial cells (HCLEC), *Phytotherapy Ressearch*, *24*, 520–524.

178. Stanković, M. S. (2011). Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts, *Kragujevac Journal of Science, 33*, 63-72.

179. Staprans, I., Rapp, J. H., Pan, X. M., Kim, K. Y., & Feingold, K. R. (1994). Oxidized lipids in the diet are a source of oxidized lipid in chylomicrons of human. *Arteriosclerosis, Thrombosis Vascular Biology*, *14*, 1900-1905.

180. Steigerwalt, R. D., Belcaro, G., Morazzoni, P., Bombardelli, E., Burki, C., & Schönlau, F. (2010). Mirtogenol® potentiates latanoprost in lowering intraocular pressure and improves ocular blood flow in asymptomatic subjects. *Clinical Ophthalmology, 4*, 471–476.

181. Stevanovic, T., Diouf, P. N., & Garcia-Perez, M. E. (2009). Bioactive polyphenols from healthy diets and forest biomass, *Current Nutrition & Food Science, 5*, 264-295.

182. Stingu, A., Volf, I., & Popa, V. I. (2009a). Chestnuts (*Castanea sativa*) extracts-a potential plants growth regulator. *Buletinul Institutului Politehnic Iasi*, *4*, 69-77.

183. Stingu, A., Volf, I., Popa, V. I. (2009b). Study of copper and cadmium accumulation by bean for phytoremediation applications. *Environmental Engineering and Management Journal, 8*(5), 1247-1252.

184. Stingu, A., Volf, I., & Popa, V. I. (2009c). Physiological changes in seedling germination and growth plant under chemical stress conditions. *Environmental Enginering and Management Journal, 8*(6), 1309-1313.

185. Stingu, A., Volf, I., & Popa I. V. (2011a). Spruce bark extract as modulator in rape plant copper bioaccumulation, *Cellulose Chemistry and Technology*, 45(3-4), 281-286.

186. Stingu, A., Stanescu, I., Volf, I., & Popa, V. I. (2011b). Hyperaccumulation of cadmium in maize plant (*Zea mays*), *Cellulose Chemistry and Technology*, *45*, (3-4), 287-290.

187. Stingu, A., Volf, I., Popa, V. I., & Gostin, I. (2012). New approaches concerning the utilization of natural amendments in cadmium phytoremediation. *Industrial Crops and Products, 35*, 53– 60.

188. Sun, B., Leandro, M. C., de Freitas, V., & Spranger, M. I. (2006). Fractionation of red wine polyphenols by solid-phase extraction and liquid chromatography. *Journal of Chromatography A, 1128*, 27–38.

189. Szakiel, A., Voutquenne-Nazabadioko, L., & Henry, M. (2011). Isolation and biological activities of lyoniside from rhizomes and stems of *Vaccinium myrtillus*. *Phytochemistry Letters*, *4*, 138–143.

190. Szakiel, A., Pączkowski, C., Koivuniemi, H., & Huttunen, S. (2012). Comparison of the triterpenoid content of berries and leaves of lingonberry *Vaccinium vitis-idaea* from Finland and Poland. *Journal of Agricultural and Food Chemistry*, *60*, 4994−5002.

191. Takikawa, M., Inoue, S., Horio, F., & Tsuda, T. (2010). Dietary anthocyanin-rich bilberry extract ameliorates hyperglycemia and insulin sensitivity via activation of AMP-activated protein kinase in diabetic mice. *The Journal of Nutrition, 140*, 527-533.

192. Tanase, C, Stingu, A., Volf, I., & Popa V. I. (2011). The effect of spruce bark polyphenols extract in combination with deuterium depleted water (DDW) on

Glycine Max L. and *Helianthus Annuus* L. Development, *Analele Stiintifice ale Universitatii „Alexandru Ioan Cuza”*, TOM XII.

193. Tanase, C., Volf, I., Vintu, S., Gradinaru, R., & Popa, I. V. (2013a). Potential applications of wastes from energy and forestry industry in plant tissue culture, *Cellulose Chemistry and Technology*, *47(7-8),* 553-563.

194. Tanase, C., Volf, I., & Popa, I. V. (2013b). Assessement of synergic regulatory actions of spruce bark extract and deuterium depleted water on maize (*Zea mays* L.) crops, *Environmental Engineering and Management Journal*, *12(6)*, 1287-1294.

195. Tapas, A. R., Sakarkar, D. M., & Kakde, R. B. (2008). Flavonoids as nutraceuticals: a review. *Tropical Journal of Pharmaceutical Research*, *7*(3), 1089-1099.

196. Teleszko, M., & Wojdyło, A. (2015). Comparison of phenolic compounds and antioxidant potential between selected edible fruits and their leaves. *Journal of Functional Foods*, *14*, 736-746.

197. Thilakarathna, S. H., & Rupasinghe, H. P. V. (2013). Flavonoid Bioavailability and Attempts for Bioavailability Enhancement. *Nutrients, 5*, 3367-3387.

198. Thorne Research (2001). Monograph on *Vaccinium myrtillus* (Bilberry). *Journal of Clinical Pharmacy and Therapeutics*, Alternative Medicine Review, 6(5), 500-504.

199. Tiwari, P., Kumar, B., Kaur, M., Kaur, G., & Kaur, H. (2011). Phytochemical screening and extraction: a review. *Internationale Pharmaceutica Sciencia*, *1*(1).

200. Toda, K., Takahashi, R., Iwashina, T., & Hajika, M. (2011). Difference in chilling-induced flavonoid profiles, antioxidant activity and chilling tolerance between soybean near-isogenic lines for the pubescence color gene. *Journal of Plant Research*, *124*, 173–182.

201. Tsao, R. (2010). Chemistry and Biochemistry of Dietary Polyphenols. *Nutrients*, *2*, 1231-1246.

202. Tumbas Šaponjac, V., Canadanovi-Brunet, J., Cetkovi, G., Djilas, S., & Cetojevi-Simin D. (2014). Dried bilberry (*Vaccinium myrtillus* L.) extract fractions as antioxidants and cancer cell growth inhibitors. *LWT - Food Science and Technology*, 1-7.

203. Uleberg, E., Rohloff, J., Jaakola, L., Trôst, K., Junttila, O., Häggman, H., & Martinussen I. (2012). Effects of temperature and photoperiod on yield and chemical composition of northern and southern clones of bilberry (*Vaccinium myrtillus* L.). *Journal of Agricultural and Food Chemistry, 60*, 10406−10414.

204. Ursini, F., & Sevanian, A., (2002). Postpradial oxidative stress. *Biological Chemistry*, *383*, 599–605.

205. Veggi, C. P., Martinez, J., & Meireles, M. A. A. (2013a). Chapter 2. Fundamentals of microwave extraction in Microwave-assisted extraction for bioactive compounds. *Food Engineering Series, 4*, 15-52.

206. Veggi, C. P., Santos, T. S., Fabiano-Tixier, A. S., Le Bourvellec, C., Meireles, M. A. A., & Chemat F. (2013b). Ultrasound-assisted extraction of polyphenols from jatoba (*Hymenaea courbaril* L.var *stilbocarpa*) bark. *Food and Public Health*, *3*(3), 119-129.

207. Viljanen, K., Kylli, P., Kivikari, R., & Heinonen, M. (2004). Inhibition of Protein and Lipid Oxidation in Liposomes by Berry Phenolics. *Journal of Agricultural and Food Chemistry*, *52* (24), 7419-7424.

208. Viljanen, K. (2005). Protein oxidation and protein-lipid interactions in different food models in the presence of berry phenolics. *Academic dissertation*, Helsinki, Finland.

209. Viljakainen, S., Visti, A., & Laakso, S. (2002). Concentrations of organic acids and soluble sugars in juice from Nordic berries. *Acta Agriculturae Scandinavica, 52*, 101-109.

210. Vilkhu, K., Mawson, R., Simons, L., & Bates, D. (2008). Applications and opportunities for ultrasound assisted extraction in the food industry. *Innovative Food Science and Emerging Technologies, 9*, 161-169.

211. Vladimir-Knežević, S., Blažeković, B., Štefan, M. B., & Babac, M. (2012). Plant polyphenols as antioxidants influencing the human health. In V. Rao (Ed.), *Phytochemicals as Nutraceuticals - Global Approaches to Their Role in Nutrition and Health* (pp. 155-180). Croatia: InTech.

212. Volf, I., Ignat, I., Neamtu, M., & Popa, V. I. (2013). Thermal stability, antioxidant activity, and photo-oxidation of natural polyphenols, *Chemical Papers*, DOI: 10.2478/s11696-013-0417-6.

213. Volf, I., Stingu, A., & Popa, V. I (2012). New natural chelating agents with modulator effects on copper phytoextraction. *Environmental Engineering and Management Journal*, *11*(2), 487-491.

214. Vučić, D. M., Petković, M. R., Rodić-Grabovac, B. B., Stefanović, O. D., Vasić, S. M., & Čomić, L. R. (2013). Antibacterial and antioxidant activities of bilberry (*Vaccinium myrtillus* L.) in vitro. *African Journal of Microbiology Research*, *7(45)*, 5130-5136.

215. Wang, C., & Zuo, Y. (2011). Ultrasound-assisted hydrolysis and gas chromatography–mass spectrometric determination of phenolic compounds in cranberry products. *Food Chemistry, 128*, 562–568.

216. Wang, L., & Weller, L. C. (2006). Recent advances in extraction of nutraceuticals from plants. *Trends in Food Science & Technology, 17*, 300–312.

217. Wang, Y., & Liu, S. (2012). Pretreatment technologies for biological and chemical conversion of woody biomass, *Tappi Journal, 11*(1), 9-16.

218. Weidner, S., Rybarczyk, A., Karamać, M., Król, A., Mostek, A, Grębosz, J., & Amarowicz, R. (2013). Differences in the phenolic composition and antioxidant properties between *Vitis coignetiae* and *Vitis vinifera* seeds extracts, *Molecules, 18*, 3410-3426.

219. Wen, D., Li, C., Di, H., Liao, Y., & Liu, H. (2005). A universal HPLC method for the determination of phenolic acids in compound herbal medicines. *Journal of Agricultural and Food Chemistry*, *53*(17), 6624-9.

220. Wissam, Z., Ghada, B., Wassim, A., & Warid, K. (2012). Effective extraction of polyphenols and proanthocyanidins from pomegranate's peel. *International Journal of Pharmacy and Pharmaceutical Sciences*, ISSN- 0975-1491, *4*(3).

221. Wrolstad, R. E., Acree, T. E., Decker, E. A., Penner, M. H., Reid, D. S., Schwartz, S. J., Shoemaker, C. F., Smith, D., & Sporns, P. (2005). Handbook of food analytical chemistry. *New Jersey: John Wiley & Sons*, (Chapter 18).

222. Wrolstad, R. E., Durst, R.W., Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science & Technology, 16*, 423–428.

223. Xia, T., Shi, S., & Wan, X. (2006). Impact of ultrasonic-assisted extraction on the chemical and sensory quality of tea infusion. *Journal of Food Engineering*, *74*, 557–560.

224. Xia, E. Q, Cui, B., Xu, X. R., Song, Y., Ai, X. X., Li, H. B. (2011). Microwave-assisted extraction of oxymatrine from *Sophora flavescens. Molecules, 16*, 7391-7400.

225. Yang, S-T., El-Enshasy, H., & Thongchul, N. (2013). Bioprocessing technologies in biorefinery for sustainable production of fuels, chemicals, and polymers. *John Wiley & Sons,* ISBN: 9780470541951.

226. Yang, B., Koponen, J., Tahvonen, R., Kallio, H. (2003). Plant sterols in seeds of two species of *Vaccinium* (*V. myrtillus* and *V. vitis-idaea*) naturally distributed in Finland, *European Food Research and Technology*, *216*, 34–38.

227. Zhang, J., Han, C., & Liu, Z. (2009a). Absorption spectrum estimating rice chlorophyll concentration: Preliminary investigations. *Journal of Plant Breeding and Crop Science, 1*(5), 223-229.

