KAUNAS UNIVERSITY OF TECHNOLOGY

FACULTY OF CHEMICAL TECHNOLOGY

DEPARTMENT OF FOOD SCIENCE AND TECHNOLOGY

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DEVELOPMENT OF EFFECTIVE PROCESSES FOR THE ISOLATION OF BIOACIVE COMPOUNDS FROM *ELEUTHEROCOCCUS* BERRIES AND BILBERRIES AND EVALUATION OF THE OBAINED PRODUCTS

Master's Degree Final Project

Supervisor

Prof. Dr. Petras Rimantas Venskutonis

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Food Science and Safety (code 621E40001)

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"DEVELOPMENT OF EFFECTIVE PROCESSES FOR THE ISOLATION OF BIOACTIVE COMPOUNDS FROM *ELEUTHEROCOCCUS* BERRIES AND BILBERRIES AND EVALUATION OF THE PRODUCTS OBTAINED"

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ABBREVIATIONS

EH	*Eleutherococcus Henryi*
ES	*Eleutherococcus Sessiliflorus*
SFE-CO_2	Supercritical Fluid Extraction
CE	Conventional Extraction
ASE	Accelerated Solvent Extraction
MHG	Microwave Hydro Diffusion and Gravity
UAE	Ultrasound Assisted extraction
TPC	Total polyphenol content
DPPH	2,2 diphenyl-1-picrylhyrazyl
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid
FCRC	Folin Ciocalteu reagent reducing capacity
TFC	Total Flavonol content
TMAC	Total Monomeric Anthocyanin Content
PRF	Polyphenol Rich Fraction
MAG	Mono acyl glycerol
DAG	Di acyl glycerol
TAG	Tri acyl glycerol
MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
SFA	Saturated fatty acids
TLC	Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
GC-FID	Gas Chromatography-Flame Ionization Detector
UV Vis.	Ultraviolet Visible spectrum
UPLC/MS	Ultraperformance Liquid Chromatography / Mass Spectrometry
HPTLC	High Performance Thin Layer Chromatography
ESI-QTOF	Electron Spray Ionization – Quadrupole Time of Flight
COSMO-RS	Conductor like screening model for Real Solvents
U.S.A.	United States of America

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SUMMARY

Supercritical fluid extraction and accelerated solvent extraction (ASE) were applied for the removal of lipids and bioactive compounds of *Eleutherococcus Henryi* and *Eleutherococcus Sessiliflorus* berries. Antioxidant capacity of extracts were evaluated by total polyphenol content (TPC), ABTS and DPPH radicals scavenging and ferric reducing antioxidant power (FRAP) assays and results were elucidated by trolox equivalent antioxidant capacity (TEAC). The fatty acid profile and tocopherol content of the lipid fraction were analyzed. Oleic and linoleic acids were predominantly found in the lipophilic fraction. The major individual tocopherol present in the lipid fraction was α-tocopherol. The oxidative stability of bioactivity rich ASE extract was evaluated by Oxipres and Rancimat assays in an emulsion system. Phytochemical screening was performed using UPLC/ESI-QTOF-MS and resulted in the identification of coumarin (scopolin), phenolic acid (chlorogenic acid), flavonol (hyperoside), anthocyanin (cyanidin-3-sambubioside). An online HPLC-UV/DPPH method was employed to identify individual radical scavengers present in ASE extract.

Bio-refining of bilberry pomace using innovative technologies such as microwave hydro diffusion and gravity extraction (MHG) and ultrasound assisted extraction (UAE) with different concentrations of ethanol/water as the solvent system was established. Bead milling was also utilized in this sequential extraction scheme to remove the lipophilic fraction. Solubility index of target polyphenols was predicted using a computational tool (COSMO-RS) and compared to experimental results obtained by *in vitro* antioxidant activity assessments. MHG extract (2 W/g) had the highest Folin-Ciocalteu reducing capacity (43.46 ± 0.48 mg GAE/ g of Extract), total flavonoid (4.17 ± 0.04 mg QE/ g of Extract), total monomeric anthocyanin content (12.19 ± 0.13 mg D3GE /g of Extract) and radical scavenging capacity (22.64 ± 2.23 mg TE/ g of Extract). In UA ethanol/water extraction the highest anthocyanin (10.41 ± 0.08 mg QE/ g of Extract) and flavonoid content (12.19 ± 0.51 mg/g of Extract) was present in ethanol (100 %) extract. These results were in good correlation with computational prediction.

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SANTRAUKA

Superkritinės skysčių ekstrakcijos ir pagreitintos ekstrakcijos tirpikliais (ASE) metodai buvo taikyti riebalų ir bioaktyvių junginių ekstrakcijai iš *Eleutherococcus Henryi* ir *Eleutherococcus Sessiliflorus* uogų. Atioksidacinis ekstraktų aktyvumas buvo įvertintas bendrojo fenolinių junginių kiekio nustatymo (BFJK), ABTS ir DPPH radikalų surišimo gebos nustatymo ir geležies jonų redukcijos antioksidacinės galios nustatymo (FRAP) metodais ir rezultatai buvo išreikšti trolokso ekvivalento antioksidacine geba (TEAC). Riebalų rūgščių sudėtis ir tokoferolių kiekis buvo įvertinti lipofilinėje frakcijoje. Oleino ir linoleno rūgštys buvo dominuojančios minėtoje frakcijoje. α-tokoferolis buvo pagrindinis tokoferolis, identifikuotas lipofilinėje frakcijoje. Bioaktyviais junginiais gausaus ASE ekstrakto oksidacinis stabilumas buvo įvertintas instrumentiniais Oksipreso ir Rancimato metodais emulsijų sistemoje. Fitocheminių junginių analizė taikant UPLC/ESI-QTOF-MS metodą lėmė kumarino (skopolino), fenolinės rūgšties (chlorogeninės rugšties), flavonolio (hyperozido), antiocianino (cyanidin-3-sambubiozido) identifikavimą. Tiesioginis (on-line) HPLC-UV/DPPH metodas buvo pritaikytas indvidualių, radikalus surišančių, junginių identifikavimui ASE ekstrakte.

Mėlynių išspaudų biorafinavimui buvo taikytos inovatyvios mikrobangų vandens difūzijos ir gravitacijos (MHG) ir ultragarsu skatinamos ekstrakcijos (UAE) technologijos naudojant skirtingų koncentracijų etanolio/vandens tirpiklių sistemą. Ekstrakcija heksanu rotacinėje maišyklėje su keraminiais rutuliukais buvo naudota pakopinėje ekstrakcijoje lipofilinės frakcijos pašalinimui. Tikslinių polifenolių tirpumo indeksas buvo prognozuojamas naudojant skaičiavimo įrankį (COSMORS) ir palygintas su tyrimų rezultatais, gautais įvertinus *in vitro* antioksidacinį aktyvumą. MHG ekstraktas (2 W/g) turėjo didžiausią Folin-Ciocalteu redukcinę gebą (43.46 ± 0.48 mg GAE/ g ekstrakto), bendrą flavonoidų kiekį (4.17 ± 0.04 mg QE/ g ekstrakto), bendrą monomerinių antiocianinų kiekį (12.19 ± 0.13 mg D3GE /g ekstrakto) ir radikalų surišimo gebą (22.64 ± 2.23 mg TE/ g ekstrakto). Ultragarsu skatinamoje etanolio/vandens ekstrakcijoje didžiausi antiocianinų (10.41 ± 0.08 mg QE/ g ekstrakto) ir flavonoidų (12.19 ± 0.51 mg/g ekstrakto) kiekiai buvo nustatyti etanoliniame (100 %) ekstrakte. Šie rezultatai gerai koreliavo su apskaičiuota prognoze.

INTRODUCTION

Knowledge of biological activity or active substances extracted from plants and its constituents have a key position in the progression of modern studies on the health promoting factors exhibited by various bioactive constituents. The effectiveness of functional foods and nutraceutical products in preventing diseases depends on preserving the stability, bioactivity, and bioavailability of active ingredients. In general, bioactive compounds isolated from plant materials might be defined as secondary metabolites that instate pharmacological or toxicological effects in human and animals and can be identified and characterized from extracts of roots, stem, bark, leaves, flowers fruits and seeds.

The quantitative and qualitative studies of the bioactive compounds from plant materials depend primarily on the extraction method and system employed. Common parameters such as the matrix properties of the botanical source, solvent, temperature, pressure and time influence the extraction conditions and the properties of bioactive compounds isolated. The extraction conditions serve as the primary component, that the bioactive plant compounds might be classified based on the type of extraction: 1. hydrophilic or polar compounds (e.g., phenolic acids, flavonoids, organic acids, sugars); 2. lipophilic or nonpolar compounds (e.g., carotenoids, alkaloids, terpenoids, fatty acids, tocopherols, steroids). Another common classification criterion is characterizing bioactive constituents according to their nature of distribution: 1. shortly distributed (simple phenols, pyrocatechols, aldehydes); 2. widely distributed (flavonoids, phenolic acids); and 3. least abundant polymers (lignin and tannin) (Beatriz Vieira da Silva et al., 2016)

Eleutherococcus henryi (Oliv.) Harms and *Eleutherococcus sessiliflorus* (Rupr. et Maxim.), belong to the Araliaceae family. Almost all anatomical parts of the plant have been consumed traditionally in different forms for its therapeutic properties in countries like China, Japan, and Korea. The majority of the berries may be consumed fresh, but berries from some medicinal plants like *Eleutherococcus* are not suitable for direct consumption due to its astringent flavor which can be attributed to its high terpenoid and lignin content. But, new innovations in food and beverage industry has paved way for manufacturers to use such biological activity rich berries in their products. A recent patent, strawberry *Acanthopanax sessiliflorus* fruit wine and brewing method thereof (Patent no: CN101649285 A, 2010) is an example.

Apart from its adaptogenic properties, the pharmacological research and analysis on Eleutherococcus Sessiliflorus fruit have acknowledged the surplus biological and health promoting functions it possesses, this includes antitumour, immunostimulating activity (Lee et al., 2003), antithrombotic and antiplatelet aggregation inhibitory activities (Song Yang et al., 2011

and Jing ling Jin et al., 2004). Several other research works on the phytochemical constituents of *Eleutherococcus sessiliflorus* fruits have indicated the presence of wide range of bioactive compounds such as terpenoids (chiisanogenin and chiisanoside), lignans (syringaresinol and pinoresinol), coumarin (scopolin), flavonol (hyperoside) and phenolic acid (protocatechuic acid) and anthocyanin (cyanidin-3-sambubioside) (Dae-Young Lee et al., 2012, Longshan Zhao et al., 2014, Sang-Jin Lee et al., 2013 and Chun-Juan Yang et al., 2013). For instance, the main anthocyanin cyanidin-3-O-sambubioside present in *E. Sessiliflorus* fruit is said to have inhibited metastasis in breast cancer cells (Lee et al., 2013). Similarly, authors Sung Keun Jung et al., 2011 in their study concluded that hyperoside, isolated from *A. Sessiliflorus*, significantly inhibits ERK activity and may be useful as a novel chemopreventive agent.

Bilberry or European wild blueberry belongs to *Vaccinium* genus and is predominantly found in North America and European countries. Bilberry is packed with a wide range of bioactive compounds such as anthocyanins, flavonols, flavan-3-ols, stilbenes, procyanidins, tannins, vitamins, phenolic and hydroxycinnamic acids (Baj et al., 1983). The high concentration of such polyphenols and secondary metabolites in wild bilberries can be attributed to their elevated environmental stress exposure which significantly modulates their phytochemical profile thereby enabling them to accumulate larger amounts of defensive phytochemicals than their cultivated relatives (Kellogg et al., 2009; Szakiel et al., 2012). Bilberry is considered to have a protective role in human health against cardiovascular disorders, advanced age -induced oxidative stress, inflammatory responses, and diverse degenerative diseases. Bilberry extracts have demonstrated a protective effect against restraint stress-induced liver damage in mice (Bao et al., 2008), cytoprotective effect against oxidative damage of intoxicated rat hepatocytes (Valentova et al., 2007) and all these effects was attributed to the antioxidant potential of its constituents.

During juice processing, a considerable amount of polyphenol-rich seeds and skins of berries are discarded resulting in a relatively lower concentration of polyphenol in juice. Enzyme-aided pressing has helped manufacturers to enhance polyphenol content in juice, yet there is a significant amount of polyphenols trapped in pomace (Aaby et al., 2013; Buchert . et al., 2005; Koponen et al., 2008). Microwave Hydro diffusion and Gravity (MHG) is a novel technology with enormous potential for a variety of extractive applications including production of aromatic juices and extracts. Extraction of different compounds with respect to microwave time from various fruits was studied by Cendres et al. (2014) and the authors concluded that at different steps of extraction certain classes of compounds were extracted with MHG. Similarly, the merits of Ultrasound-Assisted Extraction (UAE) was widely reviewed by Chemat et al. (2017) and its application includes extraction and intensification among others.

1. AIM AND OBJECTIVE OF THE THESIS

Aim: To valorise *E. Henryi* and *E. Sessiliflorus* fruits and bilberry pomace as a promising source of antioxidants, polyphenols, and other bioactive phytochemicals by using innovative extraction technologies for their recovery and various methods for evaluating antioxidant properties and composition.

This thesis gives a comprehensive idea on the phytochemical composition of the *E. Henryi* and *E. Sessiliflorus* fruits and a vivid picture of the antioxidant potential exhibited by the extracts. Thus, enabling future research activities on *Eleutherococcus* species to establish a systematic approach for its maximum utilization in food systems. Similarly, innovative extraction techniques were employed sequentially to extract target polyphenols from bilberry pomace. The anthocyanin composition in all bilberry extracts was quantified with HPTLC and compared to the results obtained by UV-Vis spectrophotometry. As final valorization step, all waste residues were subjected to bead milling in order to remove the lipophilic fraction. The fatty acid profile was determined by Gas Chromatography- Flame Ionization Detection (GC-FID) and lipid class distribution was identified by High-Performance Thin Layer Chromatography (HPTLC).

The main objectives of this thesis were

1. to employ different extraction techniques like Supercritical Fluid Extraction (SFE), Accelerated solvent extraction (ASE) to extract polar and non-polar compounds with appropriate solvents from *Eleutherococcus* berries.

2. to elucidate the antioxidant potential of the extracts obtained using in vitro antioxidant assays such as Total Polyphenol content (TPC), Radical scavenging capacities using $ABTS^{\cdot}$, $DPPH^{\cdot}$ assays and reducing power (FRAP).

3. to investigate the phytochemical composition using Ultra Performance Liquid Chromatography (UPLC) and Mass Spectrometry (MS). Total Tocopherol Content and Fatty acid profile of the non-polar fraction of the fruit extracts were also determined using High-Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) respectively.

5. to identify anthocyanin and bioactive constituents (tentative) using UPLC/ESI-QTOF-MS.

6. to identify the polyphenols responsible for free radical scavenging activity in the bioactivity rich extract using an online HPLC-UV-DPPH assay.

7. developing a new schematic approach (Fig. 3.5.) for biorefining of bilberry pomace by incorporating innovative and green extraction technologies such as MHG and UAE.

8. utilization of computational prediction software (COSMO-RS) to obtain theoretical values of solubility index of target polyphenols in different ethanol: water concentrations and its comparison to the experimental results from various *in vitro* antioxidant assays (Folin-Ciocalteu reducing capacity, total flavonoid content, total monomeric anthocyanin content and radical scavenging capacity of extracts) employed.

2. LITERATURE REVIEW

2.1. Berries as a source of functional food

Increased consumption of fruits and vegetables is recommended in dietary guidelines worldwide (World Health Organization, Food, and Drug Administration etc.,) and the intake of fruits like berries which are packed with vital nutrients and phytochemicals can prevent various diseases and disorders. Berries, make up the largest proportion of fruit that is consumed in the human diet. Berry fruits are consumed in fresh, frozen, and processed forms such as dried and canned fruits, yogurts, beverages, jams, and jellies. Berries promote significant health benefits because of their high levels of polyphenols, antioxidants, vitamins, minerals and fibers. Berry extracts are widely consumed in botanical dietary supplement forms for their potential human health benefits. Many in vitro and in vivo models including animal studies have shown that berries have anticancer, antioxidant, and antiproliferative properties. For example, strawberries and black raspberries have been identified as sources of phenolic compounds like ellagic acid, and gallic acid which have potential cancer chemopreventive activity. Berries contain high levels of a diverse range of phytochemicals, most of which are phenolic molecules. These phytochemicals include a variety of beneficial compounds, such as essential minerals, vitamins, fatty acids, and dietary fibers. Berries are an important source of provitamin A, minerals, vitamin C, and B-complex vitamins. Berry fruits contain about 15% soluble solids (mainly sugars) and their high level of fructose makes them valuable for individuals with diabetes. The high dietary fiber content is important because fruit pectin acts as an intestinal regulator (Shivraj hariram Nile & Se Won Park, 2014).

2.2. Chemical constituents of *Eleutherococcus*

Eleutherococcus berries are typically used for medicinal preparation in native regions of Korea, Japan, and China.

Figure 2.1. *Eleutherococcus Henryi* and *Eleutherococcus Sessiliflorus* berries (adapted from https://commons.wikimedia.org/wiki/File:Eleutherococcus_henryi_FruitsInflorescences_BotGardBln0906.jpg)

It is not consumed directly because of its high terpenoid and lignin content which gives it an astringent flavour. Several compounds were isolated and identified from *E. Sessiliflorus* previously. The summary of chemical compounds found in *E. Sessiliflorus* reported in various research articles were tabulated (Table 2.1.). Chiisanogenin a terpenoid and its glycoside chiisanoside was found to be the major compound of interest as it is said to have anti-inflammatory activity. Very few articles focused on the polyphenol content and composition of *E. Henryi* and *E. Sessiliflorus* berries.

Table 2.1. Chemical constituents of *E. Sessiliflorus* previously reported in the literature.