228. Zhang, L., Ying Shan, Y., Tang, K., & Putheti, R. (2009b). Ultrasound-assisted extraction flavonoids from Lotus (*Nelumbo nuficera Gaertn*) leaf and evaluation of its anti-fatigue activity. *International Journal of Physical Sciences, 4*(8), 418-422.

229. Zhang, L., Wang, Z., Wu, D., Xu, M., & Chen, J. (2011). Microwave-assisted extraction of polyphenols from *Camellia oleifera* fruit hull. *Molecules, 16*, 4428-4437.

230. Zheng, W., & Wang, Y. S. (2003). Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries, and lingonberries. *Journal of Agricultural and Food Chemistry*, *51,* 502-509.

231. Zheng, X., Xu, X., Liu, C., Sun, Y., Lin, Z., Liu, H. (2013). Extraction characteristics and optimal parameters of anthocyanin from blueberry powder under microwave-assisted extraction conditions. *Separation and Purification Technology, 104*, 17–25.

232. Zhou, B., Wu, L-M., Yang, L., & Liu, Z-L. (2005). Evidence for α-tocopherol regeneration reaction of green tea polyphenols in SDS micelles. *Free Radical Biology and Medicine, 38(1),* 78-84.

233. Zhu, L., Liu, X., Tan, J., & Wang, B. (2013). Influence of harvest season on antioxidant activity and aonstituents of rabbiteye blueberry (*Vaccinium ashei*) leaves. *Journal of Agricultural and Food Chemistry,* 61, 11477-11483.

234. Zitka, O., Sochor, J., Rop, O., Skalickova, S., Sobrova, P., Zehnalek, J., Beklova, M, Krska, B., Adam, V., & Kizek, R. (2011). Comparison of various easy-to-use procedures for extraction of phenols from apricot fruits. *Molecules, 16*, 2914-2936.

235. Żwir-Ferenc, A., & Biziuk, M. (2006). Solid phase extraction technique – trends, opportunities and applications. *Polish Journal of Environmental Studies, 15*(5), 677-690.

236. Encyclopaedia Britannica, *Vaccinium*, (2015). http://www.britannica.com/EBchecked/topic/621302/Vaccinium, (accessed May 29th 2015).

237. Euro+Med (2006-): Euro+Med PlantBase-the information resource for Euro-Mediterranean plant diversity. Published on the Internet http://ww2.bgbm.org/EuroPlusMed/ (accessed Juin 02th 2015).

238. Mirtoselect, Bilberry vs Blueberry, http://www.mirtoselect.info/bilberry-vs-blueberry/ (accessed March 10th 2013).

239. U.S. Department of Agriculture, Agricultural Research Service. USDA Database for the Flavonoid Content of Selected Foods, Release 3.1. Nutrient Data Laboratory Home Page: 2013, http://www.ars.usda.gov/nutrientdata/flav.

240. < http://www.fineli.fi/food.php?foodid=442&lang=en> (Accessed 29.05.15).

Scientific publications

Published articles:

1. **Oana-Crina Bujor**, Iulia Adina Talmaciu, Irina Volf, Valentin I. Popa, *Biorefining to recover aromatic compounds with biological properties*, Tappi Journal, **2015**, 14(3), 187-193 (**IF 0.73**).

Articles in preparation:

1. **Oana-Crina Bujor**, Carine Le Bourvellec, Irina Volf, Valentin I. Popa, Claire Dufour, Seasonal variations of the phenolic constituents in bilberry (*Vaccinium myrtillus* L.) leaves, stems and fruits and their antioxidant activity **(under review at *Food Chemistry* journal)**.
2. **Oana-Crina Bujor**, Irina Volf, Valentin I. Popa, Claire Dufour, Phenolic profile and antioxidant activity of leaf, stem and fruit extracts of lingonberry (*Vaccinium vitis-idaea* L.) at three vegetative stages (**manuscript in preparation**).
3. **Oana-Crina Bujor**, Valentin I. Popa, Claire Dufour, Inhibition of lipid oxidation by phenolic bilberry and lingonberry extracts (**manuscript in preparation**).

International communications:

1. **Oana-Crina Bujor**, Irina Volf, Valentin I. Popa, Claire Dufour, *Influence des stades végétatifs sur l'activité antioxydante des extraits de branche, feuilles et fruits d'airelle rouge,* COFrRoCA - Colloque Franco-Roumaine de Chimie Appliquée, Montpellier, France, 15-18 September 2014 (**oral presentation**).
2. **Oana-Crina Bujor**, Camelia Mihăilescu, Michèle Loonis, Irina Volf, Valentin I. Popa, Claire Dufour, *Influence of different harvest periods on the phenolic profile and content of leaf, stem and fruit extracts of bilberry*, 2nd International Conference on Chemical Engineering (ICCE 2014), Iasi, Roumanie, 5-8 November 2014 (**poster**).
3. **Oana-Crina Bujor**, Irina Volf, Valentin I. Popa, Claire Dufour, *Antioxidant activity of bilberry extracts from different morphological parts at three vegetative stages*, 2nd International Conference on Chemical Engineering (ICCE 2014), Iasi, Roumanie, 5-8 November 2014 (**oral presentation**).
4. **Oana-Crina Bujor**, Camelia Mihăilescu, Michèle Loonis, Irina Volf, Valentin I. Popa, Claire Dufour, *Phenolic constituents in Lingonberry: dynamic accumulation in leaf, stem and fruit extracts at different harvest periods*, 22nd Young Research Fellows Meeting - Biology and Chemistry: a permanent dialogue, Paris, France, 4-6 February 2015 (**poster**).
5. **Oana-Crina Bujor**, Irina Volf, Valentin I. Popa, Claire Dufour, *Inhibition of lipid oxidation by phenolic bilberry and lingonberry extracts in an in vitro digestion model*, 4th International Conference of Food Digestion, Naples, Italy, 17-19 March 2015 (**poster**).
6. **Oana-Crina Bujor**, Camelia P. Stefanache, Claire Dufour, Evelyn Wolfram, Anca Miron, Doina Dănilă, *Natural products of Ericaceae family and their nutritional and therapeutic potential - a review*, 2nd International Conference on Natural Products Utilization: from Plant to Pharmacy Shelf (ICNPU), Plovdiv, Bulgaria, 14-17 octombrie 2015 (**poster**).
7. **Oana-Crina Bujor**, Christian Ginies, Valentin I. Popa, Claire Dufour, *Phenolic extracts of bilberry: they protect lipid from oxidation in in vitro simulated digestion conditions*, 2nd Euro-Mediterranean Symposium on Fruit and Vegetable Processing, Avignon, France, 4 - 6 April 2016 (**poster**).

This article was published as a bibliographic review in the journal "Tappi Journal":

Bujor O-B., Talmaciu I.A., Volf I., Popa I.V. (2015). Biorefining to recover aromatic compounds with biological properties. *Tappi Journal*, 14(3), 187-193.

PEER-REVIEWED | **BIOACTIVE COMPOUNDS**

Biorefining to recover aromatic compounds with biological properties

OANA-CRINA BUJOR, IULIA ADINA TALMACIU, IRINA VOLF, AND VALENTIN I. POPA

ABSTRACT: Although extraction of bioactive compounds by biomass resource biorefining is challenging, interest in these compounds is increasing. This review summarizes our results in the field of secondary compounds (especially polyphenols) obtained via biorefining technology. The technology also offers the possibility to separate other compounds with industrial value or biological properties. To isolate polyphenols, different biomass sources such as spruce wood bark, *Asclepias syriaca* (a latex-bearing plant), *Crataegus monogyna* (hawthorn), chestnut shells, vine stems, and grape seeds have been used. The isolated products have been tested for their biological properties in the fields of plant and microorganism development. Experimental results confirmed the important role of polyphenols in the metabolism of different organisms. For plant development, the polyphenols were tested in the following processes: germination, plant cultivation, tissue cultures, and grafting. The influence of polyphenols can be associated with stimulation and regulation of cell differentiation, as evidenced by genetic studies and enzymes biosynthesis. Polyphenols might also participate in regulating the metabolism of different yeast strains and might inhibit the development of bacteria or fungi. Interactions between polyphenols and soil cultivation might also be used for bioremediation of arid and polluted soils.

Application: The information in this report might help industry develop biorefineries that could allow separating valuable compounds for industrial applications and for their biological activity. Wood bark along with other forestry wastes can be used to recover polyphenols. Residues from extraction can be separated into hemicelluloses, cellulose, and lignin to obtain bioproducts or compounds with biological activity.

Sustainable economic growth requires that industrial production be based on renewable raw materials. Petroleum, today's most used industrial raw material, is neither sustainable nor environmentally friendly. Although energy production can leap toward the use of alternative resources such as wind, sun, water, and biomass, material and chemical industries are fundamentally dependent on biomass. Therefore, special measures are required, such as the development of biorefineries and their implementation on a large scale. These biorefineries can be the key to the integrated production of food, feed, chemicals, materials, goods, and fuels of the future in a profitable and sustainable manner, without negative environmental impacts [1,2].

Generally, biorefining refers to fractionating biomass into various components that can possibly undergo further biological, (bio) chemical, or physical processing and separation to obtain chemicals or their precursors. By means of co-producing chemicals, the production costs of secondary energy carriers could potentially become more profitable, especially when biorefining is integrated into the existing chemicals, materials, and power industries. The accessibility of the raw material and its chemical composition will decide its use as an energy carrier versus biomass as fuels (e.g., by combustion).

In the last 2-3 decades, biomass (agricultural crops, forestry products, organic fractions of household and industrial wastes, and aquatic biomass) has attracted research and commercial interest as a renewable source of fuels and high value chemicals [3]. The success of demonstration-scale biorefineries serves as proof of recent and continuous expansion on the renewable materials processing front. In 2003 alone, approximately 31.5 million tons of bioethanol were produced worldwide, mainly from maize starch. Approximately 90% of the world's supply of ethanol is now produced from agricultural feedstocks using fermentation. These examples demonstrate the global impact that bio-refining can have if developed and adopted more comprehensively, while focusing on a variety of key molecules [4-6].

From all the valuable biomass extractives, polyphenols are a widespread group of secondary metabolites found in all plants, representing the most desirable phytochemicals because of their potential to be used as additives in food, cosmetics, medicine, and others fields. At present, there is an increased interest to recover them from plants and wastes from agricultural and food industries. For example, in our studies, sources such as vine stems, grape seeds, wood bark (spruce and pine bark), and plants such as *Asclepias syriaca* (a latex bearing plant) and *Crataegus monogyna* have been studied as important sources of phenolic compounds. This review summarizes our results obtained in the field of secondary

compounds (especially polyphenols) obtained by applying biorefining technologies.

PHENOLIC COMPOUNDS AS SECONDARY BIOACTIVE AROMATIC COMPOUNDS RECOVERED BY BIOREFINING

Phenolic compounds are among the most numerous and widely distributed groups of aromatic compounds in the plant kingdom. Of more than 8000 phenolic structures currently known, more than 6000 are the flavonoids [7,8]. From the chemical point of view, polyphenols are natural compounds with aromatic structures containing one or more aromatic rings. They are known for their valuable properties as antioxidants, antibacterials, and chelating agents. They are also used for cell division regulation, anticancer effects, anti-inflammatory effects, cardiovascular benefits, anti-diabetes potential, energy endurance enhancement, and protection against Alzheimer's disease.

Sources of vegetal biomass to isolate phenolic compounds

Phenolic compounds are considered the most abundant constituents of plants and processed foods. Some phenolic compounds are extremely widespread, others are specific to certain plant families or found only in certain plant organs or at certain development stages [9]. The main sources of phenolic compounds are fruits and vegetables, seeds, cereals, berries, beverages (wine, tea, and juices), olives, and aromatic plants, but also agricultural and industrial wastes and wood and non-wood forest resources [10-12].

In our studies, sources such as vine stems, grape seeds, and wood bark (spruce and pine) have been used to separate lignin and different phenolic compounds. Spruce and pine bark, which represent a waste in the wood industry, have been reported to contain a wide range of phenolic compounds, including stilbene glycosides [13], gallic acid, catechin, and vanillic acid [14]. Grape seeds were found to contain up to 506 mg gallic acid equivalents (GAE)/100 g of total phenolics, 193 mg GAE/100 g of total tannins, 27 mg rutin equivalent (RE)/100 g of total flavonoids, and 18 mg/100 g of total anthocyanins [15]. Besides these, other plants, such as Asclepias syriaca and Crataegus monogyna (hawthorn), have been studied as important sources of phenolic compounds [16,17]. Some polyphenols (e.g., gallic, syringic, p-coumaric, ferulic, and vanillic acids; rutin; quercitine; and catechin) are present in wood bark and other forestry residues.

Characterization of phenolic compounds separated by biorefining

Because of the structural diversity and complexity of phenolic compound in plants, extraction is the first and the most important step in the separation and characterization of these compounds. The phenolic nature of polyphenols makes them relatively hydrophilic, thus free phenolic compounds, including aglycones, glycosides, and oligomers, are extracted using water; polar organic solvents, such as methanol, ethanol, acetonitrile, and acetone; or their mixture with water [7].

Green extraction is used to protect the environment and consumers, and to enhance competition between industries through use of more ecologic (the use of co-products, biodegradability), economic (less energy and solvent consumption), and innovative processes [18]. In agreement

Raw Material	Type of Extract	Gallic Acid	Catechin	Vanillic Acid	Syringic	*p*-coumaric Acid	Ferulic Acid	Rutin	Quercetin
Spruce bark	Aqueous extract	-	31	39.4	-	-	-	-	-
	Ethanolic extract	10.2	71.9	71.9	-	-	-	-	1.39
Grape seeds	Aqueous extract	6.12	44.36	-	-	-	-	-	
	Ethanolic extract	12.54	63.60	-	-	-	-	-	2.38
Crataegus Monogyna	Aqueous extract	-	23.42	-	2.14	-	-	-	
	Ethanolic extract	10.98	89.52	-	2.95	3.59	2.25	30.32	0.64
Asclepias Syriaca	Aqueous extract	-	-	0.87	0.98	0.11	-	-	
	Ethanolic extract	0.65	-	2.94	1.94	0.40	-	2.25	0.14

I. Concentration of phenolic compounds (mg/100 g dried plant) in different sources [17].

with this green extraction approach, unconventional extraction methods such as microwave-assisted extractions (MAE) [19], ultrasound-assisted extractions [20], and techniques based on the use of compressed fluids as extracting agents, such as subcritical water extraction (SWE) [21], supercritical fluid extraction (SFE) [22], or accelerated solvent extraction (ASE) [23] are applied to separate phenolic compounds. For the investigated sources, the following polyphenols were identified: gallic, syringic, p-coumaric, ferulic and vanillic acids; rutin; quercitine; and catechin (**Table I**) [17].

Various spectrophotometric and chromatographic methods have been developed to quantify and characterize phenolic compounds from plant extracts:

- The Folin–Ciocalteu assay is a widely used spectrophotometric method for determining total phenolic content.
- Vanillin and proanthocyanidin assays have been used to estimate total proanthocyanidins.
- The pH differential method is used to quantify total anthocyanins.
- Total flavonoids content can be determined using a colorimetric method based on the complexation of phenolic compounds with Al(III) [15].

Spectrophotometric assays give an estimation of the total phenolic contents, whereas various chromatographic techniques are used for separation, identification, and quantification of individual phenolic compounds [8].

The most commonly used technique to identify phenolic compounds is high performance liquid chromatography (HPLC). The HPLC method is also used for quantitative analysis of phenolic metabolites from different plant extracts [24,25]. Identification and analysis of phenolic compounds are usually achieved by using a combination of HPLC (diode array detector), mass spectrometry, and nuclear magnetic resonance imaging [26]. Application of these methods allowed us to characterize the extracts separated from spruce wood bark and other sources (**Table II**).

APPLICATIONS OF PHENOLIC COMPOUNDS IN BIOLOGICAL SYSTEMS

Important studies in vitro and in vivo showed the possibilities to apply phenolic compounds from natural sources as antioxidant, antibacterial, and anticarcinogenic agents; plant growth regulators; amendments in bioremediation; to interact with some complexing agents [27], and as allelochemicals [28].

Phenolic compounds as plant growth bioregulators

Natural compounds with aromatic structure, such as phenolic compounds, have extremely complex roles in plant physiological processes. Phenolic compounds are important for the physiology of plants, contributing to their resistance to microorganisms, insects, and herbivorous animals. Those compounds help to preserve the integrity of plants that have continuous exposure to environmental stressors, including ultraviolet radiation, relatively high temperatures, heavy metals [29], and dehydration. They are also involved in plant growth and development cycles [17,30].

As bioregulators, phenolic compounds have an important role in the growth and development of different parts of plants: roots, stems, buds, and leaves [31]. Data from the literature provide information concerning the influence of phenolic compounds in the processes of seed germination and plant development, either as individual compounds or global extracts obtained from different plant sources. Phenolic compounds may act as stimulators and as inhibitors in plant growth, depending on the extraction procedure, concentration, and nature of extracted compounds containing the same bioactive phenolic compounds (**Table III**) [16,32-34].

The action of polyphenols of spruce bark extracts on tomato seeds shows a positive influence on the rate and capacity of germination, seedling growth, and biomass amount accumulated after germination. The stimulating effect of primary root elongation and hypocotyls, found in the presence of polyphenolic extracts at concentrations of 40 mg/L and 200 mg/L in growth medium can be compared with those

Polyphenols Sources	Analysis	Techniques
- Spruce bark - Grape seeds - *Asclepias syriaca* - Chestnut shells - *Crataegus monogyna* (hawthorn)	Extraction	Ultrasound-assisted extractions (UAE) Supercritical fluid extraction (SFE) Microwave-assisted extractions (MAE) Conventional extraction methods
	Polyphenols Separation/Identification	Mass spectrometry-high performance liquid chromatography (MS-HPLC)
	Heavy metals concentrations	Atomic absorption spectrometry (AAS)
	Plant response analysis	Ultraviolet-visible (UV-Vis) spectrometry Fourier transform-infrared (FT-IR) spectroscopy

II. Methods for characterization of extracts separated from spruce wood bark and other sources.

BIOACTIVE COMPOUNDS

Extract Type	Tested Plant	Effects of the Polyphenolic Aqueous Extracts					
		Roots Length	Stems Length	Leaves Area	Roots Dry Mass	Stems Dry Mass	Leaves Dry Mass
Chestnut shell extract	Oat seeds	_	_	~	+	_	~
	Grape seeds	+	+	~	~	+	_
Spruce bark extract	Maize seeds	+	_	~	+	_	~
+: stimulation effect. ~: no visible effect. -: inhibition effect.							

III. Effects of polyphenolic extracts in the processes of seed germination [33,34].

of auxins or citokinins [13,35]. The same effect of spruce bark extracts was demonstrated on maize callus tissue developed in culture medium containing deuterium-depleted water [31,34].

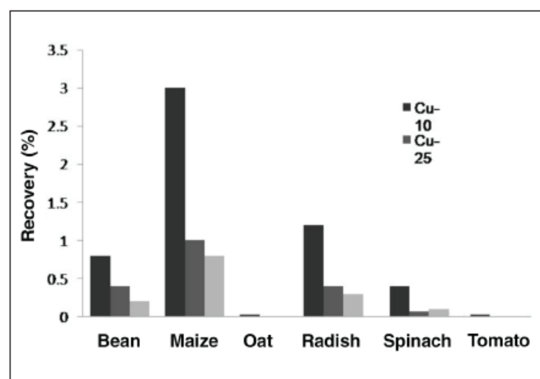
Ignat et al. [16] observed that elongation of bean plant (*Phaseolus vulgaris*) radicles was stimulated in the presence of aqueous extracts from spruce bark, *Asclepias syriaca* plant, and grape seeds obtained by sonication. They also observed an inhibitory effect on the amount of fresh biomass accumulated. In our studies, different lignins and polyphenols extracted from biomass sources (old wood and stems of *Vitis* species) were tested in model experiments to follow their actions as allelochemicals. The results of these investigations allow us to appreciate that lignins have a biostimulating effect on mitotic division in the radicular meristems of *Phaseolus vulgaris*. We concluded that this effect is induced as a result of the improvement of micromedia conditions at the plant roots level, correlated with the benefic influence of lignin on the microflora present in soil [28].

Phenolic compounds as amendments in bioremediation

Bioremediation refers to the use of green plants to remove, contain, or convert environmental contaminants (e.g., organic solvents, polychlorinated biphynols, heavy metals, polyaromatic hydrocarbons, explosives and energetic, or nutrients) into harmless compounds [36].

Two options to deal with heavy metal contaminated soil are phytoextraction and phyto-stabilization. Phytoextraction (phyto-accumulation) is a nondestructive technique, developed to remove trace elements from soil through uptake and accumulation by plants. Phyto-stabilization (phyto-immobilization) aims at establishing a vegetation cover and at promoting in situ inactivation of trace elements by combining the use of metal-tolerant plants and soil amendments that help reduce the mobility and toxicity of pollutants. [37].

In studies developed in our group, individual phenolic compounds (catechin) and phenolic extracts from spruce bark (*Picea abies*), *Asclepias syriaca* plant, chestnut shell (*Castanea sativa*), and grape seed (*Vitis vinifera*) were used



1. Use of different vegetal biomass resources in copper bioremediation (Cu-10, Cu-25, Cu-50 - copper ions concentration in testing solution - 10, 25, 50 mg/L [38,42,43]).

as modulators of copper (**Fig. 1**) and cadmium bioaccumulation in grape, bean (*Phaseolus vulgaris*), oat (*Avena sativa*), and maize (*Zea mais*) plants [29,37-42].

All extracts could properly be used in bioremediation as an alternative to synthetic chelators for in situ inactivation of heavy metal ions and being suitable in phyto-stabilization and improving the phyto-extraction process [29]. However, the bioaccumulation process depends on heavy metal concentrations and polyphenolic extracts compositions determined by raw materials [40].

Applications of phenolic compounds in microorganism development

Phenolic compounds have various defensive functions in plants, such as cell wall strengthening and repair or antimicrobial and antifungal activities [43,44]. Phenolic compounds such as catechin act on different bacterial strains belonging to different species (*Escherichia coli*, *Bordetella bronchiseptica*, *Serratia marcescens*, *Klebsiella pneumonie*, *Salmonella choleraesis*, *Pseudomonas aeruginosa*, *Staphilococcus aureus*, and *Bacillus subtilis*) by generating hydrogen peroxide and by altering the

BIOACTIVE COMPOUNDS

Reference Microbial Plant Extracts Strains	Inhibition Zones (mm)		
	Staphylococcus aureus	*Pseudomonas aeruginosa*	*Escherichia coli*
Asclepias syriaca aqueous extract	-	6	6
Asclepias syriaca ethanolic extract	-	12	6
Spruce bark aqueous extract	-	-	-
Spruce bark ethanolic extract	15	10	10
Crataegus monogyna aqueous extract	-	-	-
Crataegus monogyna ethanolic extract	15	-	-
Grape seed aqueous extract	-	-	-
Grape seed ethanolic extract	12	-	-

VI. Antimicrobial activity of spruce bark,* Crataegus monogyna, Asclepias syriaca, *and grape seed extracts. Adapted from Ignat et al. (2013) [17].

permeability of the microbial membrane [45], presenting strong bactericidal action.

Catechin (epicatechin [EC], epicatechin gallate [ECg], epigallocatechin [EGC], and epigallocatechin gallate [EGCg]), which occur in green tea and black tea, possess strong bactericidal action. We observed and identified a reactive oxygen species as the active mechanism that was generated from the catechins. EGCg reacted with the dissolved oxygen in aqueous solution, resulting in the generation of hydrogen peroxide.