S.NO.	Compound	Solvent	Molecular formula	Reference*
1	22α-hydroxychiisanogenin			
2	3,4-seco-lupan-20(30)-ene-3,28-dioic acid			
3	(1R)-1,4-epoxy-11α,22α-hydroxy-3,4-secolupan-20(30)-ene-3,28-dioic acid			
4	divaroside		$C_{42}H_{64}O_{15}$	
5	chiisanoside		$C_{48}H_{74}O_{19}$	
6	22α-hydroxychiisanoside	70% ethanol		1
7	22α-hydroxy-3,4-seco-lupa-4(23),20(30)-diene-3,28-dioic acid 3-methyl ester		$C_{31}H_{48}O_5$ / Acanthosessiligenin I	
8	3,4-seco-lupa-4(23),20(30)-diene-3,28-dioicacid 28-O-β-D-glucopyranoside		$C_{36}H_{56}O_9$ / Acanthosessilioside A	
9	(1R)-1,4-epoxy-11α-hydroxy-3,4-seco-lup-20(30)-ene-3,28-dioic acid 3-methyl ester		$C_{31}H_{49}O_6$ / Acanthosessiligenin II	
10	(1R)-1,4-epoxy-11α-hydroxy-3,4-seco-lup-20(30)-ene-3,28-dioic acid 3-methyl ester 28-O-β-D-glucopyranoside		$C_{37}H_{59}O_{11}$ / Acanthosessilioside B	
11	(1R)-1,4-epoxy-11α,22α-dihydroxy-3,4-seco-lup-20(30)-ene-3,28-dioic acid 3-methyl ester 28-O-β-D-glucopyranoside		$C_{37}H_{58}O_{12}$ / Acanthosessilioside C	
12	(1R)-1,4-epoxy-3,4-seco-lup-20(30)-ene-3,28-dioic acid 3-O-β-D-glucopyranoside		$C_{36}H_{55}O_{10}$ / Acanthosessilioside D	
13	Kenposide A		$C_{21}H_{36}O_{10}$	
14	Sacranoside B		$C_{21}H_{36}O_{10}$	
15	1-O-[(S)-oleuropeyl]-β-D-glucopyranose			2
16	(2*E*)-3,7-dimethylocta-2,6-dienoate-6-*O*-α-L arabinopyranosyl-(1→6)-β-D-glucopyranoside			
17	(3*Z*,6*E*)-3,7-dimethyl-3,6-octadiene-1,2,8-triol		`	
18	(6*E*)-7-methyl-3-methylene-6-octene-1,2,8-triol			
19	protocatechuic acid		$C_7H_6O_4$	
20	scopolin		$C_{16}H_{18}O_9$	
21	pinoresinol-4,4´-di-O-b-D-glucopyranoside		$C_{32}H_{42}O_{16}$	
22	(2S,3R,4S,5S,6R)-2-[4-[(3R,3aS,6R,6aS)-6-[3,5-dimethoxy-4-[(2S,3R,4S,5S,6R) -3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]-1,3,3a,4,6,6a-hexahydrofuro[3,4-c]furan-3-yl] -2,6-dimethoxyphenoxy]-6-(hydroxymethyl) oxane-3,4,5-triol	water-saturated n-butanol	$C_{34}H_{46}O_{18}$ / acanthoside D	3
23	hyperin		$C_{21}H_{20}O_{12}$	
24	(2S,3R,4S,5S,6R)-2-[4-[(3S,3aR,6S,6aR)-3-(4-hydroxy-3,5-dimethoxyphenyl)- 1,3,3a,4,6,6a-hexahydrofuro[3,4-c] furan-6-yl]-2,6-dimethoxyphenoxy]-6-(hydroxymethyl) oxane-3,4,5-triol		$C_{28}H_{36}O_{13}$ / acanthoside B	

25	momordin Ib			
26	3-O-[(α-L-arabinopyranosyl) -(1 → 2)]- [β-D-glucuronopyranosyl-6-Omethyl ester]-olean-12-ene-28-olic acid	Ethanol	$C_{42}H_{66}O_{13}$	4
27	(1R,11α)-1,4-epoxy-11-hydroxy-3,4-secolupane-20(30)-ene-3,28-dioic acid		$C_{30}H_{46}O_6$	
28	(1R,11α,22α)-1,4-epoxy-11,22-hydroxy-3,4-secolupane-20(30)-ene-3,28-dioic acid		$C_{30}H_{46}O_7$	
29	5 - hydroxy methylfurfural		$C_6H_6O_3$	
30	sesamin	Methanol	$C_{20}H_{18}O_6$	5
31	ursolic acid		$C_{30}H_{48}O_3$	
32	scoparone		$C_{11}H_{10}O_4$	
33	5-(5-oxo-pyrrolidin-2-ylomethyl)-furan-2-carbaldehyde	Methanol	Sessiline (nitrogenous compound)	6
34	3,5- dihydroxycinnamic acid	70% aqueous ethanol	$C_9H_8O_4$	7
35	3-(3',4'-dihydroxybenzyl)-4-[(7*S*),7-hydroxy-3,5- dimethoxybenzyl] tetrahydrofuran		Acanthosessilin A	
36	hinokinin		$C_{20}H_{18}O_6$	
37	syringaresinol		$C_{22}H_{26}O_8$	
38	pinoresinol	70% ethanol	$C_{20}H_{22}O_6$	8
39	piperitol		$C_{10}H_{18}O$	
40	xanthoxylol		$C_{20}H_{20}O_6$	
41	simplexoside		$C_{26}H_{30}O_{11}$	
42	oleanolic acid -3-O-6´-O- methyl-β-D-glucuronopyranoside			
43	oleanolic acid -3-O-β-D-glucuronopyranoside			
44	oleanolic acid -3-O-β-D-glucopyranoside			
45	oleanolic acid		$C_{30}H_{48}O_3$	9
46	chiisanogenin		$C_{33}H_{44}O_5$	
47	daucosterol		$C_{35}H_{60}O_6$	
48	β-sitosterol		$C_{29}H_{50}O$	

* References 1-9 are indicated in the reference section.

The triterpenoids that are classified as 3,4-secolupane-type are chiisanoside, chiisanogenin, 24-hydroxychiisanogenin, and 22α-hydroxychiisanogenin, etc. Chiisanoside has been reported to have antihepatotoxic, antidiabetic, antiviral effects on mitogen-induced proliferation of lymphocytes (Hyun-Ju Jung et al., 2005). The terpenoid chiisanogenin, its glycoside chiisanoside, and other oleanolic acid derivatives are predominantly found in the lipid fraction.

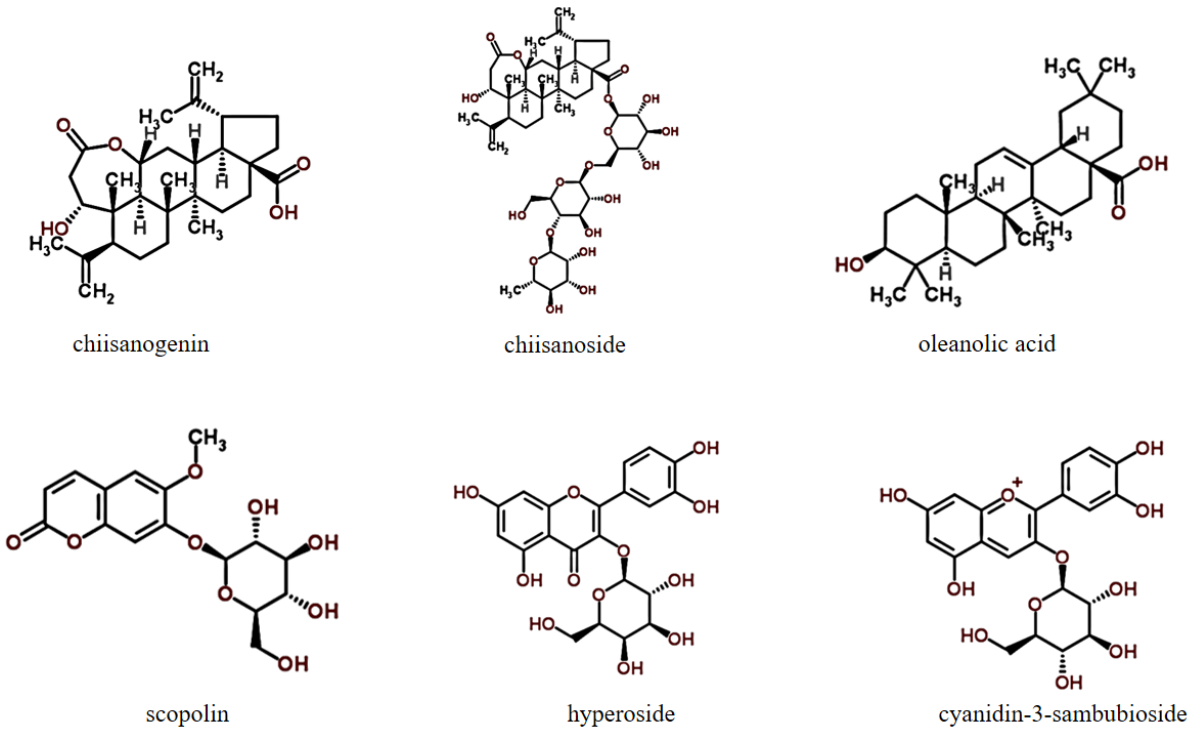


Figure 2.2. Phytochemicals found in *E. Henryi* and *E. Sessiliflorus* berries (images obtained from www.chemspider.com)

The individual concentration of five flavonoids such as rutin, hyperin, quercetin, afzelin, and kaempferol was quantified in the methanolic extracts of various A*canthopanax* species (A. chiisanensis, A. divaricatus, A. koreanum, A. senticosus, and A. Sessiliflorus). The study concluded that the major flavonoid present in A. Sessiliflorus was hyperin with 3.209 ± 0.011 mg/g of dried extract (Jeong Min Lee et al., 2013). The potential chemical constituents present in the plant material varies, with respect to geographical location, genotype, the season of harvest, sunlight exposure, altitude etc. *Eleutherococcus* comprises of a variety of phytochemicals with potential benefits, the major classes are anthocyanin, flavonol, phenolic acids, coumarins, fatty acids, sterols, terpenoids, and lignans.

2.3. Chemical constituents of *Vaccinium myrtillus* L.

Figure 2.3. *Vaccinium myrtillus* L. (adapted from http://www.tacethno.com/herbals-all/vaccinium-myrtillus-bilberry-fruit.html).

Bilberry is also known as the "longevity fruit" is a rich source of antioxidant phytochemicals with an abundant concentration of phenolic compounds, such as anthocyanins, flavonols, and phenolic acids. Other compounds such as triterpenoids, free fatty acids (Anna Szakiel et al., 2012), glycerides, sterols, and waxes are also found in bilberry. The figure 2.4. and Table 2.2. given below elucidates the phytochemical constituents of bilberry and their chemical concentrations in various bilberry extracts, by-products. Bilberry is widely consumed, mostly in its raw natural form and has a huge market in the derivative food product sector. Wherein, it is fortified it a variety of food products such as fruit pulp, yogurt, coloring extracts, and even water.

In order to obtain a vivid idea on the phytochemical composition of bilberry, ten research articles from peer-reviewed journal were selected, in which the polyphenol composition and concentration were evaluated. This, screening technique aids in obtaining a first-hand information on the conventional extraction techniques employed and the chemical constituents present in the plant material. After scrutiny, the compounds of interest are selected for theoretical prediction of their solubility index in different concentration of ethanol/water solvent system which is explained in detail in the materials and methods "computational prediction: COSMO-RS".

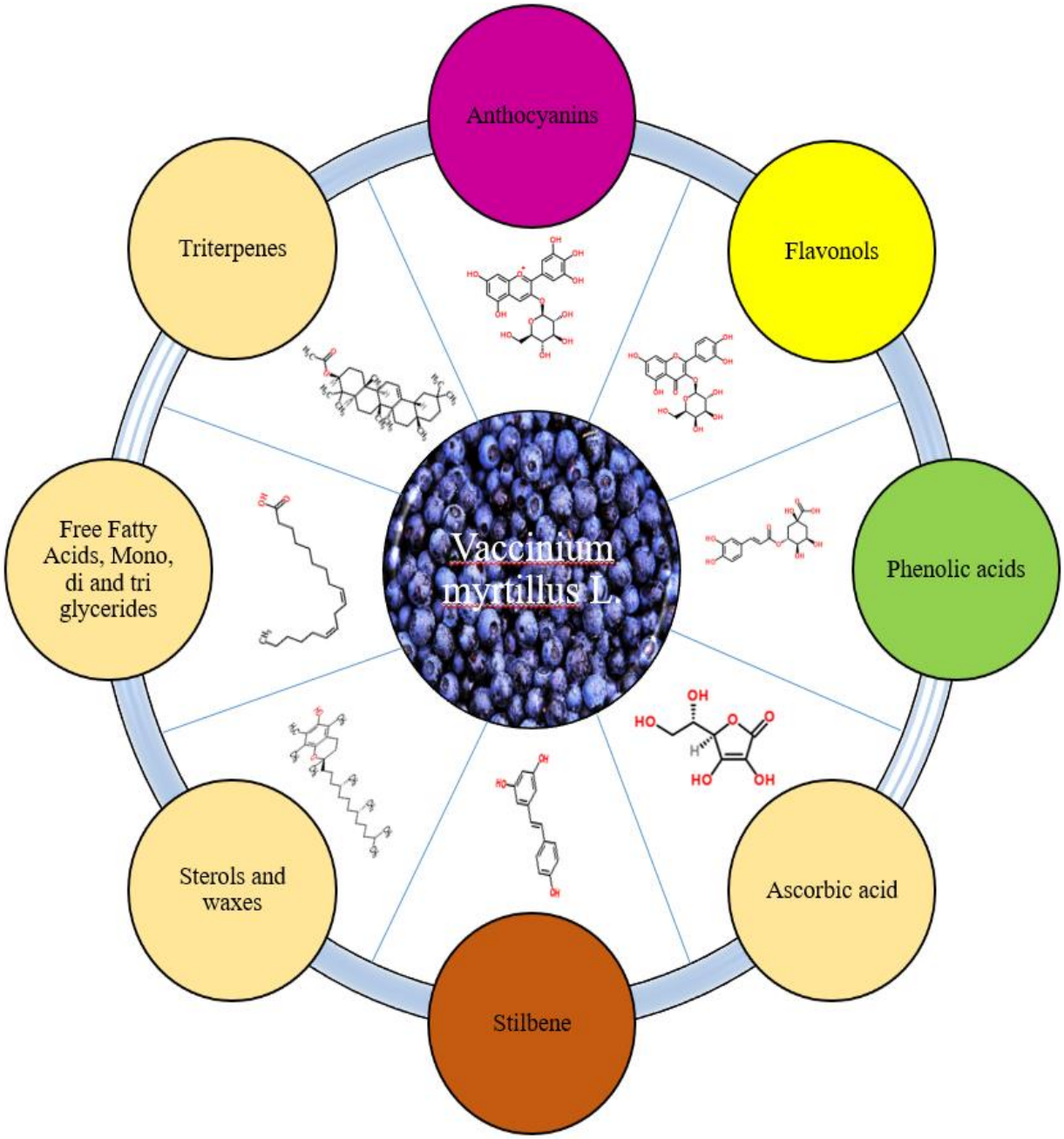


Figure 2.4. Phytochemical composition of bilberry

The figure depicts various constituents present in bilberry, it is a good mixture of essential micro and macronutrients that collectively and individually contribute to the human health benefits. Some of the known chemopreventive agents present in berries include vitamins A, C, and E, and folic acid; calcium and selenium; carotene and lutein; phytosterols such as sitosterol and stigmasterol; triterpene esters; and phenolic molecules such as anthocyanins, flavonols, flavanols, proanthocyanidins, ellagitannins, and phenolic acids.

Table 2.2. Anthocyanins found in bilberry

Anthocyanins/References	**B1**	**B2**	**B3**	**B7a**	**B7b**	**B9**
	mg/100 g FW	% (C3GE)	% (w/w, DW)	mg C3GE/100 g FW, berry	press residue	mg/100g FW
delphinidin 3-galactoside	167.1 ± 6.5	3.16	0.39 ± 0.11	43.7 ± 0.0	60.1 ± 0.6	92.1 ± 4.7
delphinidin 3-glucoside	**169.1 ± 6.6**	**3.83**	**0.37 ± 0.10**	**47.7 ± 0.5**	**81 ± 1**	**86.6 ± 10.5**
cyanidin 3-galactoside	122.6 ± 5.7	2.25	0.28 ± 0.12			48.4 ± 6.1
delphinidin 3-arabinoside	152.3 ± 6.1	3.01	0.24 ± 0.06			59.1 ± 4.8
cyanidin 3-glucoside	130.4 ± 6.2	3.81	0.26 ± 0.12	46.5 ± 0.1	57.1 ± 0.1	46.6 ± 2.4
petunidin 3-galactoside	50.0 ± 2.6	1.03	0.15 ± 0.04	14.3 ± 0.4	19.7 ± 0.4	30.1 ± 3.9
cyanidin 3-arabinoside	110.6 ± 4.9	2.04	0.15 ± 0.06	31.5 ± 0.1	38.1 ± 0.2	25.0 ± 0.7
petunidin 3-glucoside	101.9 ± 6.2	2.37	0.25 ± 0.07	30.4 ± 0.4	51.1 ± 0.9	53.4 ± 5.9
peonidin 3-galactoside	13.3 ± 1.2	0.26	0.06 ± 0.02			
petunidin 3-arabinoside	23.9 ± 1.5	1.17	0.07 ± 0.02			13.7 ± 0.8
peonidin 3-glucoside	56.7 ± 5.1	1.21	0.22 ± 0.08			41.2 ± 2.3
malvidin 3-galactoside	27.5 ± 3.3	0.87	0.22 ± 0.08			21.7 ± 3.9
peonidin 3-arabinoside	4.5 ± 0.4	0.11	0.21 ± 0.07			
malvidin 3-glucoside	67.7 ± 5.9	2.04	0.21 ± 0.07	34.3 ± 0.3	70 ± 1	50.8 ± 3.3
malvidin 3-arabinoside	12.8 ± 1.3	0.47	0.05 ± 0.01	7.8 ± 0.1	14.6 ± 0.8	
cy-3-gal + dp-3-arab				73 ± 1	92 ± 2	
pn-3-gal + pt-3-arab				14.0 ± 0.2	20.2 ± 0.8	
pn-3-glc + mv-3-gal				29.7 ± 0.3	42.8 ± 0.8	

FW- Fresh weight, C3GE – Cyanindin-3-glucoside equivalent, DW – Dry Weight, B7a- Berry, B7b- Pomace.

Table 2.3. Flavonols found in bilberry

Flavonol/References	**B1** mg/100 g FW	**B4** mg/kg FW	**B5** µg/100g DB	**B6** mg/g DE	**B7a** mg C3GE/100 g FW, berry	**B7b** press residue	**B8** mg/kg DW	**B9** mg/100g FW
Quercetin	0.8 ± 0.0	30	243.30 ± 8.67				2.2 ± 0.1	
Myricetin	0.4 ± 0.0	21	40.66 ± 1.03				36.9 ± 1.3	
Rutin	0.2 ± 0.0		51.80 ± 1.36					
Kaempferol			15.64 ± 0.62					
Umbelliferone			17.52 ± 0.69					
Quercetin-3-O-galactoside				**0.812 (0.05)**			**786 ± 38**	**4.4 ± 0.8**
Quercetin-3-O-glucoside				0.752 (0.07)			32.5 ± 1.4	3.2 ± 1.1
Quercetin-3-O-rutinoside				0.129 (0.03)				
Quercetin-3-O-glucuronide				0.018 (0.002)				
Quercetin-3-O-xyloside				0.023 (0.004)				
Quercetin-3-O-arabinoside				0.099 (0.01)				
Quercetin-3-rhamnoside							22.6 ± 1.0	
Myricetin-3-O-galactocside				0.005 (0.001)				
Kaempferol-3-Ogalactoside				0.005 (0.001)				
Kaemferol-3-O-glucoside				0.017 (0.002)				
Kaempferol-3-O-(-6-coumaroyl)-glucoside				0.014 (0.002)				
Myricetin galactoside					7.7 ± 0.4	38.3 ± 0.3		
Quercetin hexoside					13.1 ± 0.2	75.6 ± 0.2		
Quercetin pentoside					3.3 ± 0.0	24.4 ± 0.3		
Vaccino-side					16.5 ± 0.7	16.2 ± 0.0		
Esculetin							2.8 ± 0.2	
Scopoletin							0.046 ± 0.002	
Phloridzin							3.2 ± 0.1	

FW- Fresh weight, DB – Dry bilberry, DE – Dry Extract, C3GE – Cyanindin-3-glucoside equivalent, DW – Dry Weight

Table 2.4. Phenolic acids found in bilberry

Phenolic acid/References	**B1** mg/100 g FW	**B5** µg/100g DB	**B6** mg/g DE	**B7a** mg C3GE/100 g FW, berry	**B7b** press residue	**B8** mg/kg DW	**B9** mg/100g FW
chlorogenic acid	**23.1 ± 1.0**	**21.0 ± 0.84**	**0.028 (0.01)**	**7.1 ± 0.1**	**5.5 ± 0.1**		**6.1 ± 1.8**
caffeic acid	0.3 ± 0.0	15.33 ± 0.56	0.014 (0.003)			3.1 ± 0.2	
ferulic acid	0.4 ± 0.0	22.76 ± 0.81				0.44 ± 0.01	
p-coumaric acid	0.3 ± 0.0	57.87 ± 2.68	0.242 (0.02)			1.20 ± 0.05	
ellagic acid	1.2 ± 0.0	9.99 ± 0.37					
gallic acid	6.2 ± 0.3	7.24 ± 0.16					
Protocatechuic acid		19.41 ± 0.76					
Syringic acid		27.43 ± 1.13	0.274 (0.01)				
Dihydroxybenzoic acid hexoside			0.010 (0.002)				
Vanillic acid hesoside			0.014 (0.01)				
Caffeoyl hexoside			0.712 (0.07)				
Chlorogenic acid			1.201 (0.06)			1320 ± 35	
Feruloyl hexoside			0.081 (0.01)				
Unknown quinic acid derivative			2.609 (0.88)				
Ellagic acid pentoside							
Caffeic acid hexoside				6.9 ± 0.3	6.3 ± 0.0		
Neochlorogenic acid						0.70 ± 0.08	
Crypto chlorogenic acid						1.45 ± 0.05	
Salicylic acid						0.46 ± 0.02	

FW- Fresh weight, DB – Dry bilberry, DE – Dry Extract, C3GE – Cyanindin-3-glucoside equivalent, DW – Dry Weight; References are indicated in the reference section.