Ignat et al. [17] evaluated antibacterial activities of several types of phenolics extracted from spruce bark, grape seeds, *Crataegus monogyna* (hawthorn), and *Asclepias syriaca* against gram-positive and gram-negative pathogen bacteria. The results showed that spruce bark, *Crataegus monogyna*, and grape seed ethanol extracts exerted antibacterial activity (the largest inhibition zones, 12-15 mm) against gram-positive pathogenic bacteria (*Staphylococcus aureus*) (**Table IV**). Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) are less susceptible to herbal ethanolic extracts obtained from spruce bark and *Asclepias syriaca* because of smaller inhibition zones and are not susceptible to aqueous extracts.

Phenolic extracts from spruce bark, red grape seeds, and *Asclepias syriaca* plant were also tested on the cultivation of different species of *Rhodotorula spp.* yeast. Phenolic compounds in all extracts were observed to influence the development of yeast species in terms of biomass yield resulting after fermentation carotenoid pigment biosynthesis. The yeasts also used the phenolic compounds as a carbon and energy source, their concentration being reduced when the duration of cultivation was increased. When these extracts were the only carbon source, yeasts were able to metabolize them [45-47]. Lignin, another natural phenolic compound, can be used as a carbon source for the cultivation of *Rhodotorula spp.* yeast species. The results obtained are in agreement with the those mentioned previously, namely that the lignins introduced in the culture medium for the cultivation of *Rhodotorula spp.* yeasts are convenient for increasing the biomass yield and for the biosynthesis of carotenoid compounds [48].

Phenolic compounds as systems for assessing antioxidant capacity

Phenolic compounds, as with aromatic compounds, have generated great interest because of their antioxidant capacity [8,49], which is reflected in their reducing character and ability to interact with a variety of metal ions and proteins [9]. In particular, phenolic compounds exert their antioxidant activity by direct scavenging reactive oxygen species (ROS), inhibition of enzymes involved in oxidative stress, and chelation of trace metals responsible for ROS production and stimulation of endogenous antioxidant defense systems. The antioxidant properties of these compounds are of particular interest in the fields of foods (inhibition of lipid oxidation), physiology (protection against oxidative stress), and cosmetics. Antioxidant consumption has shown its effectiveness in the prevention of cancer, cardiovascular disease, osteoporosis, obesity, diabetes, and in protection against hydrogen peroxide, ultraviolet irradiation, and the effects of aging on the skin [7,21,50-52].

CONCLUSIONS

Biorefining is now more than a concept. It offers the potential for biomass complex processing to obtain a range of byproducts with energy and chemical value.

The results of our studies showed the possibilities offered by biorefining to fractionate and characterize compounds

BIOACTIVE COMPOUNDS

with aromatic structure (polyphenols and lignin) along with the other main compounds (hemicelluloses, cellulose, and lignin).

Polyphenols and lignin have important biological activity, as demonstrated in plant, animal, and microorganism metabolism. In some cases, these compounds can be used to obtain bio-based products, which can have industrial and biological value. These bio-based products could be used in pharmaceutical, cosmetics, medicine fields, and agriculture, food conservation, bioremediation, crop protection, etc. **TJ**

LITERATURE CITED

1. Kamm, B. and Kamm. M., *Chem. Biochem. Eng. Q.* 18(1): 1(2004).
2. Moshkelani, M., Marinova, M., Perrier, M., et al., *Appl. Therm. Eng.* 50(2): 1427(2013).
3. Holm-Nielsen, J.B. and Ehimen, E.A., in *Advances in Biorefineries: Biomass and Waste Supply Chain Exploitation* (K.W. Waldron, Ed.), Woodhead Publishing (Elsevier), Sawston, UK, 2014, Chap. 23, pp. 89-111.
4. Fish, S., *IGER Innovations*, "The biorefining of biorenewable," 2007, pp. 50-54.
5. Eksioglu, S.D., Acharya, A., Leightley, L.E., et al., *Comput. Ind. Eng.* 57(4): 1342(2009).
6. FitzPatrick, M., Champagne, P., Cunningham, M.F., et al., *Bioresour. Technol.* 101(23): 8915(2010).
7. Tsao, R., *Nutrients* 2(12): 1231(2010).
8. Vladimir-Knežević, S., Blažeković, B., Štefan M.B., et al., in *Phytochemicals as Nutraceuticals – Global Approaches to Their Role in Nutrition and Health* (R. Venketeshwer, Ed.), InTech, Rijeka, Croatia, 2012, pp. 155-180.
9. Cheynier, V., *Am. J. Clin. Nutr.* 81(1 Suppl): 223S(2005).
10. Ignat, I., Volf, I., and Popa, V.I., *Food Chem.* 126(4): 1821(2011).
11. Moure, A., Cruz, J.M., Franco, D., et al., *Food Chem.* 72(2): 145(2001).
12. Stevanovic, T., Diouf, P.K., and Garcia-Perez, M.E., *Curr. Nutr. Food Sci.* 5(4): 264(2009).
13. Balas, A. and Popa, V.I., *Rom. Biotechnol. Lett.* 12(3): 3209(2007).
14. Hainal, A.C., Ignat, I., Volf, I., et al., *Cellul. Chem. Technol.* 45(3-4): 211(2011).
15. Ignat, I., Stingu, A., Volf, I., et al., *Cellul. Chem. Technol.* 45(3-4): 205(2011).
16. Ignat, I., Stingu, A., Volf, I., et al., *Lucr. Stiint., Ser. Agron.* (*Scientific Papers, Agronomy Series*) 52(1): 187(2009).

ABOUT THE AUTHORS

Bujor

Talmaciu

Volf

Popa

Significant interest is being shown in recovering bioactive compounds with biological proprieties from wastes, especially those of forestry, agriculture, and food industries. In Romania, this field, with great and promising industrial applications, has not been very well explored. Because of that, we chose to develop this area and find new solutions for obtaining these valuable byproducts.

This paper is a review of the results obtained in the last 10 years by our research group. The subjects discussed are closely dependent on each other.

A challenge was to take into account the different uses for the compounds that might be obtained and the specialized knowledge in different fields (biology, medicine, agriculture, and others). This imposed a certain rigor in the assimilation of new working techniques, along with adaptation of teams with mixed specialists.

Polyphenols are compounds with multiple functionalities that were evidenced by multidisciplinary research. From our research results, we found that polyphenols hold great promise for use in medicine and pharmaceuticals. They might also be useful as precursors to obtain green fertilizers or plant crop protections and bioremediation agents, given their multiple biological properties.

Based on the information from our research, the industry can develop biorefineries that could allow separating valuable compounds that can be used for industrial applications and for their biological activity. Wood bark and other forestry wastes can be used to recover polyphenols. At the same time, it possible that the residues resulting from extraction could be used to separate hemicelluloses, cellulose, and lignin to obtain bioproducts or compounds with biological activities.

We next propose to develop a new technology that could facilitate the recovery of secondary and primary compounds step by step, which will be flexible enough to be applied to different biomass sources.

Bujor and Talmaciu are Ph.D. students in Chemical Engineering, Volf is associate professor and senior researcher, and Popa is professor of Wood Chemistry and Biotechnology, Gheorghe Asachi Technical University of Iasi, Faculty of Chemical Engineering and Environmental Protection, Iasi, Romania. Email Popa at vipopa@ch.tuiasi.ro.

17. Ignat, I., Radu, D.G., Volf, I., et al., *Cellul. Chem. Technol.* 47(5-6): 387(2013).
18. Chemat, F., Vian, M.A, Cravotto, G., et al., *Int. J. Mol. Sci.* 13(7): 8615(2012).
19. Mandal, V., Mohan, Y., and Hemalatha, S., *Pharmacogn. Rev.* 1(1): 7(2007).
20. Ghitescu, R.E., Volf, I., Carausu, C., et al., *Ultrason. Sonochem.* 22: 535(2015).
21. Dai, J. and Mumper, J.R., *Molecules* 15(10): 7313(2010).
22. Herrero, M., Mendiola, J.A., Cifuentes, A., et al., *J. Chromatogr. A* 45(1): 35(2000).
23. Kaufmann, B. and Christen, P., *Phytochem. Anal.* 13(2): 105(2002).
24. Lee, J., Rennaker, C., and Wrolstad, R.E., *Food Chem.* 110(3): 782(2008).
25. Kim, D.W., Curtis-Long, M.J., Yuk, H.J., et al., *Food Chem.* 153: 20(2014).
26. Cheynier, V., *Phytochem. Rev.* 11(2-3): 153(2012).
27. Popa, M.I., Aelenei, N., Popa, V.I., et al., *React. Funct. Polym.* 45(1): 35(2000).
28. Popa, V.I., Dumitru, M., Volf, I., et al., *Ind. Crops Prod.* 27(2): 144(2008).
29. Stingu, A., Volf, I., Popa, V.I., et al., *Ind. Crops Prod.* 35(1): 53(2012).
30. Castillo, F., Hernández, D., Gallegos, G., et al., in *Fungicides for Plant and Animal Diseases* (D. Dhanasekaran, Ed.), InTech, Rijeka, Croatia, 2012, Chap. 4.
31. Tanase, C., Volf, I., Vintu, S., et al. *Cellul. Chem. Technol.* 47(7-8): 553(2013).
32. Popa, V.I., Agache, C., Beleca, C., et al. *Crop Res.* 24(2): 398(2002).
33. Stingu, A., Volf, I., and Popa, V.I., *Buletinul Institutului Politehnic Iasi*, Tom LV(LIX), Fasc. 4: 69(2009).
34. Tanase, C., Volf, I., and Popa, V.I., *Environ. Eng. Manage. J.* 12(6): 1287(2013).
35. Balas, A., Danaila, M., Popa, V.I., et al., *Buletinul Institutului Politehnic Iasi*, Tom LI(LV), Fasc. 3-4: 124(2005).
36. Bodarlau, R., Teaca, C.A., and Popa, V.I., *Environ. Eng. Manage. J.* 1(1): 67(2002)
37. Stingu, A., Volf, I., and Popa, V., *Cellul. Chem. Technol.* 45(3-4): 281(2011).
38. Stingu, A., Volf, I., and Popa, V.I., *Environ. Eng. Manage. J.* 8(5): 1247(2009).
39. Stingu, A., Stanescu, I., Volf, I., et al., *Cellul. Chem. Technol.* 45(3-4): 287(2011).
40. Volf, I., Stingu, A., and Popa, V.I., *Environ. Eng. Manage. J.* 11(2): 487(2012).
41. Stingu, A., Ignat, I., Hainal, A., et al., "Metal elements in environment, medicine and biology," Tome IX, Romanian Academy, 90-94 (2009).
42. Stingu, A., Volf, I., and Popa, V.I., *Scientific Papers, Horticulture Series* 53(1): 77(2010).
43. Hainal, A.R., Ignat, I., Volf, I., et al., *Scientific Papers, Horticulture Series* 53(1): 603(2010).
44. Ferrazzano, F.G., Amato, I., Ingenito, A., et al., *Molecules* 16(2): 1486(2011).
45. Danaila, M., Popa, V.I, and Volf, I., *Proceedings of the 8th ILI Forum*, International Lignin Institute, Orbe, Switzerland, 2007, p. 105.
46. Hainal, A.C., Ignat, I., Volf, I., et al., *Cellul. Chem. Technol.* 45(3-4): 211(2011).
47. Hainal, A.R., Diaconescu, R., Volf, I., et al., *Rom. Biotechnol. Lett.* 17(2): 7084(2012).
48. Hainal, A.R, Capraru, A.M, Volf, I., et al., *Cellul. Chem. Technol.* 46(1-2): 87(2012).
49. Volf I., Ignat I., and Neamtu, M., *Chem. Pap.* 68(1): 12(2013).
50. Jitaru (Ciubotariu), D., "Research concerning biological active properties of polyphenolic products," Ph.D. thesis, Gheorghe Asachi Technical University of Iasi, Romania, 2005.
51. Bocancea (Tarabuta), I.P., "Studies concerning the influence of vegetal compounds on microorganisms development in the stress conditions," Ph.D. thesis, Gheorghe Asachi Technical University of Iasi, Romania, 2013.
52. Danaila, M., "Contributions to the study of influence on natural products with aromatic structure on microorganisms development," Ph.D. thesis, Gheorghe Asachi Technical University of Iasi, Romania, 2009.

This article is under review at the journal "Food Chemistry":

Title

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

Authors

Oana-Crina Bujor[a,b,c], Carine Le Bourvellec[b,c], Irina Volf[a], Valentin I. Popa[a], Claire Dufour[b,c*]

Affiliations

[a]"Gheorghe Asachi" Technical University of Iasi, Faculty of Chemical Engineering and Environmental Protection, 700050 Iasi, Romania.

[b]INRA, UMR 408 Safety and Quality of Plant Products, F-84000 Avignon, France.

[c]University of Avignon, UMR 408 Safety and Quality of Plant Products, F-84000 Avignon, France.

Corresponding author

Claire Dufour – INRA, UMR SQPOV, 228 rte de l'Aérodrome, 84914 Avignon Cedex 9, France. tel: + 33 432 72 25 15. fax: + 33 432 72 24 92. claire.dufour@avignon.inra.fr.

Abstract

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 caffeic acid derivatives and flavonol glycosides in leaves whereas flavanol oligomers represented more than half of the phenolic compounds in stems. Thioacidolysis revealed low degrees of polymerization (2 – 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 those of May leaves. The latter were relatively richer 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 maximum 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, beverage and nutraceutical ingredients due to its richness in nutritional and bioactive compounds and are 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 (Rouanet et al., 2010; Erlund et al. 2008; Mauray, Felgines, Morand, Mazur, Scalbert, & Milenkovic, 2010; Mykkänen, Huotari, Herzig, Dunlop, Mykkänen, &

Kirjavainen, 2014). 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, Petković, Rodić-Grabovac, Stefanović, Vasić, & Čomić, 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, amendments in bioremediation, allelochemicals, and plants growth regulators (Bujor, Talmaciu, Volf, & Popa, 2015).

The quality and quantity of phenolic compounds in *Vaccinium 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; Martz, Jaakola, Julkunen-Tiitto, & Stark, 2010; Jovančević et al., 2011; Uleberg et al., 2012; Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 2015). It may appear that higher phenolic contents are favored by northern latitudes, altitude and a sunny environment. In bilberry fruits, high amounts of anthocyanins, hydroxycinnamic acid derivatives and low amounts of flavonols, proanthocyanidins and coumaroyl iridoids were identified (Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic, 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 (Martz, Jaakola, Julkunen-Tiitto, & Stark, 2010; Liu, Lindstedt, Markkinen, Sinkkonen, Suomela, & Yang, 2014). A comparative study conducted by Teleszko & Wojdyło (2015) showed that phenolic compounds were found in a

markedly higher content in the leaves than in the 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 the most commonly, leaves sometimes and stems never. In this context, the primary aim of this study is to simultaneously assess the seasonal variations of phenolic compounds in leaves, stems, and fruits of bilberry collected at three different periods of vegetation. Additionally, an original analysis of the oligomeric proanthocyanidins is proposed addressing both the degree of polymerization and flavanol unit constitution. Finally, contents in total polyphenols, assessed globally by the Folin-Ciocalteu method or specifically by UPLC, and the antioxidant capacity in the DPPH test are tentatively correlated.

2. Methodology

2.1. Materials

2.1.1. Bilberry samples

Fruits, leaves and stems of wild bilberry (*Vaccinium myrtillus* L.) were collected from mountains near Borca (Neamt, Romania, coordinate: 47° 11′ 34″ N and 25° 47′ 8″ E) in May, July and September during the years 2013-2014.

Fresh bilberry fruits were frozen at -24 °C, then lyophilized in a Christ Alpha 1-4 LSC (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 through a standard sieve to a final particle size < 0.315 mm. Grinded samples were kept in a desiccator until extraction. Before extraction, the residual moisture of grinded samples was determined using a RADWAG MAX 50/1 moisture analyzer (RADWAG Balances & Scales, Poland). Residual moistures between 7% and 9.5% were found for all plant materials.

2.1.2. Chemicals and solvents

Chemicals: Gallic, chlorogenic and *p*-coumaric acids, (+)-catechin, (-)-epicatechin, 37% hydrochloric acid, anhydrous sodium carbonate, Folin & Ciocalteu's phenol reagent, DPPH (1,1-diphenyl-2-picrylhydrazyl) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France); quercetin-3-glucoside (isoquercitrin), quercetin-3-rhamnoside (quercitrin), quercetin-3-galactoside (hyperoside), procyanidin B2,

procyanidin A2, (-)-epigallocatechin and cyanidin-3-O-galactoside (ideain chloride), were purchased from Extrasynthese (Genay, France); Procyanidin C1 (Epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin trimer) was extracted from apple fruits (*Malus domestica* Borkh.) of the Kermerrien variety as described earlier (Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998).

Solvents: 96% ethanol was purchased from Chemical Company (Iasi, Roumania); HPLC-MS grade methanol and acetonitrile from Fisher Scientific (Illkirch, France); formic acid from Merck (Darmstadt, Germany); glacial acetic acid from Merck (Fontenay Sous Bois, France); toluene-α-thiol from Sigma–Aldrich. Ultrapure water (resistivity 18.2 $M\Omega.cm^{-1}$ 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 samples placed in an extraction vial fitted with a condenser was added 30 mL of aqueous 1% 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, Xu, Liu, Sun, Lin & Liu, 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 of the residual water. Dry extract (DE) refers to the dry matter after extraction and freeze-drying.

2.3. Qualitative and quantitative analyses of phenolic compounds

For UPLC/MS analyses, freshly prepared solutions of bilberry leaf and stem Dry Extracts (10 mg/mL) in water were directly used. For the bilberry 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 by using a Waters ACQUITY UPLC chromatograph (Waters, Milford, MA) coupled to an UV−vis diode-array detector and a HCT ultra ion trap mass spectrometer equipped with an electrospray ionization (Mane, Loonis, Juhel, Dufour, & Malien-Aubert (2011). Separation was carried out using a reverse-phase Acquity BEH C18 column (50 mm x 2.1 mm i.d., 1.7 µm; Waters) at 30 °C. For polyphenols, a binary solvent system was used with solvent A (0.05% formic acid in water, v/v) and solvent B (acetonitrile) at a flow rate of 1 mL/min and with the following gradient elution: 0-2 min, linear 0-3% B; 2-3 min, isocratic 3% B; 3-6 min, linear 3-5% B; 6-7 min, linear 5-6% B; 7-12.5 min, linear 6-10% B, 12.5-19.5 min, linear 10-30% B; 19.5-20.5 min, linear 30-60% B; 20.5-21 min, linear 60-100% B; 21-22 min, linear 100-0% B; 22-24 min, isocratic 0% B. For anthocyanins, a binary solvent system was used with solvent A (1% formic acid in water, v/v) and solvent B (1% formic acid in acetonitrile) at a flow rate of 0.17 mL/min

and with the following gradient elution: 0-15 min, linear 0-20% B; 15-20 min, linear 20-40% B; 20-20.5 min, linear 40-100% B; 20.5-20.6 min, linear 100-0% B; 20.6-23.6 min, isocratic 0% B. The volume of extract injected was 3 μL for phenolic compounds and 1 μL of anthocyanins.

Mass detection was conducted in both negative (for phenolic compounds) and positive (for anthocyanins) electrospray ionization modes from m/z 100 to 1000. MS conditions in the negative ion mode were as follows: capillary voltage of 2 kV, nitrogen flow rate at 12 L/min; desolvation temperature at 365 °C and nebulization pressure at 60 psi. MS conditions in the positive ion mode were: capillary voltage of 1.8 kV, nitrogen flow rate at 9 L/min; desolvation temperature at 350 °C and nebulization pressure at 40 psi.

Authentic standards in MeOH, except cyanidin-3-galactoside which was acidified with 1% HCl (v/v), were used for 6 point-calibrations. Other phenolic compounds were quantified as follows: caffeic acid derivatives, 3,4-dihydroxyphenylpropionic acid hexoside and sinapic acid hexoside as chlorogenic acid (325 nm), coumaric acid derivatives as *p*-coumaric acid (330 nm), quercetin glycosides as quercetin-3-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 and anthocyanins as cyanidin-3-galactoside (520 nm). The flavanol monomers were calculated as the sum of (-)-epicatechin and (-)-epigallocatechin, while the flavanol oligomers were reported as the sum of all the dimers, trimers, cinchonains I 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 by High-Performance Liquid Chromatography (HPLC)/Diode Array Detection (DAD)/Fluorimetric detection after thioacidolysis using a method adapted from Le Bourvellec, Bouzerzour, Ginies, Regis, Plé & Renard (2011). Procyanidins were characterized by their subunit composition and their average degree of polymerization (mDP). The HPLC apparatus was a Shimadzu LC-20AD equipped with SPD-M20A DAD detector and a RF-10AXL Fluorescence detector (Shimadzu, Kyoto, Japan). Separations were achieved as in Le Bourvellec, Bouzerzour, Ginies, Regis, Plé, & Renard (2011). Individual compounds were quantified with external standards at 280 nm for (+)-catechin, (-)-epicatechin, (+)-catechin benzyl thioether (quantified as (+)-catechin), and (-)-epicatechin benzyl thioether (quantified as (-)-epicatechin). In samples containing anthocyanins, (+)-catechin and (-)-epicatechin were specifically identified and quantified by their emission-excitation 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 & Popa (2011). An aliquot of 1 mL of the solution after extraction was further diluted in water (1:50 for leaves and stems and 1:25 for fruits) before mixing with 0.5 mL of Folin-Ciocalteu reagent (2 M), 2 mL of 10% Na_2CO_3 solution 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) 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 analyzed.

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 & 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 during 30 minutes 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 the XLStat software (version 2008.3.02, Addinsoft SARL, Paris, France).

3. Results and discussion

3.1. Phenolic profile and content of bilberry extracts

From the UPLC/MS analyses, in all the morphological parts of bilberry, 106 phenolic compounds were tentatively identified (Table 1), 62 in leaf extracts, 73 in stem extracts and 40 in fruit extracts. Additionnaly, 17 were found only in leaves, 32 only in stems, and 9 only in fruits.

(a) Caffeic acid derivatives: In leaves, caffeic acid derivatives are present in 3 to 10-fold higher levels compared to coumaric acid derivatives and only in 1 to 2-fold higher levels in fruits. Interestingly, there is no difference in stems in the contents in these two hydroxycinnamic acid derivatives. 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. The 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. The compound 51

displayed a major fragment ion at *m/z* 341 (caffeic acid hexoside) and fragments at *m/z* 191 and 173 (typical for 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 or 513 could be interpreted as an additional caffeoyl or hexosyl unit on caffeoylquinic acid. Related caffeoylquinic acid derivatives, 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 a fragmentation (*m/z* 191, 179, 161 and 135) typical for caffeoylquinic acid was observed in bilberry or lingonberry buds and leaves by Ieri, Martini, Innocenti, & Mulinacci (2013) who named it caffeoyl derivative. In this study, it was 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, Loonis, Juhel, Dufour, & Malien-Aubert, 2011). 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, Martini, Innocenti, & Mulinacci, 2013).

(b) Coumaric acid derivatives: In leaves and stems, two *p*-coumaroylquinic acids (30 and 44) were evidenced based on their major fragment ion at *m/z* 191 (quinic acid) resulting from the loss of *p*-coumaric acid. Base peak at *m/z* 191 are only produced by 3- and 5- *p*-coumaroylquinic acids (Clifford, Knight, & Kuhnert, 2005). 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 group) 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 characterized by fragment ion 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, 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. We propose that some coumaroylated and malonylated glycosides could formally be (4-O-malonyl- -*p*-coumaroyl)glycosides in agreement with the earlier identification of *p*-coumaroylhexosides. Compounds not displaying the *m/z* 249 fragment could be *p*-coumaric acid-4-O-malonylglycosides in agreement with the presence of *p*-coumaric acid-4-O-hexosides. For compounds with λ_{max} 310 nm and a shoulder at 295 nm, the possible structure could be (*p*-coumaroyl)malonylglycosides. Compounds with *m/z* 409, 411 and 451 were already found in bud and leaf extracts of bilberry by Ieri, Martini, Innocenti, & Mulinacci (2013), Liu, Lindstedt, Markkinen, Sinkkonen, Suomela, & Yang (2014) and Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic (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 monotropein (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. Finally, two *p*-coumaroyl derivatives remained unidentified (65 and 89) although the last was newly reported in stems and fruits.