After an exhaustive literature review, six compounds representing each polyphenol class were selected for their solubility index prediction in given solvent system and the compounds selected are indicated figure 2.5.

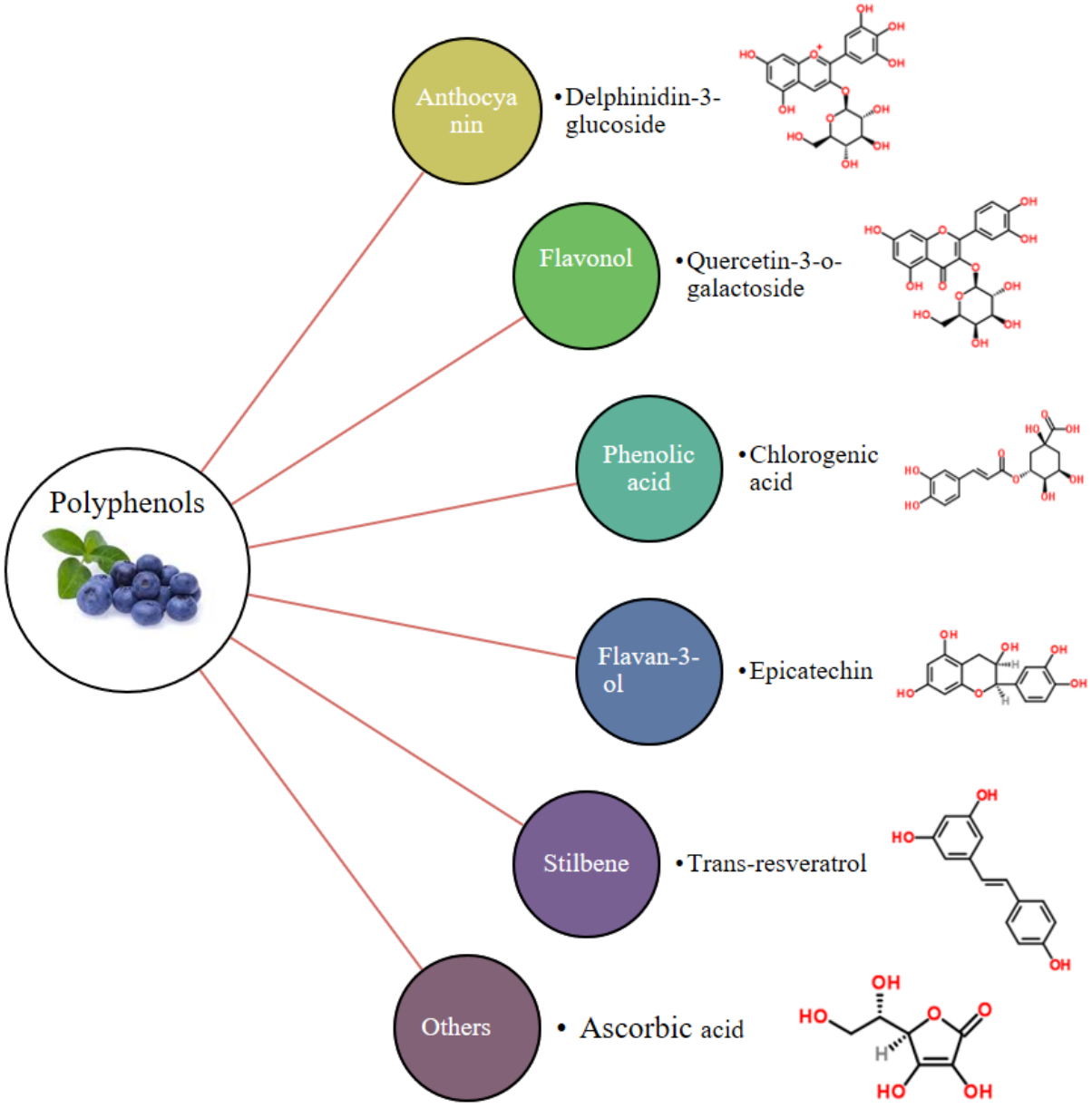


Figure 2.5. Polyphenol selection for computational prediction

Six compounds each belonging to individual polyphenol class, delphinidin-3-glucoside (Anthocyanin), quercetin-3-o-galactoside (Flavonol), chlorogenic acid (Phenolic acid), epicatechin (Flavan-3-ol), trans-resveratrol (Stilbene), ascorbic acid (Vitamin C) were selected. Trans-resveratrol and ascorbic acid were included to see the effect of extraction technique on other phytochemical that contributes to antioxidant activity and are not generally referred to as polyphenols.

2.4. Introduction to green extraction techniques

Green extraction can be defined as extraction based on the discovery and design of extraction process which will reduce energy consumption, allows the use of alternative and renewable natural products, and ensure a safe and high-quality extract/product. to facilitate green extraction three major solutions or improvisations have been identified to design and demonstrate green extraction on laboratory and industrial scale, this mainly focuses on an approach to guarantee optimal consumption of raw materials, solvents and energy: 1. improving and optimisation of existing products; 2. using non-dedicated equipment; 3. innovation in processes and procedures including identification of alternative solvents.

To consolidate all opinions and views on green extraction, this concept can be categorized into six principles

Principle 1: Innovation by selection of varieties and use of renewable plant resources.

Principle 2: Use of alternative solvents (water or agro-solvents).

Principle 3: Reduce energy consumption and employing innovative technologies.

Principle 4: Production of by-products, valorisation of waste products.

Principle 5: Reduce unit operations and favor safe, and robust operations.

Principle 6: Aim for a non-denatured and biodegradable extract without contaminants.

In general, several extraction techniques are utilized to remove, isolate, quantify valuable compounds from plant materials (Chemat et al., 2012). Conventional extraction is generally based on using petrochemical solvents, high temperature, time, energy, and cost. Selected few extraction techniques are discussed below in context to this thesis and its feasibility and inclusion in biorefining of plant materials are discussed below.

2.4.1. Supercritical Fluid Extraction with carbon dioxide (SFE-CO_2)

Supercritical fluid extraction with carbon dioxide as solvent refers to the removal of non-polar compounds from a solid substrate. During extraction, pure CO_2 (at near critical or supercritical conditions) is allowed to flow through a cylinder which contains the solid substrate. The density of the supercritical fluid is extremely sensitive to minor changes in pressure and temperature near the critical point. The advantages of using supercritical fluids in extraction are the ease of separation of extracted solute from the supercritical solvent. The separation of desired compounds without leaving toxic residues in extracts and reduced tendencies for thermal degradation are added advantages of using SFE-CO_2 system (Mohamed & Mansoori, 2002).

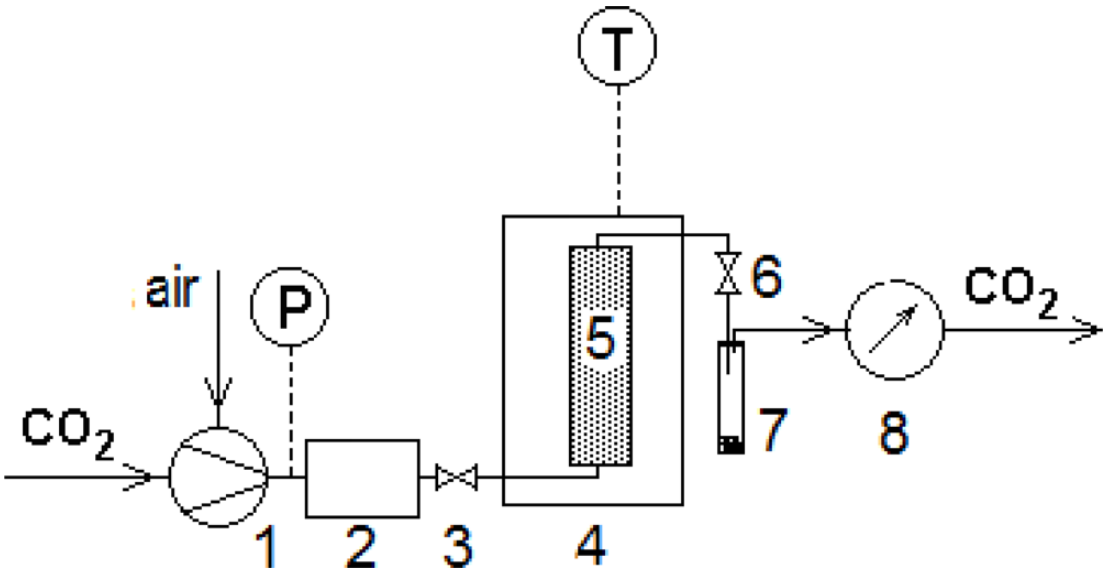


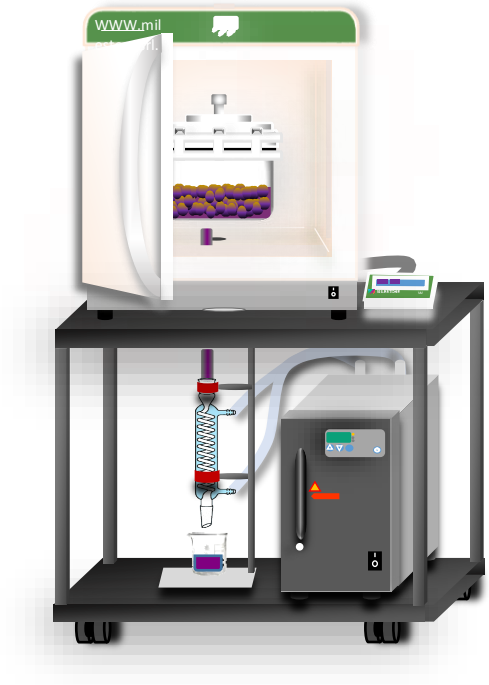
Figure 2.6. Schematic representation of supercritical fluid extraction (adapted from http://pubs.rsc.org/en/content/articlelanding/2003/gc/b208213f/unauth#!divAbstract)
1 – High-pressure pump, 2 – pressure gauge, 3 – valve, 4 – heating jacket, 5 – extraction vessel, 6 – valve, 7 – extract collection vial, 8 – flow meter.

2.4.2. Accelerated solvent extraction (ASE)

Accelerated solvent extraction combines two parameters, elevated temperatures and pressures with liquid solvents. Such elevated conditions in a closed vessel improve the recovery of desired compounds and can be completed in a short time. Elevated pressures (>1000 psi) allow for solvents to be heated at temperatures higher than their boiling point which increases diffusion rates, disrupts the strong solute–matrix interactions and decreases liquid solvent viscosity, allowing better penetration into the matrix and then improving extraction (Barros et al., 2013).

Figure 2.7. Accelerated solvent extraction system

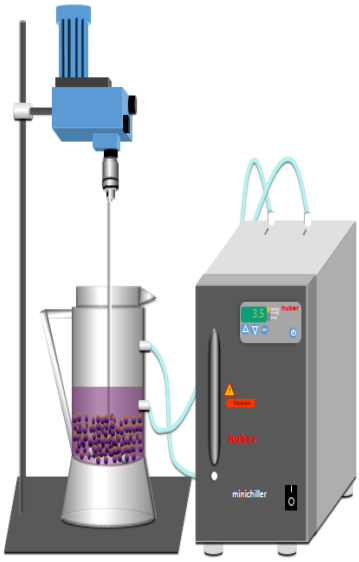
2.4.3. Microwave hydro diffusion and gravity (MHG)



During MHG, *in-situ* water in plant cells is stimulated to rotate under microwave irradiation, so immediate internal change results in a subsequent pressure increase inside plant cells, which leads to breaking down of cell walls and release of target molecules. MHG allows mass and heat transfer from inside the plant cell to the outside, while these two transport phenomena are in the opposite direction in conventional separations. This significant difference makes the temperature increase in much shorter time due to the volumetric heating effect, depending on the microwave power and the dielectric loss factor of the material being irradiated. Thus, the extract is collected at the bottom (Ying Li et al., 2013).

Figure 2.8. Microwave hydro diffusion system

2.4.4. Ultrasound Assisted Extraction (UAE)



Ultrasound-assisted extraction is based on the cavitation principle. Ultrasounds are mechanic waves able to propagate through an elastic medium. The sound wave will temporarily dislodge the molecules of the medium from their original location, which will create compression and rarefaction areas into the medium corresponding to the compression and rarefaction cycles of the sound wave Cavitation phenomena leads to high shear forces in the media. The implosion of cavitation bubbles on a product's surface results in micro-jetting which generates several effects such as surface peeling, erosion and particle breakdown (Chemat et al.,2017).

Figure 2.9. Ultrasound system

2.4.5. Bead Milling

Bead milling or mechano-chemistry is an innovative extraction and intensification technique which amplifies the recovery of desired compounds from any plant matrix. The addition of a cluster of individual ceramic beads with plant material and solvent together in an extraction tube makes it a homogenous system. When subjected to high rotational speeds (>4000 rpm) the centrifugal force and the mechanical force exerted by the beads on the sample facilitates extraction and improves the extraction rate in the solvent employed.

Figure 2.10. Bead milling ULTRA-TURREX system

Though several green extraction techniques exist, it is pertinent to understand and employ the appropriate extraction system to plant materials. These technologies can be applied individually, synergistically, sequentially to enhance productivity and quality of the product. In this thesis work, different techniques were employed based on extract required. For e.g., Supercritical fluid extraction yields the lipophilic fraction. Whereas, Microwave Hydro Diffusion and Gravity yield the polyphenol (hydrophilic) fraction. In brief, this thesis focuses on employing innovative extraction techniques to amplify the yield of desired compounds and suggests new biorefining concepts that enables valorisation of the plant material and it's by-products.

3. MATERIALS AND METHODS

3.1. Plant material

The fruits *Eleutherococcus henryi* and *Eleutherococcus sessiliflorus* were grown in Kaunas Botanical Garden of Vytautas Magnus University, Lithuania. The berries were collected and freeze-dried to remove the moisture content. The freeze-dried berries were ground in a centrifugal high-speed mill (Retsch ZM 200, Haan, Germany) with mesh size 0.2 mm. The ground berry powders were stored at -18 °C until further extraction.

Frozen bilberries (*Vaccinium myrtillus* L.) was procured from the local supermarket (Auchan) in Avignon, France. The berries at room temperature were subjected to pressing at 3000 psi (10 cycles) using a lab scale hydraulic press (R.E.U.S., Contes, France) to obtain juice and pomace. The pomace collected was stored at -18°C until further extraction.

3.2. Chemicals and reagents

Gallic acid, 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH), tetramethylchromane-2-carboxylic acid (Trolox), anhydrous sodium carbonate was purchased from Sigma–Aldrich Chemie (Steinheim, Germany); 2,4,6-tripyridyl-s-triazine (TPTZ) was from Fluka Chemicals (Steinheim, Germany); 2.0 M Folin–Ciocalteu reagent, 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), NaCl, KCl, Na_2HPO_4, and $K_2S_2O_8$ were from Merck (Darmstadt, Germany). All organic solvents were of analytical / HPLC grade and purchased from Sigma-Aldrich (Steinheim, Germany), StanLab (Lublin, Poland). Aluminium chloride (Fluka Analytical), potassium acetate, quercetin, primuline and sodium acetate were purchased from Sigma-Aldrich (USA). Potassium chloride and sodium chloride were purchased from VWR International (Leuven, Belgium). Delphinidin-3-glucoside and cyanidin-3-glucoside were purchased from Extrasynthese S.A (Genay, France). All solvents were of analytical grade and were purchased from Merck KGaA, Darmstadt, Germany.

3.3. Extraction of lipophilic fraction

3.3.1. Supercritical fluid extraction (SFE- CO_2)

The SFE- CO_2 extraction of ground *Eleutherococcus* berries was performed in supercritical fluid extractor Helix (Applied Separation, USA) were 20 g of samples was weighed and loaded into extractor vessel. The volume of consumed CO_2 was measured by a ball-float rotameter and a digital mass flow meter in standard liters per minute (SL/min) at standard parameters: P_{CO2} = 100 kPa, T_{CO2} = 20 °C, ρ_{CO2} = 0.0018 g/ml. (Kraujalis & Venskutonis, 2013). The extraction parameters set were as given below: static extraction time 10 min, dynamic extraction time 120 min, extraction temperature 50 °C, pressure 55 Mpa and CO_2 flow rate 1-2 SL/min. Experiments were carried out in triplicates and yield was calculated.

3.3.2. Soxhlet extraction

The *Eleutherococcus* berries (20 g each) were placed in cellulose extraction thimbles and subjected to extraction in a Soxhlet extractor (Behr Labor-Technik, Düsseldorf, Germany) as described in AOAC method (1995). Hexane was used as a solvent for an extraction period of 3 h. The solvent was removed using Büchi rotary vacuum evaporator (Flawil, Switzerland) at 30 °C. All extractions were carried out in triplicates, to compare the yields of the non-polar fraction obtained by supercritical fluid extraction.

3.3.3. Bead milling

Bilberry biomass including raw material to all residues obtained from different treatments was subjected to ULTRA-TURRAX® Tube Drive Extraction (UTTD) extraction to investigate the lipophilic fraction distribution. The biomass (Bilberry, Pomace, MHG residues (1;1.5;2 W/g) and all UAE Ethanol/Water (100:0; 80:20; 60:40; 40:60; 20:80; 0:100) residues were dried in a hot air oven at 30 °C for 2 days prior to milling. Extraction by bead milling was performed using ULTRA-TURRAX® Tube Drive (UTTD, Ika, Germany) operating in a 20-mL tube with 20 g of ceramic beads, 0.3 g of dried biomass and 15 mL of hexane. The drive tube was operated at 4000 rpm for 60 min. The hexane phase was recovered and stored at -4 °C until analysis.