(c) Flavonol glycosides: In stems and leaves, quercetin glycosides were present in considerable amounts from May to September (Table 3), whereas in fruits 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. The berry characteristic quercetin-3-O-(4''-(3-hydroxy-3-methylglutaryl))-α-rhamnoside (100) was identified in all the morphological parts. Its structure was confirmed using NMR experiments by Ek, Kartimo, Mattila, & Tolonen (2006) who found it in lingonberry fruit and leaves. This compound is newly described in bilberry fruit while it was evidenced in bilberry buds and leaves by Hokkanen, Mattila, Jaakola, Pirttilä, & Tolonen (2009) and Ieri, Martini, Innocenti, & Mulinacci (2013). It was quantified in leaves in July and September (4th 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).

(d) Flavanols: In stems, flavanols were present from May to September (Table 1) although they were more abundant in July and September (Table 3). 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 in trace amounts 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 in a quantifiable amount 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 quantifiable amounts in leaves from July and September while not in May. Furthermore, an (epi)catechin derivative (13) with parent ion at *m/z* 405 was also detected in leaves. Last, 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 from July and September (59 and 60) and fruits from 2014 (99). In leaves from May, cinchonains were either not found or not fragmented indicating a lower content compared to July and September. They thus appear to be specifically biosynthetized from spring to summer.

(e) Anthocyanins: Both aqueous and ethanolic fruit extracts were found to contained high levels of anthocyanins (Table 3). 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 (supplementary material). In lingonberry, cyanidin-3-galactoside appeared as the major anthocyanin (Mane, Loonis, Mora, Caris-Veyrat, & Dufour, 2011), whereas delphinidin-3-O-galactoside and delphinidin-3-O-glucoside were predominant in bilberry fruit extracts as previously determined by Može et al. (2011) and 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 and anthocyanin profiles were found in bilberry leaves, stems, and fruits 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 (Martz, Jaakola, Julkunen-Tiitto, & Stark, 2010) whatever the period and the year of harvest, their level ranging between 67 and 79% of the Dry Extract weight (Table 2). Their seasonal evolution differed between years 2013 and 2014. Chlorogenic acid (18) contributed for more than half of the Dry Extract weight and its relative content varied between 55% (May 2013) and 75% (July 2013) (Table 3). Althouh leaves are exposed to light, the *cis* isomer represented less than 2% of the chlorogenic acid pool.

Flavonol glycosides were present in lesser contents in May compared to *p*-coumaric acid derivatives although this ranging became opposite 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, Lindstedt, Markkinen, Sinkkonen, Suomela, & Yang (2014) also observed no flavonol variation between July and September 2013 for leaves collected in Finland while Martz, Jaakola, Julkunen-Tiitto, & Stark (2010) observed a slight increase for leaves submitted to light (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-galactoside (62) from July to September in year 2013 and July 2014 (3-8%).

The second and third 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%).

p-Coumaric acid derivatives appeared at their highest levels in May while decreasing during the season. The other main contributor to this group is *p*-coumaroyl monotropein (70) ranking 4th in May 2013 and May 2014, although this ranking largely decreases 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. There were opposite seasonal variations between flavanol groups and inter-annual effects for (-)-epicatechin in September. Overall, there were seasonal and inter-annual variations for all the phenolic sub-groups studied.

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 (both ≥14%). Ranking third is dimer B2 (32) for all the 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 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). As for leaves, inter-annual variations were observed for most groups in the stem extracts. 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, Jaakola, Julkunen-Tiitto, & Stark, 2010; Uleberg et al., 2012. The year effect was however higher for stems than for leaves, suggesting that abiotic stress clearly influences polyphenol biosynthesis. Genetic control is rather observed in the type of polyphenol predominantly synthetized as observed with flavanol oligomers in stems.

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 3). 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).

3.3. Characterization of flavan-3-ol oligomers

Flavanol oligomers could appear as the second 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 3) where no type-A or type B oligomers, but only cinchonain II (59) were quantified in leaves (1.1-1.4 mg/g DE for the same period). mDP ranging from 2.9 to 4.5 suggests the presence of small-size oligomers which should not have escaped UPLC. Epicatechin was the only constituting unit of flavanol oligomers with 22 to 35% of terminal unit and the rest as extension units.

In stems, flavanol oligomers appeared as the first phenolic group as already observed in UPLC quantification. The range was between 48 and 70 mg/g DE after thioacidolysis and between 40 and 71 mg/g DE by UPLC. Moreover, flavanol monomers and oligomers evolved similarly along seasons in 2013 and 2014 independently of the methods. One should note that flavanol monomers are constituted by epicatechin and epigallocatechin in UPLC analysis (4-12 mg/g DE) while by epicatechin only in the thioacidolysis method (5-18 mg/g DE). There is thus more epicatechin titrated by the thioacidolysis method. Additionally, the oligomer contents appeared underestimated after thioacidolysis in accordance with type-A oligomers being incompletely degraded as well as the lack of quantification of (epi)gallocatechin units contained in oligomers. mDP ranging between 2 and 3 were also slightly underestimated in view of the two A-type and B-type trimers (45 and 46) that were predominant by UPLC. Last, 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 provided in the thioacidolysis method when no oligomers were quantified by UPLC, mostly because of co-elution with anthocyanins. mDP remained low (2-3). Surprisingly, catechin appeared

as a terminal unit only (9%) when epicatechin was the sole extension unit. Finally, 55% aqueous ethanol was twice as efficient at extracting monomeric and oligomeric flavanols although this difference was not evidenced for the different phenolic classes evaluated by UPLC.

3.4. Antioxidant activity of bilberry extracts

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

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% for leaves, stems and fruits, respectively). When expressed in mg of gallic acid per gram of DE (year 2014), the TPC values were in the same range or higher as 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. 2A and B). 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 for 20% to the phenolic pool in May and only

7% in July and September. Besides, *p*-coumaric acid derivatives remained at constant rates in stems (11-14%) and in fruits (4-5%) from May to September.

The DPPH scavenging activities of bilberry extracts (Fig. 1) showed two contrasted seasonal variations between 2013 and 2014 as already observed for TPC and the Sum of phenolic compounds. 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. Last, the annual effect on the DPPH antioxidant activities is similar to that exhibited for the TPC values except for the September stem sample.

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. 2C and D).Catechin extension or terminal units in dimer B3 and trimer C2 are known to transfer an electron or a H-atom towards the DPPH radical although they appear slightly less reactive than monomeric catechin. The graph patterns are similar to the ones observed for Sum of phenolic compounds vs TPC. The difference in 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. 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), which belong to caffeic acid derivatives and flavonol glycosides, was higher than that of salicylic acid (0.26) and tyrosine (0.38), which are structurally related to *p*-coumaric acid (Everette, Bryant,

Green, Abbey, Wangila, & Walker, 2010). Tryptophan, ascorbic acid, as well as Cu(II), Fe(II), and Zn(II) complexes also react with the Folin-Ciocalteu reagent although more weakly than most phenolic compounds. These substances are generally present in plant aqueous extracts and they may thus contribute to the overall antioxidant activity.

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. 2E and F). 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-centered radical 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 45 new compounds which are in bold in Table 1.

Quantitative analysis allowed to accurately determines the three or four more important contributors in the following groups: caffeoyl derivatives, *p*-coumaroyl derivatives, flavon glycosides, anthocyanins, and flavanol monmers and oligomers. In general, these major contributors did not show any seasonal variations. The most important variations were however outlined between May and July suggesting the appearance (flavanol oligomers in leaves, cinchonains I and II in stems from July and Sept) or disappearance of minor compounds (*p*-coumaroyl derivatives in leaves from May). The intra-annual variations for the various phenolic groups were generally different for years 2013 and 2014. Finally, the phenolic content was highly correlated to the antioxidant activity in leaf, stem and fruit extracts of bilberry. Results from this study indicate that all the morphological parts of bilberry are suitable for valorization 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 herbal supplements. The harvest period can be refined based on the desired phenolic structures and their potential health effect. The stability of the collected dry matter remains however to be assessed to determine its optimal shelf life.

Acknowledgements

O. C. Bujor thanks the Erasmus+ Programme for the student mobility grants. We gratefully thank Mrs Camelia Mihăilescu (Faculty of Chemical Engineering and Environmental Protection, Iasi, Romania) and Mrs Michèle Loonis (INRA-SQPOV Unit, Avignon, France) for their respective guidance in plant extraction and UPLC/MS analyses, respectively. We also thank Dr. Gina Tiron from National Meteorological Administration - Moldova Meteorological Center of Iasi, for providing the meteorological data.

Conflict of interest statement

The authors declare no conflict of interest.

References

1. Akerström, A., Jaakola, L., Bång, U., & Jäderlund, A. (2010). Effects of latitude-related factors and geographical origin on anthocyanidin concentrations in fruits of *Vaccinium myrtillus* L. (bilberries). *Journal of Agricultural and Food Chemistry*, *58*, 11939–11945.

2. Bujor. O-B., Talmaciu, I. A., Volf, I., Popa I. V. (2015). Biorefining to recover aromatic compounds with biological properties. *Tappi Journal*, *14(3)*, 187-193.

3. Chanforan, C., Loonis. M., Mora, N., Caris-Veyrat, C., & Dufour, C. (2012). The impact of industrial processing on health-beneficial tomato microconstituents. *Food Chemistry*, *134*, 1786–1795.

4. Clifford, M. N., Knight, S., & Kuhnert, N. (2005). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MS^n. *Journal of Agricultural and Food Chemistry*. *53*, 3821-3832.

5. Ek, S., Kartimo, H., Mattila, S., & Tolonen, A. (2006). Characterization of phenolic compounds from lingonberry (*Vaccinium vitis-idaea*). *Journal of Agricultural and Food Chemistry*, *54*, 9834-9842.

6. Erlund, I., Koli, R., Alfthan, G., Marniemi, J., Puukka, P., Mustonen, P., Mattila, P., & Jula, A. (2008). Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol. *The American Journal of Clinical Nutrition, 87*, 323–31.

7. Everette, J. D., Bryant, Q. M., Green, A. M., Abbey, Y. A., Wangila, G. W., & Walker, R. B. (2010). A thorogh study of reactivity of various compounds classes towards the Folin-Ciocalteu reagent. *Journal of Agricultural and Food Chemistry*, *58(14)*, 8139–

8144.

8. Goupy, P., Dufour, C., Loonis, M., & Dangles, O. (2003). Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *Journal of Agricultural and Food Chemistry, 51*(3), 615-622.

9. Guyot, S., Marnet, N., Laraba, D., Sanoner, P., Drilleau, J.-F. (1998). Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of polyphenols in different tissue zones of a french cider apple variety (*Malus domestica* Var. Kermerrien). *Journal of Agricultural and Food Chemistry, 46*, 1698-1705.

10. Hainal, A. C., Ignat, I., Volf, I., Popa, I. V. (2011). Transformation of polyphenols from biomass by some yeast species. *Cellulose Chemistry and Technology, 45* (3-4), 211-219.

11. Hokkanen, J., Mattila, S., Jaakola, L., Pirttilä, A. M., & Tolonen, A. (2009). Identification of polyphenols from lingonberry (*Vaccinium vitis-idaea* L.), bilberry (*Vaccinium myrtillus* L.) and hybrid bilberry (*Vaccinium x intermedium* Ruthe L.) leaves. *Journal of Agricultural and Food Chemistry, 57*, 9437–9447.

12. Ieri, F., Martini, S., Innocenti, M., & Mulinacci, N. (2013). Phenolic distribution in liquid preparations of *Vaccinium myrtillus* L. and *Vaccinium vitis idaea* L. *Phytochemical Analysis, 24*, 467–475.

13. Jovančević, M., Balijagić, J., Menković, N., Šavikin, K., Zdunić, G., Janković, T., & Dekić-Ivanković M. (2011). Analysis of polyphenols in wild populations of bilberry (*Vaccinium myrtillus* L.) from Montenegro. *Journal of Medicinal Plants Research*, *5(6)*, 910-914.

14. Lätti, A.K., Jaakola, L., Riihinen, K. R., & Kainulainen, P. S. (2010). Anthocyanin and flavonol variation in bog bilberries (*Vaccinium uliginosum* L.) in Finland. *Journal of Agricultural and Food Chemistry*, *58*, 427–433.
15. Le Bourvellec, C., Bouzerzour, K., Ginies, C., Regis, S., Plé, Y., & Renard, M. G. C. C. (2011). Phenolic and polysaccharidic composition of applesauce is close to that of apple flesh. *Journal of Food Composition and Analysis, 24*, 537–547.
16. Liu, P., Lindstedt, A., Markkinen, N., Sinkkonen, J., Suomela, J-P., & Yang, B. (2014). Characterization of metabolite profiles of leaves of bilberry (*Vaccinium myrtillus* L.) and lingonberry (*Vaccinium vitis-idaea* L.). *Journal of Agricultural and Food Chemistry, 62*, 12015−12026.
17. Ma, Y-T., & Cheung, P. C. K. (2007). Spectrophotometric determination of polyphenols by enzymatic and chemical methods - a comparison of structure-activity relationship. *Journal of Agricultural and Food Chemistry*, *55*, 4222-4228.
18. Mane, C., Loonis, M., Juhel, C., Dufour, C., & Malien-Aubert, C. (2011). Food grade lingonberry extract: polyphenolic composition and *in vivo* protective effect against oxidative stress. *Journal of Agricultural and Food Chemistry*, *59*, 3330–3339.
19. Martz, F., Jaakola, L., Julkunen-Tiitto, R., & Stark S. (2010). Phenolic composition and antioxidant capacity of bilberry (*Vaccinium myrtillus*) leaves in Northern Europe following foliar development and along environmental gradients. *Journal of Chemical Ecology, 36*, 1017–1028.
20. Mauray, A., Felgines, C., Morand, C., Mazur, A., Scalbert, A., & Milenkovic, D. (2010). Nutrigenomic analysis of the protective effects of bilberry anthocyanin-rich extract in apo E-deficient mice. *Genes Nutrition*, *5,* 343–353.

21. Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F., & Veberic, R. (2015). A comparison of fruit quality parameters of wild bilberry (*Vaccinium myrtillus* L.) growing at different locations. *Journal of the Science of Food and Agriculture*, *95*, 776–785.

22. Može, Š., Polak, T., Gašperlin, L., Koron, D., Vanzo, A., Poklar Ulrih, N., & Abram, V. (2011). Phenolics in slovenian bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.). *Journal of Agricultural and Food Chemistry, 59(13)*, 6998-7004.

23. Mykkänen, O. T., Huotari, A., Herzig, K-H., Dunlop, T. W., Mykkänen, H., Kirjavainen, P. V. (2014). Wild blueberries (*Vaccinium myrtillus*) alleviate inflammation and hypertension associated with developing obesity in mice fed with a high-fat diet. *PLoS ONE, 9*(12), 1-21.

24. Prencipe, F. P., Bruni, R., Guerrini, A., Rossi, D., Benvenuti, S., & Pellati, F. (2014). Metabolite profiling of polyphenols in *Vaccinium* berries and determination of their chemopreventive properties. *Journal of Pharmaceutical and Biomedical Analysis*, *89*, 257-267.

25. Rouanet, J. M., Décordé, K., Del Rio, D., Auger, C., Borges, G., Cristol, J.-P., Lean, M. E. J., & Crozier, A. (2010). Berry juices, teas, antioxidants and the prevention of atherosclerosis in hamsters. *Food Chemistry*, *118*(2), 266-271.

26. Teleszko, M., & Wojdyło, A. (2015). Comparison of phenolic compounds and antioxidant potential between selected edible fruits and their leaves. *Journal of Functional Foods*, *14*, 736-746.

27. Uleberg, E., Rohloff, J., Jaakola, L., Trôst, K., Junttila, O., Häggman, H., & Martinussen I. (2012). Effects of temperature and photoperiod on yield and

chemical composition of northern and southern clones of bilberry (*Vaccinium myrtillus* L.). *Journal of Agricultural and Food Chemistry, 60*, 10406−10414.

28. Vučić D. M., Petković M. R., Rodić-Grabovac B. B., Stefanović O. D., Vasić S. M., & Čomić L.R. (2013). Antibacterial and antioxidant activities of bilberry (*Vaccinium myrtillus* L.) *in vitro. African Journal of Microbiology Research*, *7(45)*, 5130-5136.

29. Zheng, X., Xu, X., Liu, C., Sun, Y., Lin, Z., & Liu, H. (2013). Extraction characteristics and optimal parameters of anthocyanin from blueberry powder under microwave-assisted extraction conditions. *Separation and Purification Technology, 104*, 17–25.

Figures:

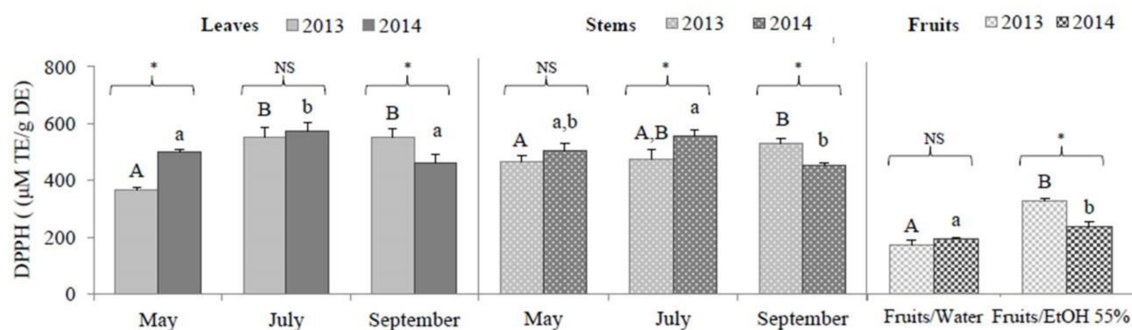


Fig. 1. Influence of different harvest periods on DPPH radical scavenging activity in leaves, stems and fruit extracts of bilberry (mean±SD, n = 3-4). Different letters indicate a significant difference between three periods of vegetation at $p < 0.05$: capital letters are used to compare the samples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

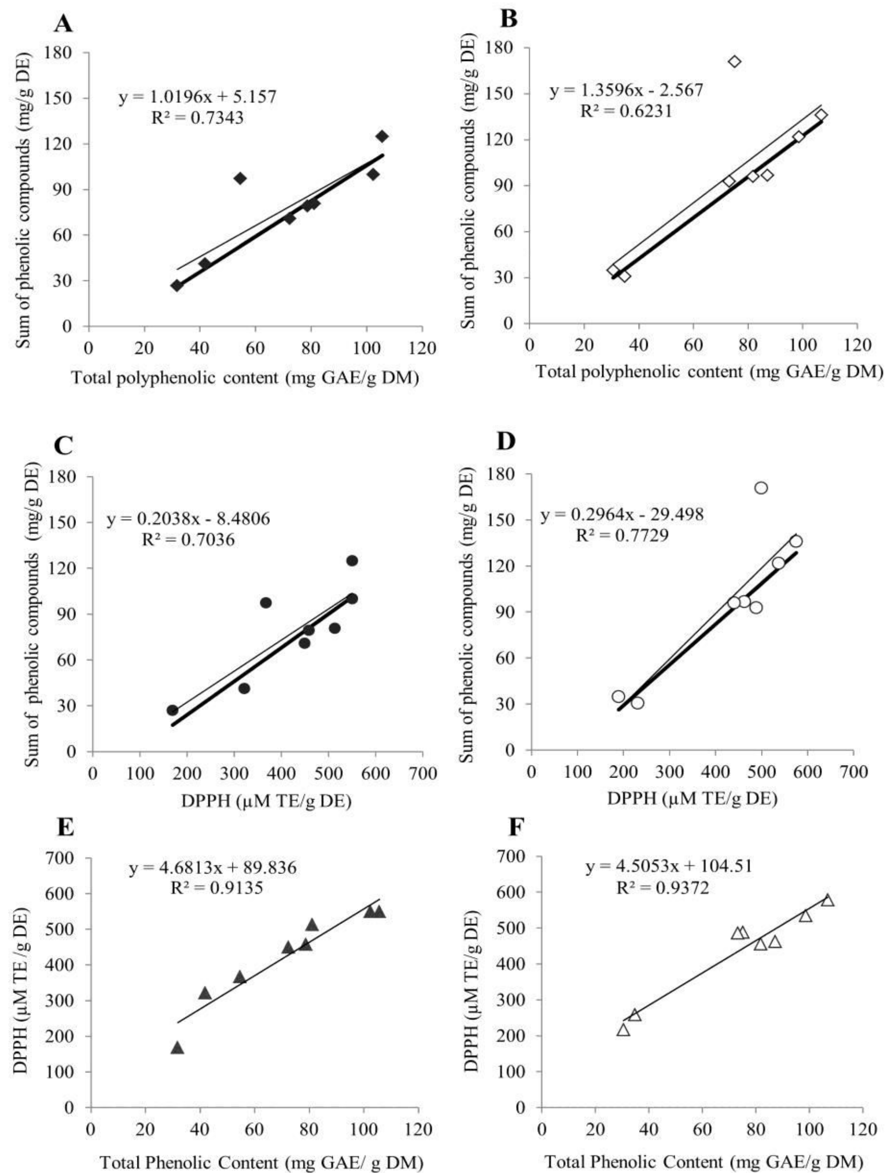


Fig. 2. Correlation between the Sum of Phenolic Compounds and the Total Phenolic Content (A: samples from 2013; B: samples from 2014), between the Sum of Phenolic Compounds and DPPH radical scavenging activity (C: samples from 2013; D: samples from 2014), and between the Total Phenolic Content by the Folin-Ciocalteu method and the DPPH radical scavenging activity (E: samples from 2013; F: samples from 2014). Thin line is the linear regression for all samples. Solid line is the linear regression without leaf from May.

Table 1. Phenolic compounds identified by UPLC/ESI-MSn 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, 575 MS^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}	

No.	t_R (min)	λ_{max} (nm)	$[M – H]^–$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.
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	Coumaroylquinic acid (1)[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)	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,	B-type trimer (2)	L[a], S	L, S	L, S	S[a]	L[a], S	S

No.	t_R (min)	$λ_{max}$ (nm)	[M – H]⁻ (*m/z*)	MS² fragments (*m/z*)	Proposed structure	2013 May	2013 July	2013 Sept.	2014 May	2014 July	2014 Sept.
				695, 587, 577, 575, 451, 425, 407, 289, 287,							
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]
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		337	191, 163	Coumaroylquinic acid (2)	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,	B-type trimer (3)	L[a], S	L, S	L, S	L[a], S	L, S	L[a], S

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.
				451,449, 425, 407, 287							
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

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.
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[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},	L	L	L, F_{H_2O},	L

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.
							F_{EtOH}			F_{EtOH}	
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]
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 malonyldihexose (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 malonyldihexose (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,	***p*-Coumaroyl malonylhexoside (3)**	L, S	L, S, F_{H_2O},	L, S	L, S	L, S, F_{H_2O},	L, S

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.
				119			F_{EtOH}			F_{EtOH}	
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, & Tolonen (2009).

[d]Lätti, Jaakola, Riihinen, & 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

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 (a)***	**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 as well as cinchonains II in stems, only cinchonains I+II 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
	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 parantheses correspond to compound number in Table 1. [b]Mean for $n = 3$.

Table 4. Flavan-3-ol composition and mDP in bilberry leaves, stems and fruits determined by HPLC following thioacidolysis.