3.4. Extraction of polyphenol fraction

3.4.1. Solvent extraction

The free polyphenol from the *Eleutherococcus* berries was extracted with different solvents such as water, methanol, and acetone: water (30:70, v/v). Extracts were obtained by adding 40 mL of respective solvent to 0.2 g of different berry samples (Freeze dried fruit powder, Soxhlet residue, and SFE-CO_2 residue) and shaken at 200 rpm for 2 h (Sklo Union LT, Teplice, Czech Republic). The extracts were filtered (Whatman 1 filter paper), centrifuged and stored at -18 °C until further analysis. From preliminary analysis (data not included) it was determined that the extracts had to be diluted before antioxidant activity determination due to higher concentrations. Therefore, the extracts were diluted three-fold with the respective extraction solvents and subjected to different antioxidant assays.

3.4.2. Accelerated solvent extraction

The residue of *E. Henryi* and *E. Sessiliflorus* berries after SFE – CO_2 were subjected to accelerated solvent extraction performed in a Dionex ASE 350 system (Dionex, Sunnyvale, CA) to obtain the bound phenolics in the fruit matrix. Eight grams of residual powder was mixed with 5 g of diatomaceous earth and placed in 34 mL stainless-steel cells. Sequential extraction was executed by employing solvents of increasing polarity, such as acetone, acidified ethanol/water mixture (80:20, v/v) and water. The extraction conditions were: pressure was set at 10.3 MPa, extraction time 15 mins and temperature 70 °C. Solvents were evaporated using a rotary vacuum evaporator at 40 °C and water extracts were lyophilized. All extracts were stored at -18 °C and extractions were carried out in duplicates to compare the yield.

3.4.3. Microwave hydro diffusion and gravity

A patented (Chemat et al., 2008) MHG apparatus (Milestone ETHOS-X microwave laboratory oven) was used for MHG extraction (Zill-e-Huma et al., 2011). The sample, bilberry pomace 300 g was placed in an extraction vessel in the reactor. Three levels of microwave power 300 W (1 W/g), 450 W (1.5 W/g) and 600 W (2 W/g) was employed for MHG extraction.

From preliminary analysis, it was found that bilberries, when subjected to MHG produce two extract fractions: (1) colored Fraction (*in situ* water with polyphenols) and (2) colorless fraction (only *in situ* water). Therefore, only the colored fraction was collected and used for all analyses. Post MHG, the solid residues were labeled MHG residues and stored at -18 °C until further extraction. The temperature, time and weight of extracts collected were recorded. All extractions were performed in triplicate. The ideal extraction conditions (Table. 3.1.) were selected based on two parameters, specific energy (E) and productivity (Pr). All extracts obtained from MHG were frozen and lyophilized to identify the global yield after microwave treatment. The freeze-dried extracts were reconstituted in methanol prior to all analysis.

Table 3.1. Specific energy and productivity at different power levels

Power (P, W/g)	1	1.5	2
Specific Energy (E, J/g)	960	810	780
Productivity (Pr, g/s)	0.102	0.182	0.232

3.4.4. Ultrasound-assisted extraction

An ultrasonic extraction reactor PEX 1 (R.E.U.S., Contes, France) with 24 kHz input power was used for UAE (Pingret et al., 2012) with different Ethanol/Water concentrations as the solvent system. The MHG residue obtained with the optimal extraction conditions was utilized for UAE. The time for sonication was selected based on initial analysis, in which 30 g of MHG residue was extracted with 300 mL (100 % Ethanol) for 10,20,30,40,50 and 60 min respectively (data not included). The highest yield was obtained at 30 min of sonication. Hence, 30 min of sonication time was employed for all subsequent extraction with the different Ethanol/Water concentrations. The following were the extraction condition under which UAE was performed: 30 g MHG residue in 300 mL solvent and 30 min. All extracts obtained from UAE were frozen and lyophilized to identify the global yield after ultrasound treatment. The freeze-dried extracts were reconstituted in methanol prior to all analysis.

3.5. Spectrophotometric *in vitro* antioxidant assays

The following antioxidant activity assays were performed for *Eleutherococcus* extracts.

3.5.1. $ABTS^{\bullet+}$ radical decolourisation assay ($TEAC_{ABTS}$)

For ABTS assay, the method proposed by Re et al. (1999), was employed with few modifications. Initially, phosphate buffered saline (PBS) solution was prepared by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of ultrapure water and if the pH was lower than 7.4, it was adjusted with NaOH. Stock ABTS•+ solution (2 mM) was prepared by dissolving reagent in 50 mL of phosphate-buffered saline (PBS). ABTS•+ was produced by reacting 50 mL of stock solution with 200 µL of potassium persulfate ($K_2S_2O_8$) solution, and the mixture was allowed to stand for 15 h in the dark at room temperature. Prior to analysis, ABTS•+ solution was diluted with PBS to obtain the absorbance of 0.800 ± 0.030 at 734 nm. Thirty µL of extracts (*Eleutherococcus*) were mixed with 3 mL of ABTS•+ solution respectively and allowed to react for 2 h in dark condition. PBS was used as a blank. The absorbance was measured spectrophotometrically at 734 nm (Spectronic Genesys 8, NY, USA). The standard calibration curve was linear between 0.05 to 0.2 mg/mL of trolox ($y = 202.85x + 3.193$; $R^2 > 0.99$). Results were calculated and expressed in mg Trolox Equivalent (TE)/ g dry extract.

3.5.2. $DPPH^{\bullet}$ radical scavenging assay ($TEAC_{DPPH}$)

Radical scavenging capacity (RSC) of the extracts was determined by the method reported by Brand-Williams et al. (1995) with slight modifications. Stock Solution was prepared by dissolving 48 mg $DPPH^{\bullet}$ in 200 mL methanol. The working solution was brought to a final absorbance of 0.8 ± 0.02 units at 515 nm. Fifty µL of extract/standard were added to 2 mL of methanolic $DPPH^{\bullet}$ solution and rest for 2 h in the dark. The absorbance of the mixture was measured and their corresponding inhibition percentages were used to calculate the radical scavenging capacity. Methanol, Water and Acetone/Water (30:70) were used as blank for the respective extracts. The standard curve was linear between 0.0187 to 0.3 mg/mL of trolox ($y = 324.75x – 0.6316$; $R^2 > 0.99$). The results were expressed in mg Trolox Equivalent (TE)/ g dry extract.

3.5.3. Ferric-ion Reducing Antioxidant Power ($TEAC_{FRAP}$)

The FRAP assay was carried out according to Benzie and Strain (1996) with few modifications. The fresh working FRAP reagent was prepared by mixing 10 mM 4,6-tripryridyls-triazine (TPTZ) solution in 40 mM HCl, 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) and acetate buffer (300 mM, pH 3.6), at 1:1:10 (v/v/v) volumetric ratio. Fifty µL of extracts (*Eleutherococcus*) were

allowed to react with 1.5 mL of FRAP reagent (37°C) to which 150 µL dH_2O was added. The mixture was kept in the dark for 2 h at room temperature and absorbance was measured at 593 nm. H_2O was used as a blank. The standard curve was linear between 0.025 to 0.2 mg/ml of trolox ($y = 6.3988x - 0.0308$; $R^2 > 0.99$). Results were expressed in mg Trolox Equivalent (TE)/g of dry extract.

3.5.4. Total Phenolics content (TPC)

TPC was determined by (Folin & Ciocalteu, 1927) method with some modifications. Briefly, the working solution was prepared by diluting 1 ml of commercial Folin – Ciocalteu reagent to 10 – fold (v/v) with distilled water. The extracts (250 µL) were added to 1.5 mL of the working FC reagent. Exactly after 5 mins 1 mL of 7.5% Na_2CO_3 was added to the mixture and allowed to equilibrate for 2 h in the dark at room temperature. A series of Gallic acid solutions within the concentration range of 0.006 to 0.2 mg/mL was used to obtain the calibration curve ($y = 4.1935x + 0.1743$; $R^2 > 0.99$). Distilled water was used as a blank. The absorbance of the mixture was measured at 765 nm and TPC was expressed in Gallic acid equivalent (GAE)/ g of the dry extract.

3.5.5. Antioxidant assays of polyphenol fraction by colorimetry

Colorimetry assays were performed to identify the antioxidant activity of bilberry extracts. The Folin-Ciocalteu reagent reducing capacity (FCRC) was measured to determine the reducing capacity of the MHG and UAE extracts. Briefly, 20 µL of extract / Gallic acid standard was allowed to react with 80 µL of 7.5 % Na_2CO_3 in a 96-well microplate and placed in SPECTROstar Omega microplate reader with UV-Vis spectrophotometer. 100µL of FC reagent were added to all wells previously equilibrated with extract and Na_2CO_3. The absorbance of the mixture was recorded at 750 nm for every 5 min over a period of 60 min with distilled water as blank at 25 °C. Results were expressed in mg of Gallic Acid Equivalent (GAE) per gram of extract.

The Total Flavonol Content (TFC) was determined by $AlCl_3$ assay (Chang et al., 2002) with Quercetin as standard. The standard solution or extract (500 µL) were mixed with 1.5 mL of 95% ethanol, 100 µL of 10% $AlCl_3$, 100 µL of 1M potassium acetate and 2.8 mL of distilled water. The mixture was allowed to equilibrate at room temperature for 45 min after which absorbance was measured at 415 nm in a spectrophotometer (Biochrom, Libra S22, UK). Results were expressed in mg of Quercetin Equivalent (QE) per gram of extract.

Total monomeric anthocyanin content (TMAC) was determined using pH differential method (Lee et al., 2005) with Delphinidin-3-glucoside as standard. 200µL of Standards or

extracts 200 µL were added to 2 mL of potassium chloride buffer and similarly, 200 µL of extracts to 2 mL of sodium acetate buffer. The absorbance was measured spectrophotometrically at 520 and 700 nm. Results were expressed in mg of Delphinidin-3-glucoside Equivalent (D3GE) per gram of extract.

DPPH Radical scavenging capacity (RSC) of the extracts were measured with trolox as standard. 50 µL of (0.5mM) methanolic DPPH solution was added to 50 µL of extracts or trolox in a microplate and the absorbance was read at 520 nm for every 5 min over a period of 60 min. All experiments were carried out in triplicate and the final results expressed in mg of Trolox Equivalent per gram of extract.

3.6. Analysis of the composition of polyphenol and lipophilic fractions

3.6.1. Fatty acid profiling by gas chromatography (GC-FID)

Fatty acid composition of the lipophilic fractions obtained from Soxhlet extraction and SFE - CO_2 were subjected to gas chromatography (GC) analysis. Fatty acid methyl esters (FAMEs) were prepared according to the official AOAC method (1995) with slight modifications. Briefly, 0.5 g of sample was taken in 50 mL test tube and 4 mL of (0.5 N) sodium methoxide was added. Esterification was performed by heating the mixture under reflux for 10 min, then 5 mL of methanolic boron trifluoride (BF_3) was added and the mixture was refluxed for 2 min. Finally, 5 mL of hexane was added and the mixture was refluxed for 2 min. The aliquot of the hexane phase was removed and diluted to 10-fold before analysis. FAMEs were analyzed on an HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) equipped with a flame ionization detector and 100 m length 0.25 mm (i.d.), 0.20 µm film thickness fused silica capillary column SP™ - 2560 (Supelco, Bellefonte, PA, USA). Analysis parameters were as follows: injection temperature 220 °C; detector's temperature 240 °C; split ratio 100:1; oven temperature was programmed in three ramps from 80 °C to 135 °C at 4 °C/min, from 135 °C to 185 °C at 4 °C/min, and from 185 °C to 240 °C at 4 °C/min and held isothermal for 5 min; carrier gas, helium at a flow rate of 20 cm^3/s. The compounds were identified by comparing their retention times to those of a commercial FAME mixture (Kraujalis, Venskutonis et al., 2013).

3.6.2. Tocopherol quantification by high-performance liquid chromatography (HPLC)

Quantitation of tocopherol in the lipophilic fraction was executed by HPLC analysis as described in (Gruszka and Kruk, 2007) with some modifications. The lipophilic extracts were diluted to a concentration of 0.1% with acetonitrile: methanol: dichloromethane (72:22:6, v/v/v). HPLC system (Perkin-Elmer Series 200, USA) equipped with C30 reverse-phase column (particle

size 5 m, 250 mm × 4.6 mm) applying isocratic elution with the dilution solvent mixture as mentioned above was used. Injection volume was 20 µl and the flow rate was 1 ml/min. Tocopherols were detected using fluorescence detector at 290 nm excitation and 330 nm emission; the analytes eluted in 40 min: α-T at 13.5 min, β-T at 11.5 min, γ-T at 11.0 min and δ-T at 9.5 min (Kraujalis and Venskutonis, 2013). Tocopherol content was identified by comparing the retention time of peaks from the sample to those of pure standard solutions, which were prepared at different concentrations (0 to 10 µg/mL) using mobile phase. Chromatographic conditions were same as mentioned before. The calibration curve (peak area versus injected amount) was used to determine the tocopherol content. Analyses were performed in duplicates.

3.6.3. UPLC-QTOF/MS analysis

Tentative compound identification was performed on an Acquity UPLC system equipped with binary solvent delivery apparatus, an autosampler with 10 µL sample loop, a photodiode array (PDA) detector, a column manager and a data manager to run the compass acquisition and data software (Waters, Milford, MA, USA) combined with a Bruker maXis UHR–TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). An Acquity BEH C18 column (1.7 µm, 50 × 2.1 mm, i.d.) maintained at 25 °C with a mobile phase composed of eluent A (0.4% v/v formic acid in ultrapure water) and eluent B (acetonitrile) were used. Initially, the mobile phase was 100 % eluent A followed by a linear increase from 0% to 100% eluent B over a period of 9 min and maintained at for 1 min. Finally, the initial conditions were re-introduced over 1 min, and the equilibration time was 1 min. The flow rate was 0.4 mL/min and the effluent was monitored at 254 nm. The effluent from the PDA detector was introduced directly into the UHR-TOF with an ESI source. Instrument control and data acquisition were achieved using the Compass 1.3 (HyStar 3.2 SR2) software. MS experiments were performed in negative ionization mode. The capillary voltage was maintained at +4000 V with the end plate offset at −500 V. Nitrogen was used as the drying and nebulizing gasses at 10.0 L/min and 2.0 bar pressure. Nitrogen was introduced into the collision cell as the collision gas (Vilma Kraujalyte et al., 2015). The peaks were tentatively identified by the characteristic MS fragmentation patterns, accurate masses and comparison with digital libraries such as Chemspider and Metlin.

3.6.4. Identification of anthocyanin

Anthocyanin identification was performed as described by (Claudia C. Gras et al., 2015) with few modifications. Two grams of *E. Henryi* and *E. Sessiliflorus* (SFE-CO_2 residue) were extracted with 10 mL solvent mixture of water/methanol/HCl (50/50/0.1; v/v/v). The mixture was centrifuged and re-extracted three times and the supernatants were pooled. The aliquots were

passed through a 0.45 µm cellulose membrane filter via a syringe and loaded into HPLC vials and subjected to UPLC analysis. The UPLC conditions were the same as above with a different mobile phase system with Eluent A being 5% formic acid in ultrapure water and methanol as eluent B. The initial mobile phase was 15% eluent B at 0 min and a linear gradient to 25% eluent B in 2 min followed, 50% eluent B in 3 min, 100% eluent B in 5 min and finally brought down to 15% eluent B over 7 min. MS experiments were done in positive ionization mode.

3.6.5. On-line HPLC/UV/DPPH and HPLC/MS assay

Compounds possessing free radical scavenging capacity in the bioactivity rich extract (ASE, 70 % ethanol/water) were identified using an on-line HPLC-UV/DPPH method. This was performed using a Waters HPLC system with Waters 1525 binary pump (Milford, MA, USA), Rheodyne 7125 manual injector (Rheodyne, Rohnert Park, CA, USA) with 20 µL injection loop and Discovery HS C_{18} 250 × 0.46 cm (5 µm) analytical column (Supelco Analytical, Bellefonte, PA, USA). The linear binary gradient was formed at a constant flow rate of 0.8 mL/min using solvent A (0.4% formic acid in ultrapure water) and solvent B (100% methanol). The analysis was initialized with 0% B, then increased to 10% in 1 min, to 50% B in 45 min, 100% B in 50 min and held for 3 min. The column was washed with solvent B and brought back to its initial conditions. Compounds eluted from the column were detected with Waters 996 photodiode array detector within the range of 230 to 500 nm. Post-UV detection, a freshly prepared 5×10^{-6} M $DPPH^{\bullet}$ solution in methanol was added to the main eluent at a flow rate of 0.6 mL/min using Agilent 1100 series quaternary pump (Agilent Technologies, Inc. Santa Clara, CA, USA). The mixture was introduced into a 15 m (0.25 mm ID) reaction coil made of PEEK (polyetheretherketone) tubing (Interchim, Frankfurt, Germany). The decrease of absorbance after the reaction of radical scavengers with $DPPH^{\bullet}$ was detected photometrically as a negative peak at 515 nm with variable-wavelength Shimadzu SPD–20A UV detector (Shimadzu Corporation, Kyoto, Japan).

For the identification of chromatographic peaks, the HPLC system was coupled to a quadrupole mass detector Micromass ZQ (Waters, Milford, MA, USA) operating in ESI negative ionization mode. Previously described solvent gradient was used. The eluting constituents were identified by comparing their *m/z* values with the ones obtained from UPLC-QTOF/MS analysis (Lina Grunovaite et al., 2016 & Pukalskaine et al., 2015).

3.6.6. Anthocyanin quantitation by High-Performance Thin Layer Chromatography

HPTLC plates silica gel 60 F_{254} were pre-developed with a mixture of chloroform/methanol (2/1, v/v) and was dried at 110 °C for 60 min on the TLC plate heater (CAMAG, Muttenz,

Switzerland). About, 10 mg of anthocyanin standards (Delphinidin-3-O-glucoside chloride and Cyanidin-3-O-glucoside chloride) were dissolved in 10 mL acidified methanol (0.5% HCl) to obtain standard solutions. For MHG and UAE extracts, 50 mg of each were dissolved in 10 mL acidified methanol as well. All sample and stock solutions were stored in the dark at -20 °C until analysis. The analysis was performed on silica gel 60 F_{254} 20 × 10 cm HPTLC plates (Merck, Darmstadt, Germany) with an ATS 5 automatic TLC sampler (CAMAG). Development was performed in an ADC 2 automatic developing chamber (CAMAG) with a mixture of ethyl acetate-methyl ethyl ketone-formic acid-water (7:3:1.2:0.8; v/v/v/v) as solvent (S. Kruger et al., 2013). Anthocyanins were quantified by a CAMAG 3 TLC scanning densitometer at a measurement wavelength of 555 nm. Anthocyanin quantitation was performed in duplicate and all data recorded was processed with winCATS software (CAMAG). CAMAG TLC Visualizer was used to capture the image of plates analyzed.

3.6.7. Analysis of fatty acid methyl esters (FAME) by GC-FID

FAME was prepared from the lipophilic fraction using acid-catalyzed transmethylation as described by Morisson et al. Triheptadecanoin (C17:0 TAG) was used as internal standard. 1mL of acidified methanol (5%) solution was added to the known volume of lipid extracted. The mixture was then heated for 90 min at 85 °C. Later, the mixture was cooled down to room temperature, to which 1.5 mL of sodium chloride (0.9%) solution and 1 mL of n-hexane were added. The mixture was transferred to a vial and vigorously shook for 1 min. Briefly, 800 µL of the organic layer was recovered and transferred to small vials before being injected into GC-FID for analysis. Fatty acid methyl esters were separated, identified and quantified by gas chromatography coupled with a flame ionization detector (GC-FID). The analysis was performed in an Agilent (Kyoto, Japan) gas chromatograph. The instrument was equipped with a BD-EN14103 capillary column 30 m × 320 µm × 0.25 µm (Agilent), and the velocity of the carrier gas (He) was 33 $cm.s^{-1}$. Two µL of sample was injected in split mode (split ratio 1:20), and the injector temperature was set at 250 °C. The oven temperature was initially 50 °C for 1 min and then progressed at a rate of 20 °C/min from 50 °C to 180 °C and then increased from 180 °C to 220 °C at a rate of 2 °C/min. The temperature was then held at 230 °C for 10 min. FAME in each extract was identified by retention time and comparison with purified FAME standards (Sigma Co., St. Louis, MO, USA).