Morphological part extracts	Period of vegetation	Procyanidin characterization				Flavanol oligomers (mg/g DE)	mDP
		Terminal units (%)		*Extension units (%)*			
		CAT	*EC*	*CAT*	*EC*		
Leaf extracts							
	May 2013	–	34.7 ± 4.5(A)	–	65.3 ± 16.6(A)	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(B)	–	70.1 ± 2.3(B)	32.4 ± 2.2(B)	3.4 ± 0.4(A)
	July 2014	**–**	**22.1 ± 0.3(b)**	**–**	**77.9 ± 1.2(b)**	**25.5 ± 1.4(b)***	**4.5 ± 0.1(b)***
	September 2013	–	26.3 ± 2.3(C)	–	73.7 ± 0.8(C)	23.8 ± 0.6(C)	3.8 ± 0.2 (A)
	September 2014	**–**	**28.0 ± 3.7(c)***	**–**	**72.0 ± 1.7(c)***	**12.7 ± 0.6(c)***	**3.6 ± 0.3(a)**
Stem extracts							
	May 2013	2.17 ± 0.11(A)	36.1 ± 1.8(A)	–	61.7 ± 3.0(A)	51.7 ± 2.1(A)	2.6 ± 0.0(A)
	May 2014	**–**	**32.6 ± 0.1(a)***	**1.61 ± 0.37(a)**	**65.8 ± 0.4(a)***	**60.0 ± 4.3(a)***	**3.1 ± 0.0 (a)***
	July 2013	3.10 ± 0.87(A)	37.5 ± 1.1(B)	–	59.4 ± 0.4(B)	63.6 ± 0.3(B)	2.5 ± 0.1(B)
	July 2014	**–**	**33.7 ± 0.6(b)***	**1.20 ± 0.17(a)**	**65.1 ± 0.9(a)***	**69.6 ± 2.3(b)***	**3.0 ± 0.0(b)***
	September 2013	5.86 ± 1.06(B)	36.5 ± 1.0(B)	–	57.6 ± 1.9(B)	60.3 ± 0.3(C)	2.4 ± 0.0 (C)
	September 2014	**7.90 ± 0.28**	**38.1 ± 0.9(c)**	**2.66 ± 2.60(a)**	**51.4 ± 2.0(a)***	**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 ($p < 0.05$).

Supplementary material:

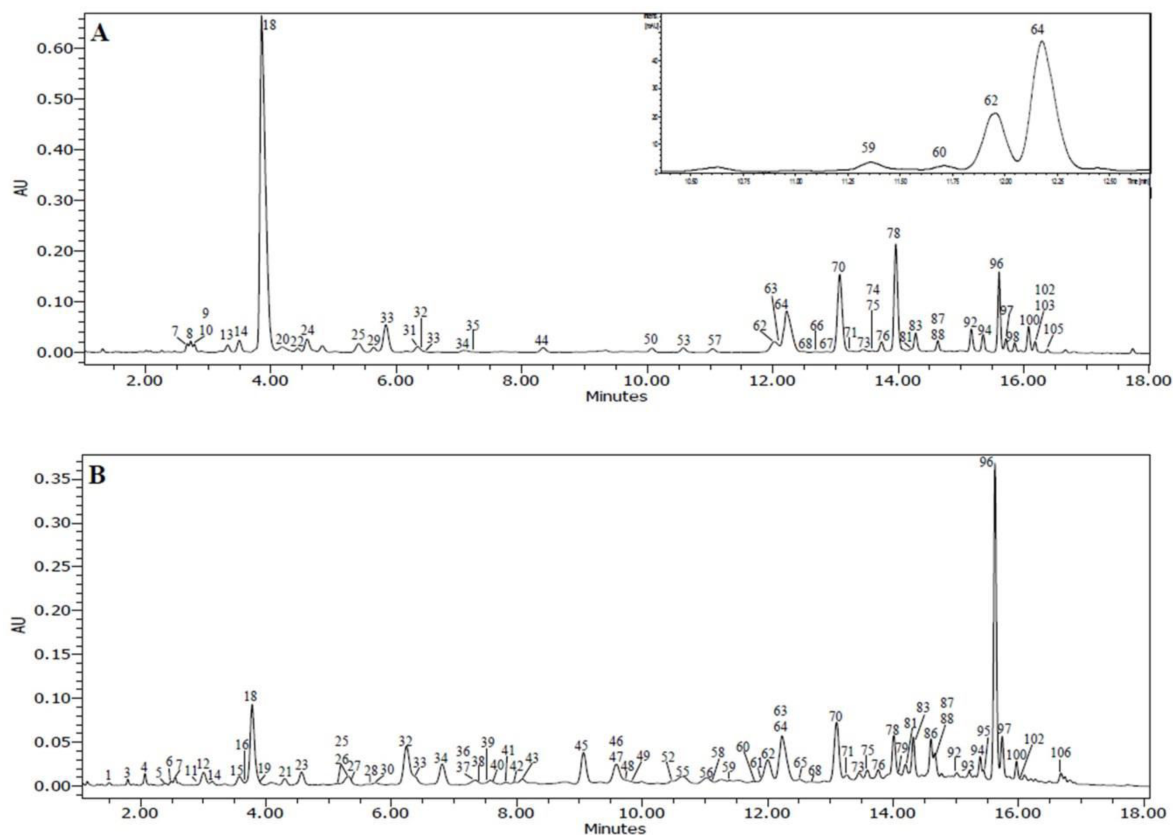


Fig. 3. Chromatographic phenolic profile of leaf (A) and stem (B) extracts of bilberry at 280 nm.

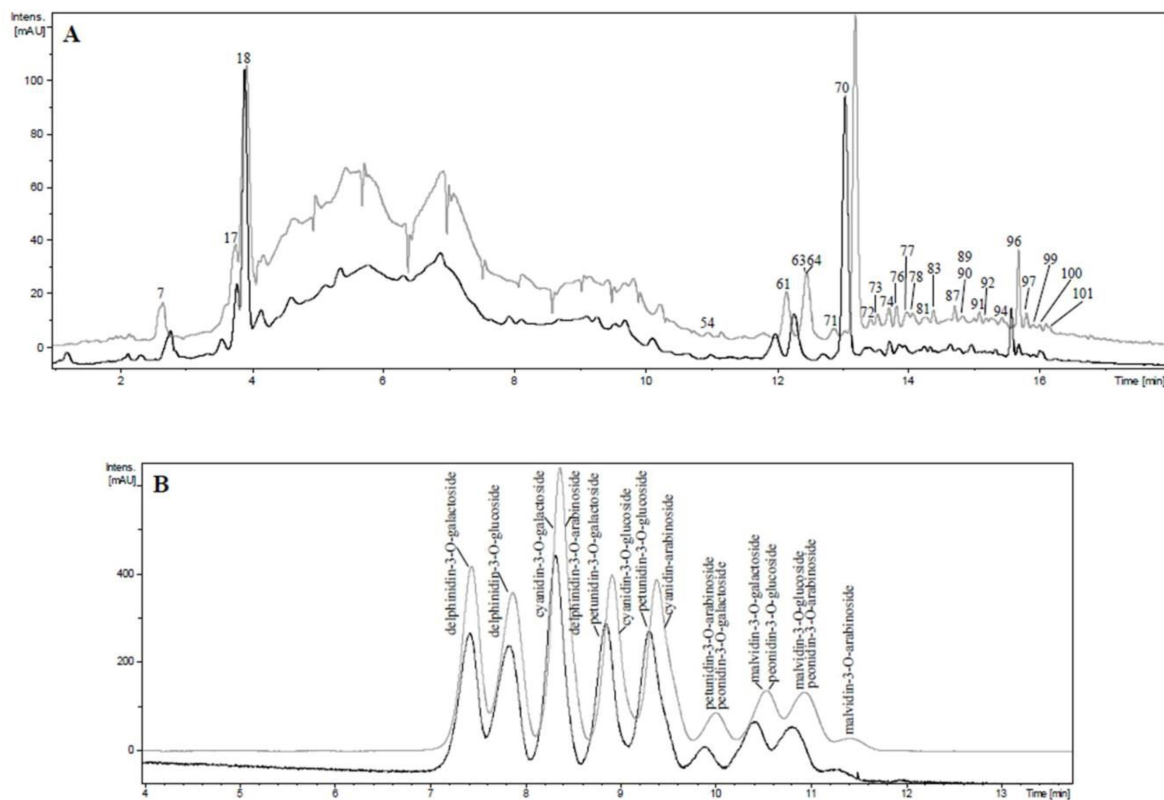


Fig. 4. Chromatographic profile at 280 nm (A) and at 520 nm (B) of aqueous (black) and ethanolic (grey) fruit extracts.

Table 5. Anthocyanins identified by UPLC/ESI-MS in fruits extracts of bilberry.

t_R (min)	λ_{max} (nm)	$[M - H]^+$ (*m/z*)	MS^2 fragments (*m/z*)	Proposed structure[a]	2013	2014
7.5	278, 522	465	303	Delphinidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.0	280, 522	465	303	Delphinidin-3-O-Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.4	278, 520	449	287	Cyanidin-3-O-Gal (std)	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.4		435	303	Delphinidin-3-O-Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
8.9	278, 518	449	287	Petunidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.1		479	317	Cyanidin-3-O-Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.4	278, 520	479	317	Petunidin-3-O- Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
9.6		419	287	Cyanidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.0	278, 524	449	317	Petunidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.0		463	301	Peonidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.5	278, 523	493	331	Malvidin-3-O-Gal	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
10.6	278, 523	463	301	Peonidin-3-O- Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
11.0	276, 526	493	331	Malvidin-3-O- Glc	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
11.0		433	301	Peonidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}
11.4	270, 528	463	331	Malvidin-3-O- Ara	F_{H_2O}, F_{EtOH}	F_{H_2O}, F_{EtOH}

Gal: galactoside; Ara: arabinoside; Glc: glucoside; F_{H_2O}: aqueous fruit extract; F_{EtOH}: ethanolic fruits extract; [std] compounds were identified by comparison with standard; [a] identified according to Može et al. (2011), Mane, Loonis, Juhel, Dufour, & Malien-Aubert, 2011 and Mikulic-Petkovsek, Schmitzer, Slatnar, Stampar, & Veberic (2015).

Table 6. Weather informations for the Borca region (Romania) during the study period.

month	year	average temperature (°C)	maximum temperature (°C)	minimum temperature (°C)	average humidity (%)	average soil temperature (°C)	maximum soil temperature (°C)	minimum soil temperature (°C)	total precipitations
February	2013	-0.8	10.8	-8.7	84	-2	5	-15.3	24.7
	2014	**-0.3**	**16.2**	**-18.3**	**86**	**-1.3**	**10.2**	**-22**	**5.3**
March	2013	0.4	13.6	-10.6	75	-0.3	20.6	-12.9	31.0
	2014	**7**	**20.9**	**-1.2**	**69**	**6.9**	**34.5**	**-2.5**	**29.2**
April	2013	11	29.9	-0.7	66	12	46.4	-1.6	45.2
	2014	**9.4**	**21.8**	**0.1**	**76**	**10.9**	**37.5**	**-0.3**	**95.5**
May	2013	16.8	30.5	5.9	64	19.8	52.4	4.4	51.6
	2014	**14.7**	**28.3**	**2.3**	**71**	**17.9**	**49.7**	**0.6**	**139.4**
June	2013	18.9	31.5	8.3	76	21.6	53.0	6.7	233.4
	2014	**17.4**	**29.6**	**8.2**	**72**	**20.1**	**52.9**	**5.5**	**59.8**
July	2013	19.9	32.7	10.2	68	23.5	56.8	7.6	50.0
	2014	**20.1**	**30.6**	**10.1**	**-**	**22.4**	**48.7**	**9.8**	**240**
August	2013	19.8	31.2	9.1	68	23.6	54.5	8.3	51.6
	2014	**19.9**	**32.5**	**7.8**	**72**	**22.9**	**52.9**	**6.0**	**44.8**
September	2013	13.5	25.7	3.3	72	14.1	42.3	1.2	50.4
	2014	**15.2**	**26.8**	**0.3**	**71**	**17.9**	**43.6**	**-1.2**	**19.2**