3.6.8. Lipid composition by HPTLC

Pre-development of the plate was done similar to that of the protocol followed in anthocyanin quantitation. Chloroform was used as a solvent to prepare stock solutions, about 10

mg of each lipid was dissolved in 50 mL solvent (0.2mg/ml). A known quantity of lipophilic fraction was dissolved in 20 mL chloroform. Extracts were loaded as a spot onto 20 × 10 cm Silica gel 60 F254 HPTLC plates using an ATS 5 automatic TLC sampler. The HPTLC silica gel plates were developed with a mixture of solvents in an ADC 2 automatic developing chamber. The eluent to separate neutral lipids was a mixture of n-hexane/diethyl ether / glacial acetic acid in a ratio of 70:30:2 *v*/*v*/*v* to a height of 7 cm from the origin. After drying, the plate was dipped for 2 seconds in a reagent (10 mg of primuline, 160 mL of acetone, 40 mL of distilled water), then scanned using a TLC Scanner. The lipid classes present in the lipophilic fraction were quantified by densitometer with identification against known neutral lipid standards (Breil et al., 2016).

3.7. Miscellaneous analysis methods

3.7.1. Determination of colour by tristimulus colorimetry

A Chromameter CR-400/410 (Konica Minolta, Osaka, Japan) was used to determine the colour of the extracts measured as per the CIE (Commission International d'Eclairage) system, three-dimensional measurements (L^*/ a^*/ b^*) of reflected light were done (Van den kerckhove et al., 2001) by placing the samples in 5 cm (Dia) Petri plate which was illuminated with D65 – artificial daylight (10 standard angles) as recommended by manufacturer. The E index is calculated from the equation $E = (L^{*2} + a^{*2} + b^{*2})^{1/2}$ (Vaida et al., 2015). All measurements were done in triplicates.

3.7.2. Effect of extracts on oxidative stability of oil and emulsion

Rapeseed oil (RO) with initial peroxide value of 2.78 m Eq/Kg was used and emulsion (EM) of oil/water type was prepared using rapeseed oil/water (70/30) to which an emulsifier

Figure 3.1. ASE extracts in emulsion system

TWEEN 20 was added to obtain a homogeneous mixture. The acidified ethanol-water extract (0.1%) was fortified with oil and emulsion to assess their oxidative stability by accelerated oil stability methods such as Oxipres and Rancimat.

3.7.2.1. Evaluation of antioxidant properties in oxipres apparatus

Five grams of RO, treated with the extract as mentioned above was placed in a reactor tube with the temperature set at 120 °C and saturated with oxygen at 0.5 MPa pressure in an oxipres apparatus (Mikrolab, Aarhus, Denmark). Reduction in pressure was observed indicating the oxidation of oil at elevated temperature. The induction period (IP) was recorded and protection factor (PF) was calculated using the following formula: ($PF = IP_X/IP_K$); where IP_X is the induction period with extract (h); IP_K is the induction period without the extract (h). Similarly, PF for emulsion mixtures was calculated and tabulated.

3.7.2.2. Evaluation of antioxidant properties in rancimat apparatus

A Metroham 873 Biodiesel Rancimat apparatus (Switzerland) was used to assess the oxidative stability of RO and EM at 120 °C. The electrical conductivity of deionized water increases as the fatty acids are oxidized and secondary reaction products are formed. The preparation of samples was similar to that of the procedure followed in the oxipres method. The IP was recorded and PF values were calculated. All experiments were carried out in duplicates.

3.7.3. Computational Prediction: COSMO-RS

The Conductor-like Screening Model for Real Solvents (COSMO-RS) is a computational prediction model based on the electrostatic interaction between the solute and a solvent, which calculates the thermodynamic properties for solvation. Developed by Klamt and co-workers (2005) it is a known powerful tool for molecular description and solvent screening based on quantum-chemical approach. This prediction was applied for bilberry pomace,

COSMO-RS prediction is a two-step procedure. First, the microscopic step is where simulation is performed in a virtual conductor environment for the molecules. In the given environment, molecule induced polarization charge density is achieved on the surface (σ-surface). Therefore, the solute molecule is converted to its energetically optimal state in the conductor with respect to its geometry and electron density, via the quantum calculation self-consistency algorithm. Secondly, a macroscopic step which is an integrated sequential approach established to determine the σ-profile, which is a 3D distribution of the polarization charges on the surface of each molecule converted into a surface composition function. Thus, enabling a wide array of data on the molecular polarity distribution of the molecule. The thermodynamics of the molecular interactions that were based on the obtained σ-profile were used to calculate the chemical potential of the surface segment (σ-potential) using COSMOthermX program (version C30 release 13.01).

The standard quantum chemical methods, triple zeta valence polarized basis set (TZVP) was used in this study. The σ-potential can be associated with the affinity of the solvent to the solute.

Table 3.2. Solvent selection based on COSMO-RS relative solubility of target polyphenols

Solvent / Solute % / log10(x_RS)	D3G	Q3oG	EC	CA	TR	AA
Ethanol (100 %)	0	0	0	0	0	0
E/W (90 / 10)	-0.80	-0.34	-0.35	-0.30	-0.39	-0.12
E/W (80 / 20)	-1.63	-0.69	-0.70	-0.61	-0.79	-0.23
E/W (70 / 30)	-2.48	-1.04	-1.05	-0.92	-1.18	-0.33
E/W (60 / 40)	-3.35	-1.41	-1.40	-1.25	-1.59	-0.42
E/W (50 / 50)	-4.27	-1.81	-1.77	-1.61	-2.01	-0.52
E/W (40 / 60)	-5.25	-2.27	-2.16	-2.02	-2.46	-0.63
E/W (30 / 70)	-6.33	-2.82	-2.61	-2.52	-2.95	-0.77
E/W (20 / 80)	-7.56	-3.50	-3.12	-3.12	-3.51	-0.95
E/W (10/ 90)	-8.97	-4.36	-3.74	-3.89	-4.15	-1.21
Water (100 %)	-10.60	-5.46	-4.50	-4.87	-4.92	-1.56
Solubility Index	0 to -1	High	-1 to -2	Medium	> -2	Low

D3G (Delphinidin-3-Glucoside), Q3oG (Quercetin 3-o-Galactoside), EC (Epicatechin), CA (Chlorogenic Acid), TR (Trans-resveratrol), AA (Ascorbic Acid), E/W (Ethanol/Water)

In this work, the model is based on the prediction of the chemical potential of individual solute in the solvent system (Ethanol/Water). The solutes were selected after exhaustive literature review and each solute corresponds to an individual class of polyphenol. Calculation of the relative solubility of target polyphenols Delphinidin-3-glucoside (Anthocyanin), Quercetin-3-galactoside (Flavonol), Epicatechin (Flavan-3-ol), Chlorogenic acid (Phenolic acid), Trans-resveratrol (Stilbene) and Ascorbic Acid (Vitamin C) in different ethanol/water ratios were elucidated by implementing this COSMO-RS model in COSMOtherm software (C30 1401, CosmothermX14, COSMOlogic GmbH &Co. KG). The relative solubility is calculated from the following equation:

$$\log_{10}(x_j) = \log_{10}\left[\frac{\exp\left(\mu_j^{pure} - \mu_j^{solvent} - \Delta G_{j,fusion}\right)}{RT}\right] \text{ (Equation 1)}$$

μ_j^{pure}: chemical potential of pure compound j (Joule/mol)

$\mu_j^{solvent}$: chemical potential of j at infinite dilution (Joule/mol)

$\Delta G_{j,fusion}$: free energy of fusion of j (Joule/mol)

x_j : solubility of j (g/g solvent).

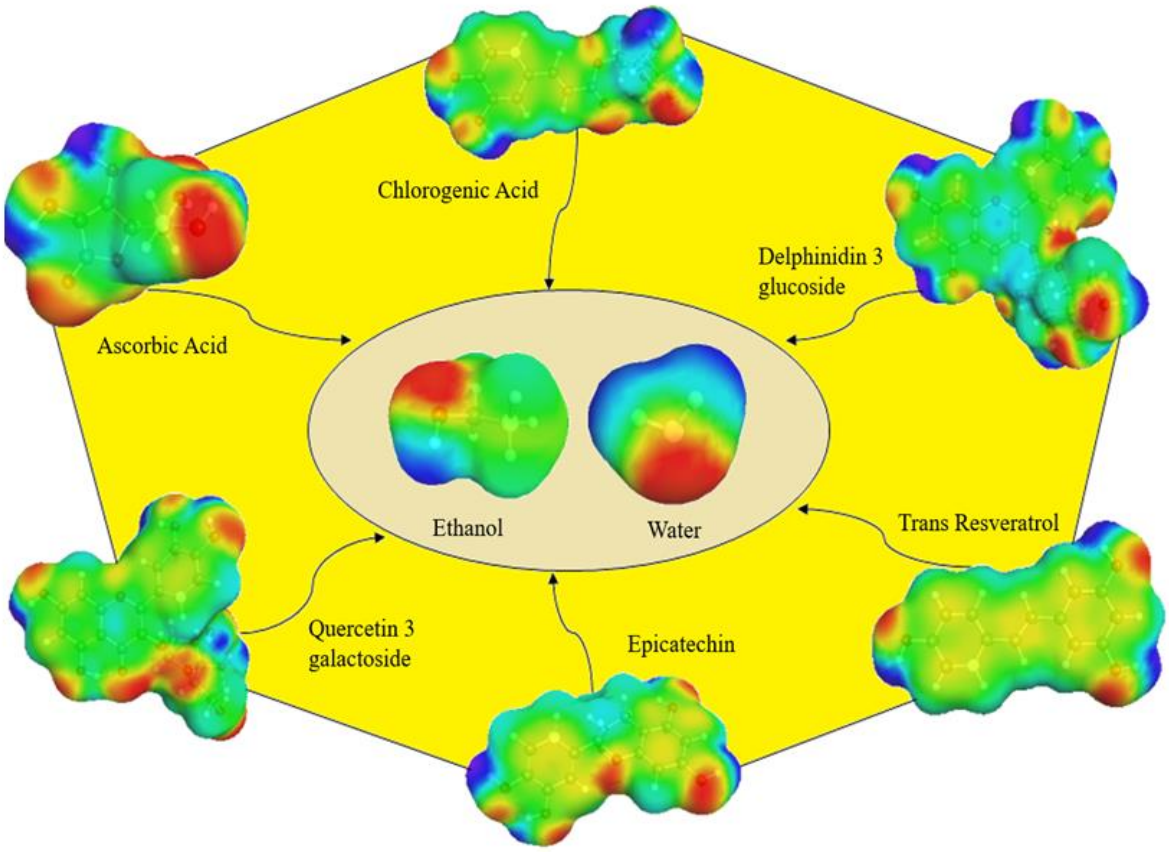


Figure 3.2. 3-D model of solute and solvent

Relative solubility is always calculated in infinite dilution. The logarithm of the best solubility is set to 0 and all other solvents are ranked relatively to the best or reference solvent (Table. 3.2).

Figure 3.3. depicts the individual parameters and the sequential order in which COSMO-RS prediction occurs. In this case, the prediction is explained with an example (Delphinidin-3-glucoside). Initially, the "SMILES" of the desired solute/compound is given as input in the COSMOtherm software and the appropriate values are assigned for temperature, model etc. The software utilizes the SMILES entry to generates a 3D model of the structure with its polarization charges called the σ – surface. The σ – surface is used to predict the σ – profile of the compound and similarly, the σ – profile is used to predict the σ – potential of the compound. Combined data of all solute and solvent compounds are generated and thus COSMO-RS prediction is established.

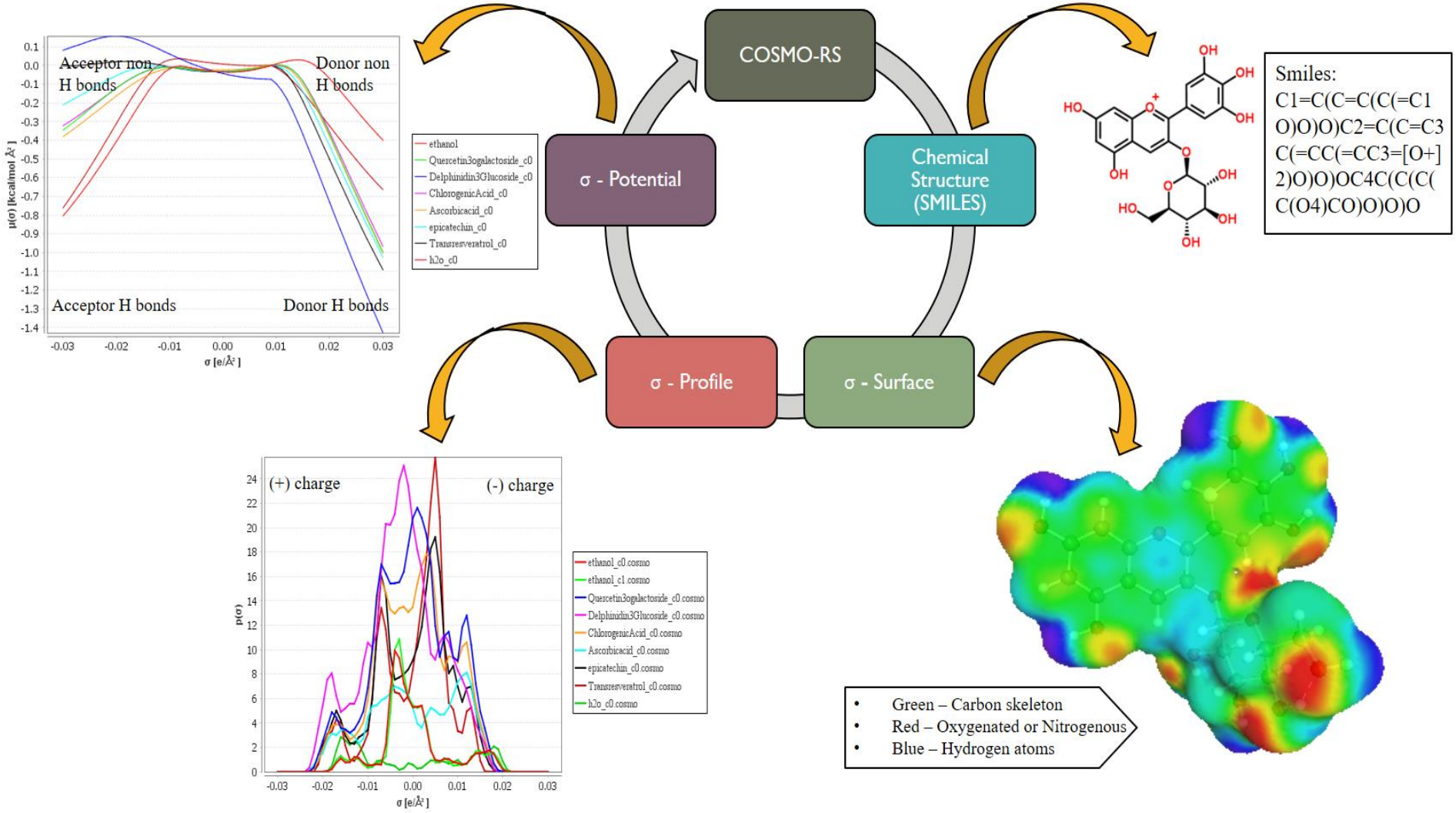


Figure 3.3. Pictorial representation of COSMO-RS components

3.8. Design of experiment

Two different biorefining schemes were adopted for this thesis. The first scheme (Fig 3.4.) for *Eleutherococcus* berries, in which the lipophilic fraction was initially removed by Soxhlet and supercritical fluid extraction with hexane and carbon dioxide as the respective solvents. The fatty acid methyl ester profile was determined by GC-FID and tocopherol concentration was estimated by HPLC analysis. Colorimetry was performed to determine the impact of extraction conditions on the colour profile of fruit material. Conventional solvent extraction with solvents of increasing polarity such as methanol, water, and acetone/water were used. The residue from supercritical fluid extraction was subjected to accelerated solvent extraction with solvents of increasing polarity such as acetone, ethanol/water, and water. Antioxidant activities (Trolox equivalent antioxidant assays) were performed and bioactivity rich extract was identified. Phytochemical screening to identify chemical constituents present in the extracts were performed using UPLC-MS analysis. Individual radical scavengers present in the extract was also identified using an online HPLC-UV/DPPH assay.

The second biorefining scheme (Fig 3.5.) adapted for bilberry pomace was executed by incorporating innovative and green extraction technologies such as MHG and UAE. These innovative extraction techniques were employed sequentially to extract target polyphenols from bilberry pomace. The scope of the thesis also includes the utilization of computational prediction software (COSMO-RS) to obtain theoretical values of solubility index of target polyphenols in different ethanol: water concentrations and its comparison to the experimental results from various *in vitro* antioxidant assays (Folin-Ciocalteu reducing capacity, total flavonoid content, total monomeric anthocyanin content and radical scavenging capacity of extracts) employed. The anthocyanin composition in all extracts was quantified with HPTLC and compared to the results obtained by UV-Vis spectrophotometry. As final valorization step, all waste residues were subjected to bead milling to remove the lipophilic fraction. The fatty acid profile was determined by Gas Chromatography- Flame Ionization Detection (GC-FID) and distribution of lipids by High-Performance Thin Layer Chromatography (HPTLC).

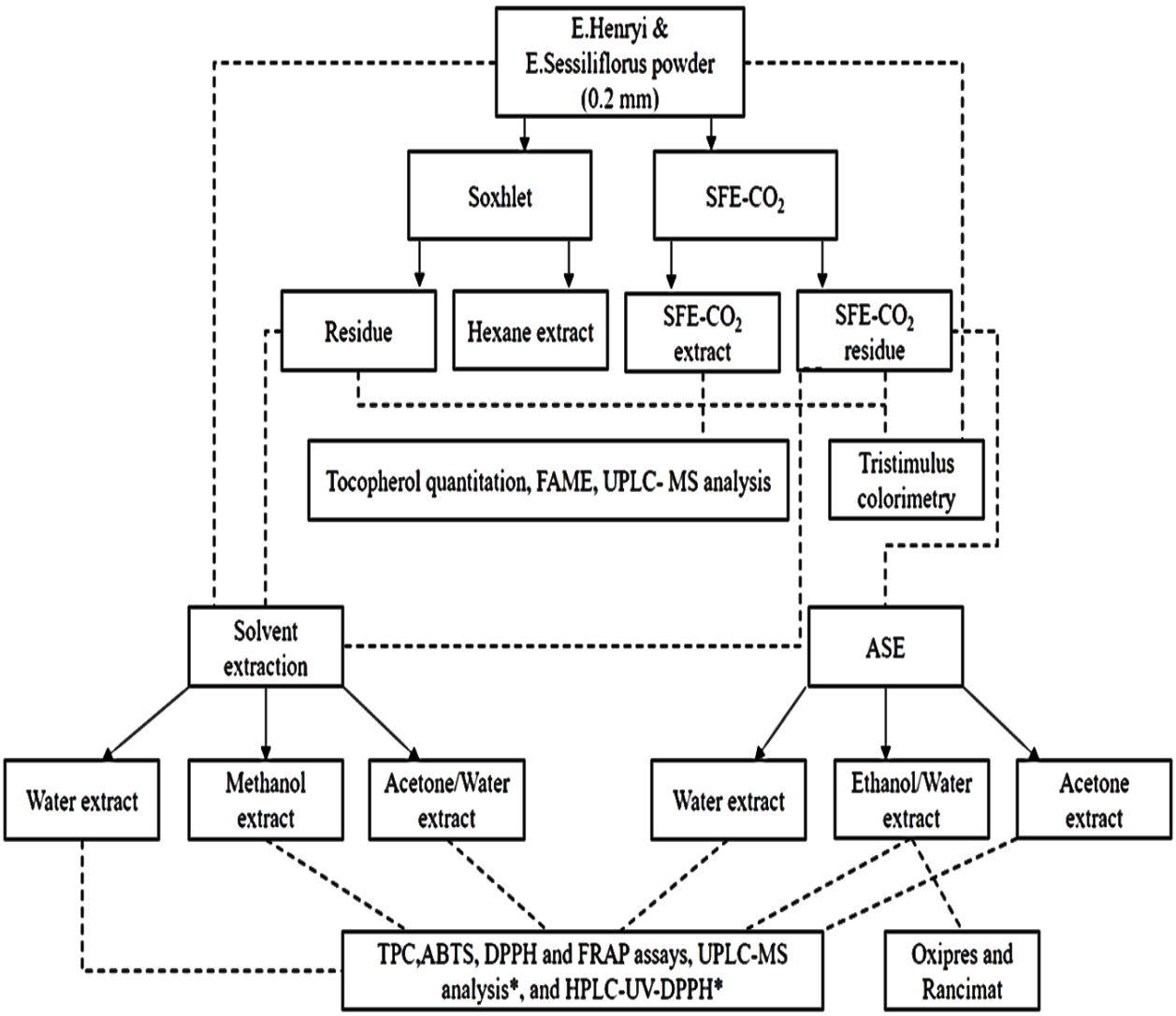


Figure 3.4. Graphical representation of experimental scheme for *Eleutherococcus*

Fig 3.6. depicts the different extraction techniques, spectrophotometric analysis, chromatography and spectrometry analysis employed to both plant materials *Eleutherococcus* and bilberry. Under miscellaneous analysis, the assays that do not fall under previously mentioned categories are stated. COSMO-RS is computational prediction parameter; therefore, it also falls under the Miscellaneous category. This general assortment helps in differentiating the procedures adopted for different plant materials and is intended to make comparisons easy for future reference.

3.9. Statistical analysis

All analysis was performed in duplicates or triplicates and the calibration curves were generated with Microsoft Excel 2013, and results are represented as mean ± standard deviation.

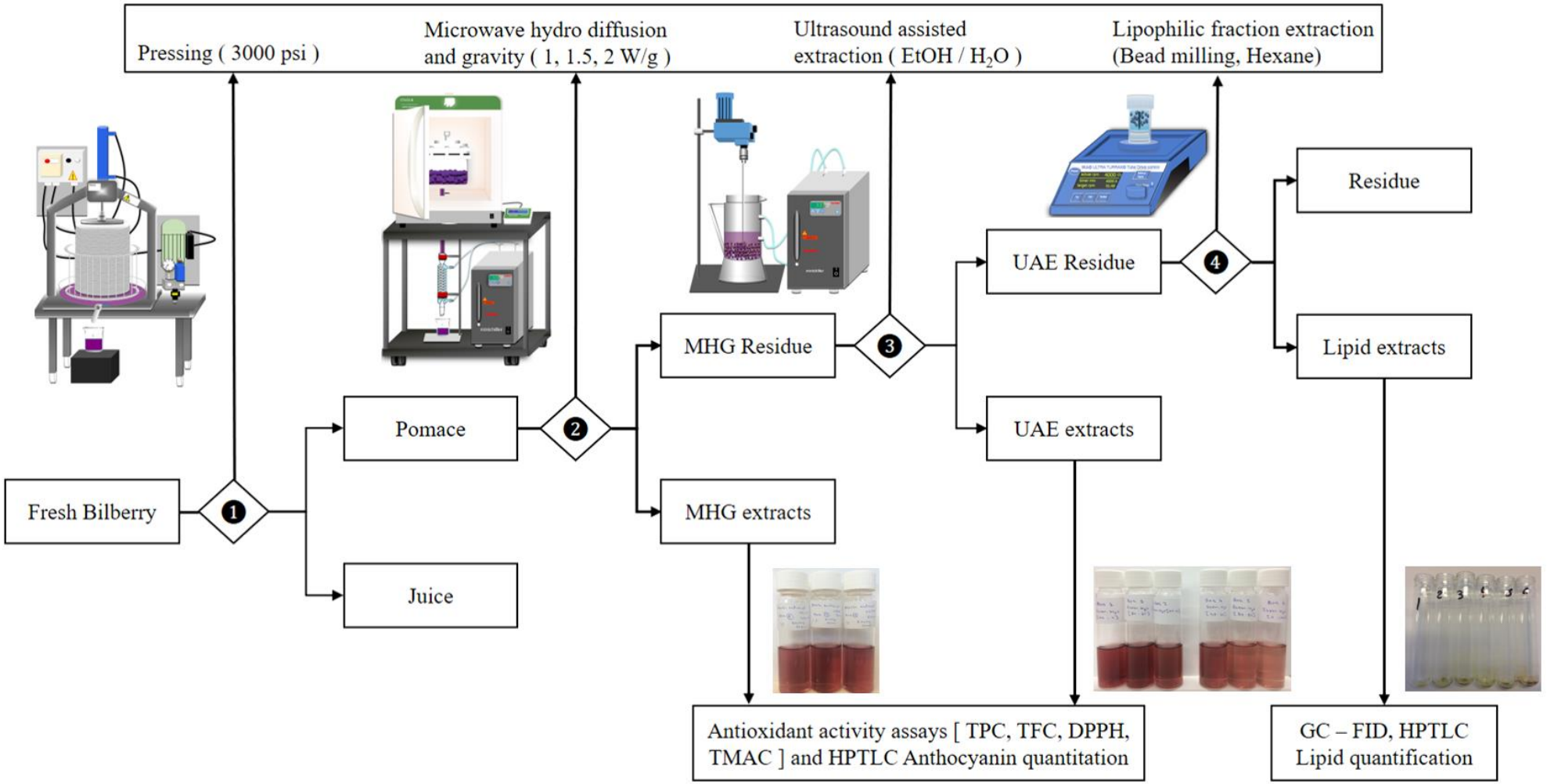
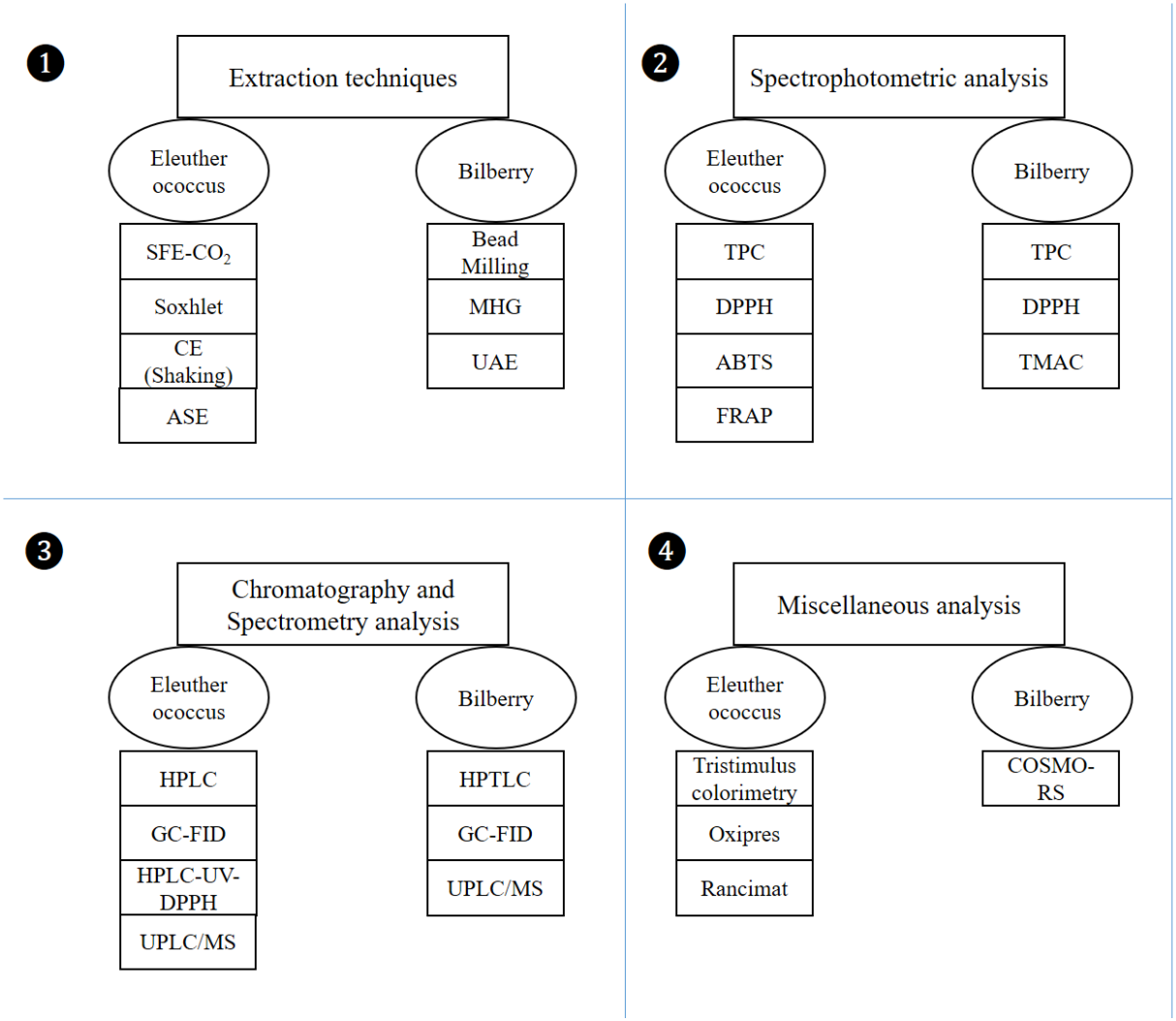


Figure 3.5. Graphical representation of experimental scheme for bilberries



- $SFE\text{-}CO_2$ – Supercritical Fluid Extraction
- CE – Conventional Extraction
- ASE – Accelerated Solvent Extraction
- MHG – Microwave Hydro Diffusion and Gravity
- UAE – Ultrasound Assisted extraction
- TPC – Total polyphenol content
- DPPH – 2,2 diphenyl-1-picrylhyrazyl (Radical Scavenging Capacity)
- ABTS – 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (Radical Scavenging Capacity)
- HPLC – High Performance Liquid Chromatography (Tocopherol quantitation)
- GC-FID – Gas Chromatography-Flame Ionization Detector (Fatty Acid Methyl Ester)
- Online HPLC-UV-DPPH – Identification of DPPH Radical Scavengers
- UPLC/MS – Ultraperformance Liquid Chromatography / Mass Spectrometry
- HPTLC – High performance Thin Layer Chromatography
- COSMO-RS – Conductor like screening model for Real Solvents

Figure 3.6. Experiment and assay classification in this thesis

4. RESULTS AND DISCUSSION

4.1. Extraction yields and total phenolic content (TPC)

The SFE-CO_2 extract yields of EH and ES fruit powder were 4.69 ± 0.41 and 5.40 ± 0.44 % respectively. Soxhlet extraction with hexane (EH: 6.43 ± 0.11 and ES: 7.57 ± 0.42 %) as the solvent resulted in relatively higher yields than supercritical extraction with CO_2. The phenolic content (Table 4.1.) of the lipophilic fraction in ES was higher than the EH extracts. Soxhlet extracts of both the fruits had a higher yield, phenolic content and exhibited higher antioxidant activity when compared to SFE-CO_2 extracts. Author, Asilbekova, and others reported that the oil content from the fruit of *E. Sessiliflorus* was 7.2 % with hexane as solvent.

Solvent extraction with solvents of different polarity such as methanol, water, acetone/water was performed to provide the comprehensive data on the antioxidant activities of the sample. The influence of lipid extraction techniques and its effect on the fruit material in terms of antioxidant potential was also analyzed. The data (Table 4.1.) reveals that there is no significant or adverse impact on the sample material due to soxhlet and supercritical extraction. The phenolic content, the antioxidant profile of the fruit material was similar to that of their corresponding residues obtained from soxhlet and supercritical fluid extraction. The highest antioxidant activity was recorded in the water extract of EH, SFE-CO_2 residue (31.78 ± 0.23 mg GAE/g DPM) and the lowest in the methanol extract of ES, soxhlet residue with (14.55 ± 0.12 mg GAE/g DPM). The water and acetone/water extracts had higher phenolic content than the methanol extracts owing to the higher polarity of the solvent.

Sequential extraction in ASE system with acetone, ethanol/water and water were performed to facilitate complete removal of soluble compounds from the fruit material (SFE-CO_2 residue) at 70 °C. The yield of ASE ethanol/water extracts from EH was 50.88 ± 4.07 %, followed by water extracts from EH with a yield of 4.38 ± 0.18 %. Similarly, ES had a yield of 33.94 ± 0.97 % (ASE, ethanol/water), 3.81 ± 0.27 % (ASE, water). The yield was significantly higher in ethanol/water as solvent and *E. Henryi* had a higher yield than *E. Sessiliflorus* suggesting the total removable solids is higher in *E. Henryi* fruits. From table 4.1., it is evident that the water extracts in both fruits had relatively higher phenolic content and antioxidant activity than ethanol/water extracts. Yet, ethanol/water extract was considered the bioactivity rich fraction as the yields were significantly higher. It is of paramount importance to note that only ethanol/water doesn't completely remove the soluble fraction and further extraction with water is required for exhaustive extraction of soluble compounds from the fruit material. Though, the global yield was higher in the acidified ethanol/water extracts it was interesting to find that the water extracts in the sequential

extraction (ASE) had the overall highest phenolic content and exhibited higher antioxidant activity when compared to all other extracts. The highest phenolic content was recorded in the water extracts (EH: 240.43 ± 4.30 and ES: 195.61 ± 6.08 mg GAE/g DE). The authors (Daniel Zaluski et al., 2015) reported that the yield for ultrasonicated 75 % ethanol extracts from freshly dried *E. Henryi* fruits cultivated in Poland was 27.4 %, the total phenolic content was 4350mg/100 g of extract and an EC_{50} (Anti-DPPH*) of 0.2mg/ml. The variation in the result can be attributed to several parameters including different geographical location, the difference in extraction technique and solvent ratio etc.

4.2. Antioxidant properties of *Eleutherococcus* extracts

All extracts including the ethanol/water and water extracts from ASE were analyzed for its antioxidant activity by $TEAC_{ABTS}$, $TEAC_{DPPH,}$ and $TEAC_{FRAP}$. The highest antioxidant activity was recorded in the $TEAC_{ABTS}$ water extracts (EH: 562.88 ± 27.17 and ES: 468.01 ± 17.96 mg TE/g DE) followed by the ethanol/water extracts (EH: 421.35 ± 19.93 and ES: 309.36 ± 15.43 mg TE/g DE). The Trolox Equivalent Antioxidant Capacity (TEAC) for the ASE extracts in different assays are in the following order, $TEAC_{ABTS} > TEAC_{DPPH} > TEAC_{FRAP.}$ The TEAC for extracts from conventional extraction with solvents of increasing polarity didn't follow any particular trait (Table 4.1.). Overall, EH hydrophilic extracts displayed higher antioxidant activity than the ES extracts in all assays. These results supplement the fact that extraction techniques play a crucial role in eluting polyphenols from plant matrix and thereby enhancing the potential antioxidant activity of the extracts. In this case, ASE extracts displayed higher (> 10-fold) antioxidant activity than conventional extraction. Whereas, in the lipophilic fraction the antioxidant activity exhibited by the EH and ES hexane extracts were slightly higher than their corresponding CO_2 extracts.

4.3. The impact of soxhlet and supercritical fluid extraction on colour

In order to identify the effect of lipid extraction on the fruit material, colorimetry assay was performed on the raw material freeze dried fruit powder, soxhlet residue and SFE-CO_2 residue of *E. Henryi* and *E. Sessiliflorus* fruits. The CIELAB system provides the value of Luminosity (L*, black white component), Chromaticiness coordinates a* (+red to – green component) and b* (+yellow to – blue component). The L*, a*, b*, and index E values are presented in table 4.2. The reduction in luminosity L* and index E values indicates the loss of

Table 4.1. The yields and antioxidant activities (TPC, ABTS, DPPH, FRAP) of *E. Henryi* and *E. Sessiliflorus* fruits extracted with solvents of different polarity.

Extraction technique employed	Sample	Solvent	Yield (%)		TPC (mg GAE / g DE)		ABTS (mg TE / g DE)		DPPH (mg TE / g DE)		FRAP (mg TE / g DE)	
			EH	ES	EH	ES	EH	ES	EH	ES	EH	ES
Soxhlet	Fruit powder	HX	6.43 ± 0.11	7.57 ± 0.42	17.03 ± 0.33	19.65 ± 0.38	3.43 ± 0.12	3.17 ± 0.05	2.99 ± 0.21	2.44 ± 0.08	-	-
SFE - CO_2	Fruit powder	CO_2	4.69 ± 0.41	5.40 ± 0.44	16.36 ± 0.43	18.87 ± 0.89	2.57 ± 0.16	2.70 ± 0.28	1.83 ± 0.16	2.10 ± 0.05	-	-
ASE 70 °C	SFE-CO_2 residue	AC	7.44 ± 0.41	6.34 ± 0.37	-	-	-	-	-	-	-	-
		ET/W	50.88 ± 4.07	33.94 ± 0.97	178.30 ± 9.66	130.14 ± 6.33	421.35 ± 19.93	309.36 ± 15.43	190.41 ± 6.93	131.47 ± 4.53	185.10 ± 13.29	154.23 ± 6.95
		W	4.38 ± 0.18	3.81 ± 0.27	240.43 ± 4.30	195.61 ± 6.08	562.88 ± 27.17	468.01 ± 17.96	213.05 ± 9.45	168.31 ± 3.12	207.73 ± 9.52	170.65 ± 1.54
					TPC (mg GAE / g DPM)		ABTS (mg TE / g DPM)		DPPH (mg TE / g DPM)		FRAP (mg TE / g DPM)	
					EH	ES	EH	ES	EH	ES	EH	ES
Shaking	Fruit powder	ME	-	-	21.92 ± 0.17	15.02 ± 0.15	32.93 ± 0.27	18.37 ± 0.69	30.68 ± 1.67	20.70 ± 1.18	34.46 ± 0.14	21.08 ± 0.11
		H_2O	-	-	27.23 ± 0.17	17.00 ± 0.15	33.27 ± 0.39	22.09 ± 0.57	25.12 ± 0.56	15.60 ± 0.28	27.26 ± 0.16	18.19 ± 0.12
		AC/W	-	-	29.29 ± 0.19	20.03 ± 0.15	48.36 ± 1.18	33.35 ± 0.35	37.46 ± 0.75	27.96 ± 0.29	37.28 ± 0.51	29.45 ± 0.15
	Hexane residue	ME	-	-	21.30 ± 0.22	14.55 ± 0.12	37.28 ± 0.73	23.46 ± 0.39	33.64 ± 1.38	25.02 ± 1.30	39.00 ± 0.23	28.98 ± 0.16
		H_2O	-	-	27.60 ± 0.13	20.30 ± 0.19	31.43 ± 0.13	22.66 ± 0.27	24.59 ± 0.13	16.89 ± 0.83	27.47 ± 0.08	20.93 ± 0.14
		AC/W	-	-	29.38 ± 0.15	20.94 ± 0.07	42.85 ± 0.55	34.18 ± 0.53	35.42 ± 0.27	28.83 ± 1.12	35.79 ± 0.14	28.62 ± 0.09
	SFE - CO_2 residue	ME	-	-	23.31 ± 0.22	16.26 ± 0.10	43.45 ± 0.54	24.72 ± 0.53	36.92 ± 2.14	25.57 ± 2.95	43.29 ± 1.14	30.11 ± 0.96
		H_2O	-	-	31.78 ± 0.23	19.68 ± 0.29	31.60 ± 0.42	23.41 ± 0.57	24.94 ± 0.64	17.33 ± 0.40	29.38 ± 0.15	23.19 ± 0.02
		AC/W	-	-	29.17 ± 0.17	19.38 ± 0.24	45.43 ± 0.79	30.85 ± 0.35	37.49 ± 0.23	25.77 ± 0.76	37.50 ± 0.18	24.38 ± 0.23

Values represented as the mean ± standard deviation (n = 3). HX-Hexane; CO_2-Carbon dioxide; AC-Acetone; ET/W-Ethanol/Water (80/20: v/v) acidified; ME-Methanol; H_2O-Water; AC/W-Acetone/Water (30/70: v/v); DE – Dry Extracts, DPM – Dry Plant Material.

lightness in soxhlet and SFE-CO_2 residues, this signifies that such these extraction techniques do alter the colour profile of the berry fruits.

The residues from soxhlet extraction and supercritical fluid extraction were measured for their colour index and compared to the freeze-dried berry powder. The tristimulus colorimetry helps us understand the effect of extraction techniques on the sample material when subjected to further extraction. As both soxhlet and supercritical extraction are utilized to remove the lipophilic fraction, the magnitude of index E was relatively similar. From table 4.2. it is evident that the index E values are higher for freeze dried berry powder (23.42 ± 0.07, 24.33 ± 0.04) for *E. Henryi* and *E. Sessiliflorus* respectively.

Table 4.2. Tristimulus colorimetry

Samples (*E. Henryi*)	**L***	**a***	**b***	**Index E**
Freeze dried fruit powder	23.29 ± 0.07	2.44 ± 0.02	(-0.17 ± 0.01)	23.42 ± 0.07
Soxhlet residue	23.27 ± 0.24	2.50 ± 0.01	(-0.30 ± 0.02)	23.41 ± 0.24
SFE - CO_2 residue	23.22 ± 0.06	2.27 ± 0.02	(-0.19 ± 0.01)	23.33 ± 0.06

Samples (*E. Sessiliflorus*)	**L***	**a***	**b***	**Index E**
Freeze dried fruit powder	24.22 ± 0.05	2.12 ± 0.07	0.71 ± 0.04	24.33 ± 0.04
Soxhlet residue	23.65 ± 0.10	2.68 ± 0.01	(-0.31 ± 0.02)	23.80 ± 0.10
SFE - CO_2 residue	23.99 ± 0.15	2.11 ± 0.05	0.67 ± 0.01	24.09 ± 0.14

*Results are expressed as mean ± standard deviation (n = 3)

4.4. Fatty Acid composition and tocopherol content

The fatty acid profiles (GC-FID) of *E. Henryi* and *E. Sessiliflorus* fruit extracts obtained by conventional and supercritical fluid extraction (CO_2) were compared. The hexane extracts from soxhlet extraction and SFE- CO_2 extracts had a similar fatty acid composition, in total 16 fatty acids (Table 4.3.) were identified in the lipophilic fractions of both the berries. Oleic and Linoleic acid were major constituents in the lipophilic fractions analyzed. The content of oleic acid alone was > 50%, together oleic acid and linoleic acid constituted ≥ 87% of the fatty acid composition in the lipophilic fraction. Which implies that the lipophilic fraction of the EH and ES fruits is a good source of Mono and Poly Unsaturated Fatty Acids (MUFA / PUFA). Palmitic acid was the major saturated fatty acid with (EH: 4.18 %, 3.83 %, and ES: 4.24%, 3.30 %) in hexane and SFE-CO_2 extracts respectively. The SFE- CO_2 extracts had relatively higher % of oleic acid (EH: 56.39

%, ES: 56.46 %) than their respective hexane extracts (EH: 53.05 %, ES: 53.61 %). Conversely, the linoleic acid concentration was higher in hexane extracts (EH: 34.57 %, ES: 36.93 %) when compared to their SFE-CO_2 extracts (EH: 34.39 %, ES: 34.51 %). Author (Daniel Zaluski et al.,2017) in his study found 16 fatty acids in *E. Sessiliflorus* fruit, among them oleic acid (53.73 ± 1.74 %) and linoleic acid (34.73 ± 0.42 %) were present in higher amounts, this result was in good correlation with the data obtained.

The tocopherol content of the lipophilic fraction was quantified by HPLC (Table 4.4.). The SFE- CO_2 extracts had relatively higher tocopherol content than their respective hexane extracts in both fruits. The total tocopherol content in EH extracts (HE: 3.06, SFE- CO_2: 3.10; mg T/g of extract) was higher than that of the ES extracts (HE: 2.49, SFE- CO_2: 2.80; mg T/g of extract). In all the extracts α-tocopherol was the predominant constituent followed by γ-tocopherol and δ-tocopherol, except for the ES SFE- CO_2 extract in which γ-tocopherol was the highest (1.0198 ± 0.07 mg/g) followed by α-tocopherol (0.9869 mg/g). It was interesting to note that β-tocopherol was absent in the lipophilic fraction of the berries. Supercritical fluid extraction results in higher yields of tocopherol when compared to that of conventional extraction (Lina Grunovaite et al., 2016 and Paulis Kraujalis et al., 2013). Tocopherol might be the prime phytochemical responsible for the antioxidant activity exhibited by the lipophilic fraction, as phytochemical screening by UPLC-MS resulted in the identification of terpenes and terpenoid derivatives (Table 4.6.).

Table 4.3. Fatty Acid composition of the lipophilic fractions from *E. Henryi* and *E. Sessiliflorus*.

Fatty Acid	**Chain Length & Group**	***E. HENRYI***		**E. SESSILI**	
		HE	**SCE - CO_2**	**HE**	**SCE - CO_2**
Palmitic	C16:0 / SFA	4.18	3.83	4.24	3.30
Palmitoleic	C16:1 / MUFA	0.15	0.13	0.12	0.16
Stearic	C18:0 / SFA	0.79	0.71	0.63	0.61
Oleic	C18:1n9c / MUFA	53.05	56.39	53.61	56.46
Linolelaidic	C18:2n6t / TFA	0.14	0.15	0.14	0.15
Linoleic	C18:2n6c / PUFA	34.57	34.39	36.93	34.51
Arachidic	C20:0 / SFA	0.21	0.19	0.15	0.16
γ - Linolenic	C18:3n6 / PUFA	0.13	0.12	0.11	0.10
cis - 11 - Eicosenoic	C20:1 / MUFA	0.24	0.28	0.22	0.27
Linolenic (ALA)	C18:3n3 / PUFA	0.07	0.07	0.07	0.08
Heneicosanoic	C21:0 / SFA	1.59	1.13	1.30	0.94
cis - 11,14 - Eicosadienoic	C20:2 / PUFA	0.13	0.04	0.11	0.03
Behenic	C22:0 / SFA	0.96	0.56	0.55	0.55
cis - 11,14,17 - Eicosatrienoic	C20:3n3 / PUFA	0.54	0.24	0.35	0.31
Arachidonic (ARA)	C20:4n6 / PUFA	0.13	0.06	0.05	0.04
Lignoceric	C24:0 / SFA	0.74	0.29	0.33	0.32
Other		2.52	1.57	1.23	2.15
	∑ SFA	8.47	6.71	7.21	5.89
	∑ MUFA	53.44	56.79	53.94	56.89
	∑ PUFA	35.57	34.92	37.62	35.07

Values given are means of duplicate results * Composition in %.

Table 4.4. Concentration of Tocopherols (mg/g) in the lipophilic fraction of *E. Henryi* and *E. Sessiliflorus*

Description	**α - T**	**γ - T**	**δ - T**	**Total Tocopherol**
E. Henryi Hexane extract	1.7811 ± 0.07	0.6237 ± 0.04	0.6526 ± 0.04	3.0573
E. Henryi SFE CO_2 extract	1.7049 ± 0.03	0.7376 ± 0.08	0.6531 ± 0.02	3.0956
E. Sessiliflorus Hexane extract	0.9209 ± 0.02	0.8715 ± 0.18	0.6936 ± 0.11	2.4859
E. Sessiliflorus SFE CO_2 extract	0.9869 ± 0.03	1.0198 ± 0.07	0.7915 ± 0.06	2.7982

Values given are an average of duplicate results ± standard deviation.

Table 4.5. Oxidative stability of ASE 80 % ethanol extract in emulsion system

Extracts in emulsion	Oxipres		Rancimat	
	IP (hrs)	PF	IP (hrs)	PF
Control	2.38 ± 0.02	1.00	4.41 ± 0.03	1.00
E. Henryi (0.1%)	2.53 ± 0.01	1.07	4.53 ± 0.02	1.03
E. Sessiliflorus (0.1%)	2.90 ± 0.03	1.22	4.56 ± 0.02	1.03

Values given are an average of duplicate results ± standard deviation.

4.5. Antioxidant effect of bioactivity rich extract in emulsion

EH, and ES extracts (ASE, EtOH/H_2O) were incorporated into oil/water emulsion system and their oxidation inhibitory effects were quantified (Table 4.5.). In simpler terms, Oxipres is the measure of reduction in pressure of oxygen in the chamber that is purged with oxygen and subjected to heating at high temperature (110° C). Rancimat is the magnitude of difference in electrical conductivity due to the formation of carboxylic acids when the lipid system is exposed to high temperature. The protection factor of ASE extract (0.1 %) in RO is not included, as the results were no different from that of the control samples, this can be attributed to the poor solubility of the extracts in pure oil. The PF of extracts in emulsion were negligible, the ES extract had a PF of 1.22 in the oxipres system. The limited or absence of phenolic acids in EH and ES samples could be the possible reason for such low protection factor exhibited by the extracts. Also, phenolic antioxidants are effective in enhancing the induction period when added to oil that hasn't deteriorated to great extent. However, they are ineffective in retarding the degree of decomposition of already deteriorated lipids (Mabarouk & Dugan 1961). Incorporating ASE extracts imparted good colour to the emulsion and can act as a delivery system for anthocyanins in lipid-water systems.

4.6. Phytochemical screening using UPLC-QTOF/MS and HPLC-UV/DPPH

The lipophilic fraction (SFE-CO_2) and bioactivity rich ASE (ethanol/water) extract from both the berries were screened for its phytochemical composition. Table 4.6. summarizes the identified compounds with its corresponding molecular formula, retention time (RT) and mass to charge ratio (m/z). As negative ionization was employed, the mass of the compounds is given in its deprotonated molecular configuration $[M-H]^-$. The identification of compounds was facilitated by comparison of the spectral data obtained with digital libraries Chemspider, Metlin databases and literature data.

It was interesting to note that both *E. Henryi* and *E. Sessiliflorus* had similar phytochemical constituents. Compound **1** had an m/z value of 483.3114 with molecular formula $C_{30}H_{44}O_5$ and was identified as chiisanogenin (H.J. Jung et al., 2005, Yang C. et al.,2010). Compound **3** had an m/z value of 485.3268 with corresponding molecular formula $C_{30}H_{46}O_5$ correlating to a chiisanogenic acid derivative. Compound **4**,**6** was identified as linoleic acid (m/z 279.2323) and hydroxy linoleic acid (m/z 295.2278) respectively with fitting molecular formula $C_{18}H_{32}O_2$ and $C_{18}H_{32}O_3$ this supports our data obtained by FAME. However, compound **6** was only found in *E. Henryi* SFE-CO_2 extract. Compound **2**,**5** with m/z

Table 4.6. Tentative compound identification UPLC-QTOF/MS

No.	Compound	Molecular Formula	Solvent	Sample	RT (UPLC)	m/z, $[M-H]^-$
1	Chiisanogenin	$C_{30}H_{44}O_5$		EH, ES	5.71	483.3114
2	NI	$C_{31}H_{46}O_7$		EH, ES	5.86	529.3167
3	Chiisanogenin acid derivative	$C_{30}H_{46}O_5$	SFE-CO_2	EH, ES	6.02	485.3268
4	Linoleic acid	$C_{18}H_{32}O_2$		EH, ES	8.21	279.2323
5	NI	$C_{37}H_{70}O_6$		EH, ES	8.24	609.5084
6	Hydroxylinoleic acid	$C_{18}H_{32}O_3$		ES	6.23	295.2278
7	NI	$C_{13}H_{24}O_{13}$		EH	0.38	387.1144
8	Scopolin	$C_{16}H_{18}O_9$		EH, ES	1.64	353.0877
9	NI	$C_{26}H_{28}O_{15}$		EH, ES	1.82	579.1358
10	Chlorogenic acid (3-o-caffeoyl quinic acid)	$C_{16}H_{18}O_9$		EH, ES	2.13	353.0877
11	Hyperoside	$C_{21}H_{20}O_{12}$		EH, ES	2.36	463.0883
12	NI	$C_{25}H_{24}O_{12}$	ASE,	EH, ES	2.74	515.1194
13	NI	$C_{49}H_{76}O_{21}$	EtOH/H_2O	EH, ES	3.59	999.4799
14	NI	$C_{42}H_{66}O_{14}$		ES	4.10	793.4374
15	NI	$C_{13}H_{18}O_3$		EH, ES	4.32	221.1181
16	NI	$C_{14}H_{18}O_4$		EH, ES	4.55	249.1131
17	NI	$C_{36}H_{56}O_{10}$		EH, ES	4.72	647.3797
18	NI	$C_{36}H_{56}O_9$		ES	5.51	631.3847
19	Chiisanogenin	$C_{30}H_{44}O_5$		EH, ES	5.86	483.3109

EH – E. Henryi, ES - *E. Sessiliflorus*; RT – retention time, m/z – mass to charge ratio, $[M-H]^-$ - mass in negative ionization mode; NI – Not Identified.

the value of 529.3167 and 609.5084 could not be identified but could be terpenoid compounds as previous studies and literature data strongly suggests that the presence of wide range of terpenoid and triterpenoid compounds in Eleutherococcus species (Lee et al.,2012, Yang et al.,2009, Yang et al.,2013)

Only three compounds were identified in ASE ethanol/water extract, of which compound **8**,**10** gave the exact m/z value of 353.0877 and fitting molecular formula $C_{16}H_{18}O_9$. Therefore, compound **8** was identified as scopolin (Zhao et al.,2014) and compound **10** as chlorogenic acid

(3-o-caffeoyl quinic acid). Compound **11** was identified as hyperoside (Zhao et al.,2014) with a molecular formula of $C_{21}H_{20}O_{12}$ and gave an m/z value of 463.0883 thus proving to the only major flavonol glucoside present in the extracts. It was interesting to find traces of chiisanogenin present in the ethanol-water extracts (compound **19**) which implies supercritical fluid extraction and accelerated solvent extraction with chloroform doesn't completely remove chiisanogenin and another exhaustive extraction process with an appropriate solvent is required for isolating the compound. Compounds **7,9,12,13…18** couldn't be identified with the spectral data obtained.

The anthocyanin profile of *E. Henryi* and *E. Sessiliflorus* was identified with UPLC-QTOF/MS. Both samples had only one peak with m/z value 581.1501 and fitting molecular ion formula $C_{26}H_{29}O_{15}$ and identified as cyanidin-3-sambubioside.

Compounds contributing to the radical scavenging capacity of the bioactivity rich ASE ethanol/water extracts were identified by an online HPLC-UV/DPPH assay (Fig. 4.1.). Combined UV (positive peaks) and $DPPH^{\cdot}$ quenching (negative peaks) chromatogram are represented in figure 4.1 a, b with corresponding peaks (**1,2,3** and **4)** depicting the individual RSC of the compounds present in *E. Henryi* and *E. Sessiliflorus* respectively. Peak **1**, was not detected in UPLC screening but had a mass similar to that of quinic acid. Based on its mass data peak **1** could be quinic acid derivative. Peak **2,3,4** was identified as chlorogenic acid, hyperoside, and cyanidin-3-sambubioside respectively. The RSC of individual antioxidants depend mainly on their chemical structure, the cumulative number of hydroxyl groups which can effectively donate hydrogen or proton to the radical. Author, Osman (2011) in his paper vividly explained the reaction mechanism of epicatechin and DPPH radical in alcoholic solvents. Furthermore, it was suggested that the reaction of phenols with $DPPH^{\cdot}$ in alcoholic solvents may be an electron transfer reaction rather than hydrogen atom transfer (Foti et al.,2004).

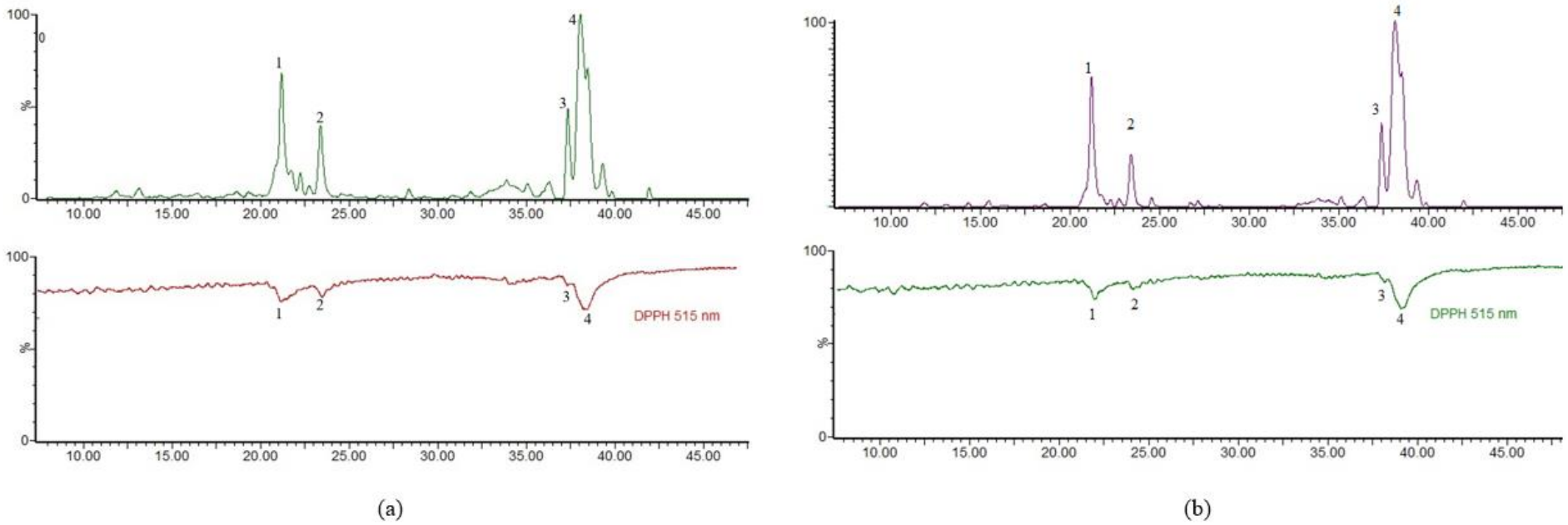


Figure 4.1. Online HPLC – UV – DPPH of extracts

(a) ASE 80% ethanol/water extract of *E. Henryi*

(b) ASE 80% ethanol/water extract of *E. Sessiliflorus*

1 – Unknown (quinic acid), 2 - Chlorogenic acid, 3 - Hyperoside and 4 - Cyanidin-3-sambubioside.

4.7. Microwave extraction yield and temperature profile

Extraction of biomolecules from plant material using microwave hydro diffusion technique is a patented process (Chemat et al.., 2010). The concentration of microwave power on the plant matrix results in rapid increase in temperature, nearing the boiling point of water as it is the major constituent. This accelerated increase initiates evaporation off the *in situ* water which in turn leads to the rupture of cells thereby facilitating the release of in situ water which acts as a carrier of free polyphenols present in the berries.

Fig. 4.3. depicts the temperature profile in the microwave system at different powers (1;1.5;2 W g^{-1}). As microwave power increased the sample temperature with respect to its corresponding time increased. The time required to reach 90 °C decreased with increase in microwave power thus reducing the total extraction time. The total extraction time refers to the time required to collect only the colored (polyphenol-rich) fraction. Therefore, the yield of MHG represents the obtained Polyphenol-Rich Fraction (PRF).

The yield of individual microwave power is shown in Fig. 4.2. it is evident that the time required to obtain the desired polyphenol fraction reduced substantially with an increase in power. Microwave power 300 W or 1 W g^{-1} required nearly 16 min to produce the PRF whereas, 450 W or 1.5 W g^{-1} took 9 min and 600 W or 2 W g^{-1} required only 6.5 min to yield the PRF. It was interesting to note that the distinction to obtain the PRF was better at higher powers, as the transition between the PRF and *in situ* water fraction was precise. Based on the specific power and productivity of MHG extraction the appropriate power 2 W g^{-1} was chosen (Table 3.1.) for further extraction. MHG extracts possessed a pleasing characteristic aroma regardless of the processing condition. Lyophilization of PRF resulted in pasty extracts owing to the high sugar content in pomace.

The temperature profile gives us a clear understanding of heat generation due to microwave irradiation. The steep increase in temperature at higher powers, 450 and 600 W resulted in a substantial yield increase. This facilitated in lowering the processing time, thereby reducing energy cost and conversely enhancing productivity.

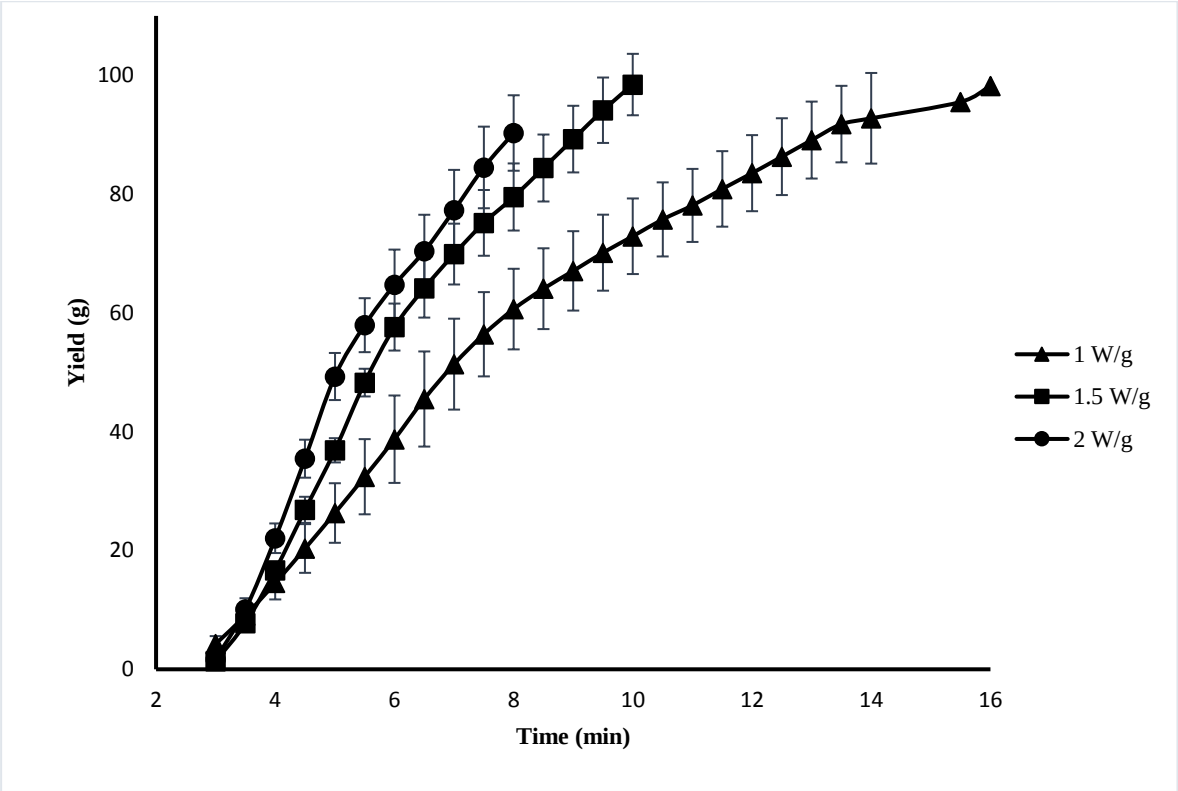


Figure 4.2. MHG extract yield profile under different microwave power

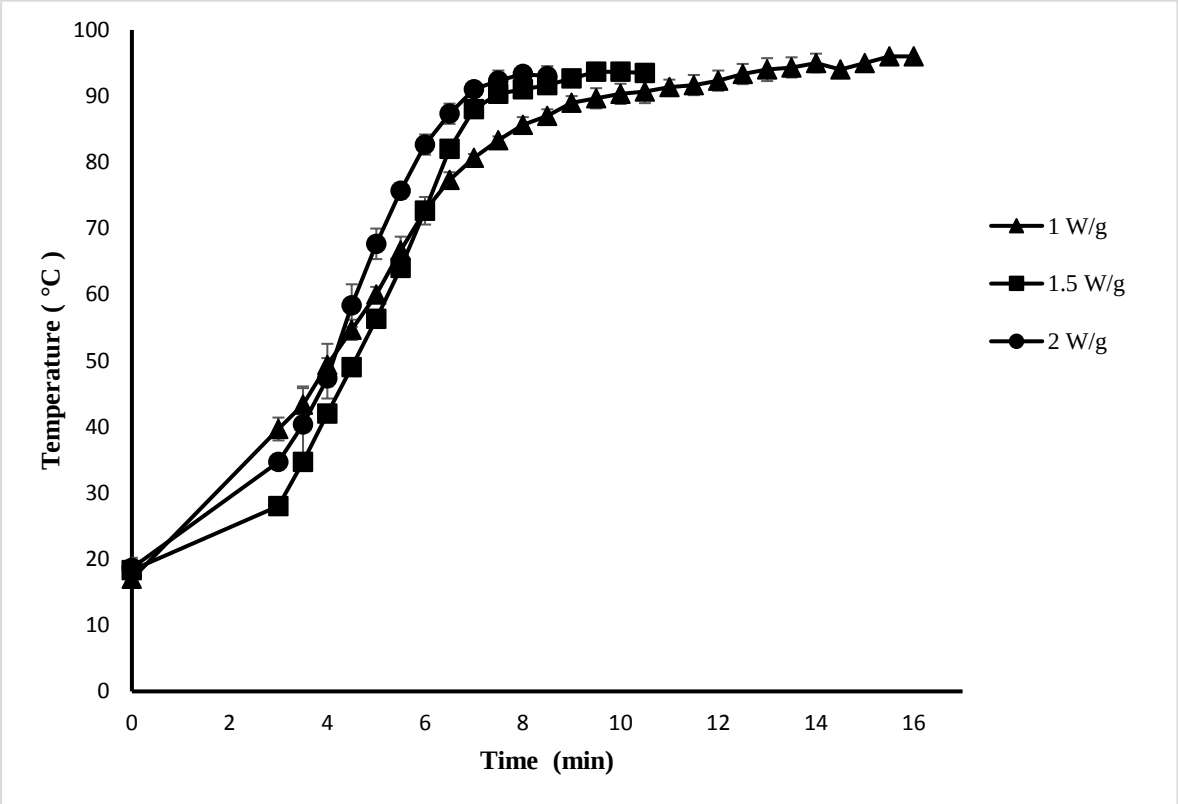


Figure 4.3. Temperature index during MHG extraction at different microwave power

4.8. Effect of microwaves on antioxidant activity of extracts

The highest phenol, flavonoid, anthocyanin content and radical scavenging capacity were found in MHG 600 W (2 W g^{-1}) extracts. From, Fig. 4.4. it is evident that no particular trait corresponding to the microwave power was observed as 1;1.5 W g^{-1} extracts had relatively lower values. The concentration of anthocyanin was higher than that of flavonols in all MHG extracts with an average of 10.38 mg D3GE and 3.55 mg QE per gram of extract.

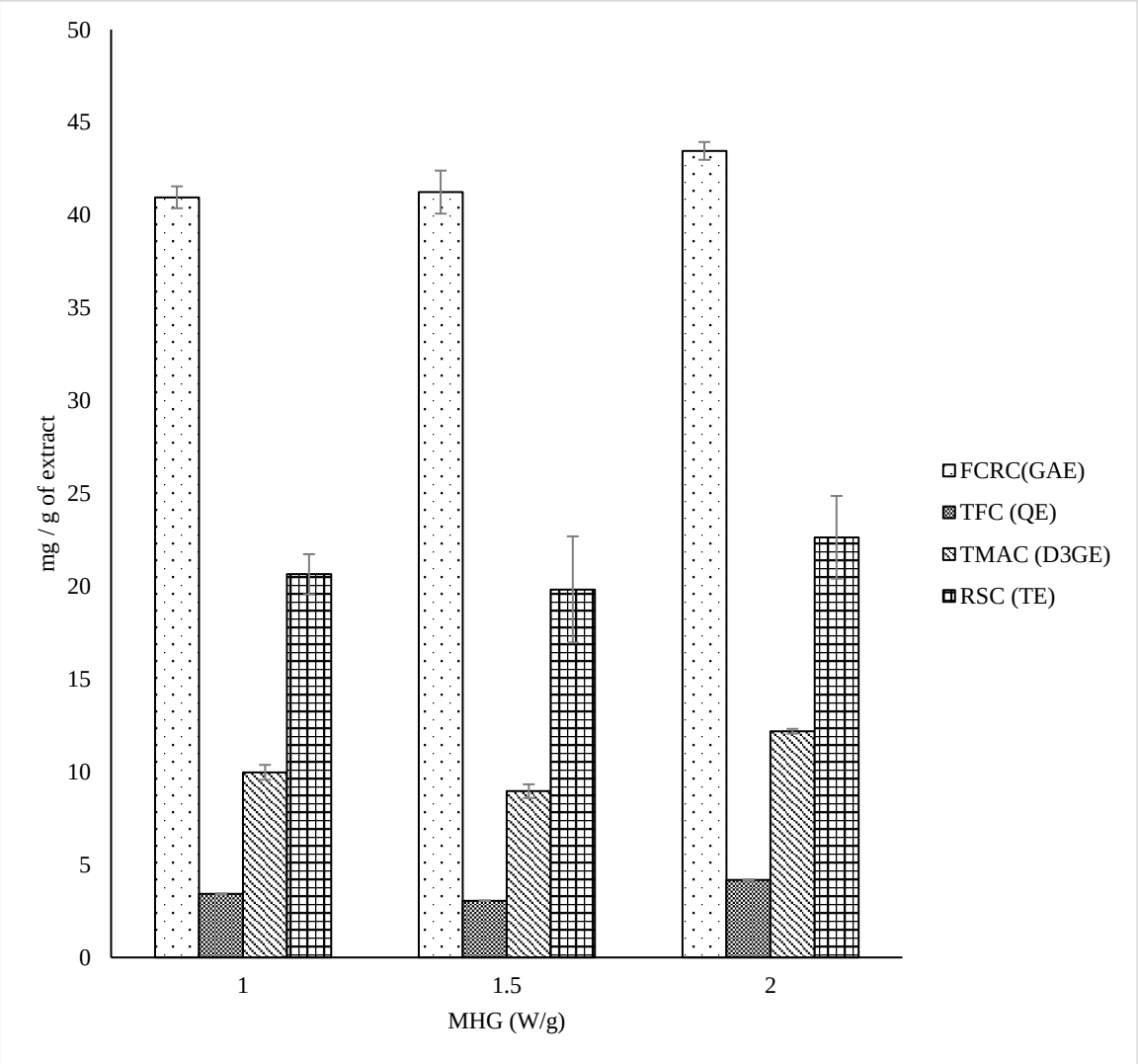


Figure 4.4. Effect of microwave power on antioxidant activity of extracts

4.9. Effect of Ultrasound Assisted Extraction (UAE)

MHG residue (2 W g^{-1}) was subjected to UAE at maximum amplitude wherein different ethanol/water concentrations were employed to extract trapped polyphenols. The yield of extracts decreased with a decrease in ethanol concentration in the solvent mixture. Ethanol: Water (100:0) had the highest yield of 7.45 ± 0.18 % whereas the lowest was found in Ethanol: water (0:100) with 5.11 ± 0.17 %. The general trend in lower yields can be attributed to decreasing ethanol concentration in the solvent system.

4.9.1. Effect of solvent on reducing and radical scavenging capacity of extracts and on flavonoid and anthocyanin content

Ethanol/water extracts at different concentrations had varying reducing and free radical scavenging capacity (Fig. 4.5.). Folin-Ciocalteu reagent reducing capacity of the extracts in decreasing order EtOH/H_2O (80:20) > EtOH/H_2O (60:40) > EtOH/H_2O (40:60) > EtOH/H_2O (100:0). Sanchez-Rangel et al. (2013) proposed that the Folin-Ciocalteau reducing power of any extract is not limited to the presence of polyphenols but also depends on the reducing sugar and ascorbic acid content of the fruit extracts.

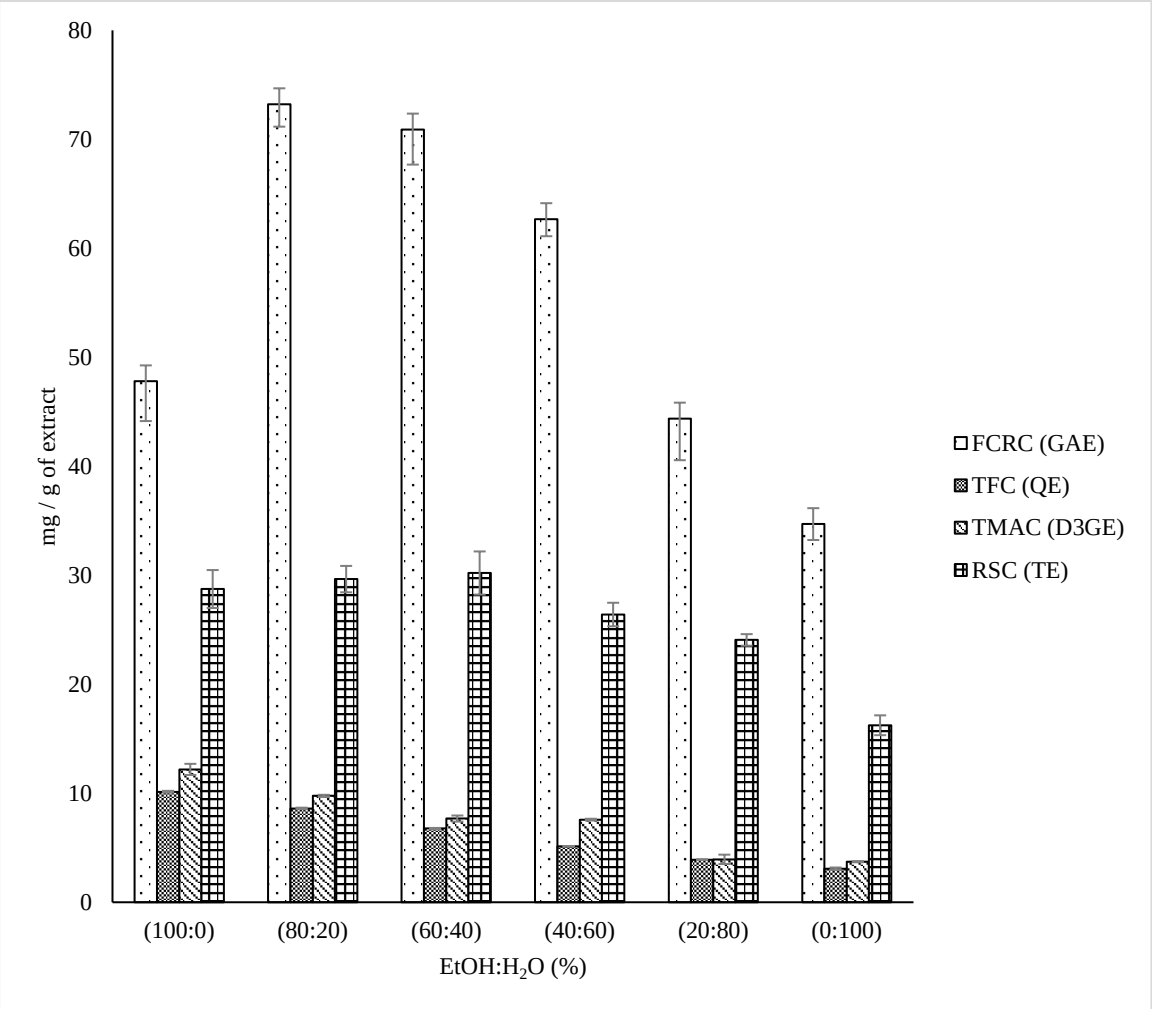


Figure 4.5. Effect of solvent on antioxidant properties of ultrasound assisted ethanol/water extraction

As both reducing sugars and ascorbic acid have the highest impact on hampering the accuracy of the FC reducing assay. The steep increase in the reducing power of ethanol: water (80:20) extract when compared to 100 % ethanol extract values could be attributed to the theory stated above. The highest radical scavenging capacity was exhibited by extracts in EtOH/H_2O (60:40) followed by EtOH/H_2O (80:20) with 30.21 ± 1.98 and 29.65 ± 1.21 mg Trolox Equivalent

per gram of extract. EtOH/H_2O (80:20), EtOH/H_2O (0:100) had the lower and lowest reducing and scavenging capacity respectively. Though low concentrations of ethanol in the solvent system enhances the polarity, the bioactivity of their respective extract tends to be significantly lower.

EtOH/H_2O (100:0) extracts had the highest flavonoid and anthocyanin content with 10.14 ± 0.08 mg QE and 12.19 ± 0.51 mg D3GE per gram of extract which was three-fold higher than that of EtOH/H_2O (0:100) extracts. Ethanol concentration in the solvent system had a direct correlation to the flavonoid and anthocyanin concentration (Fig. 4.5.).

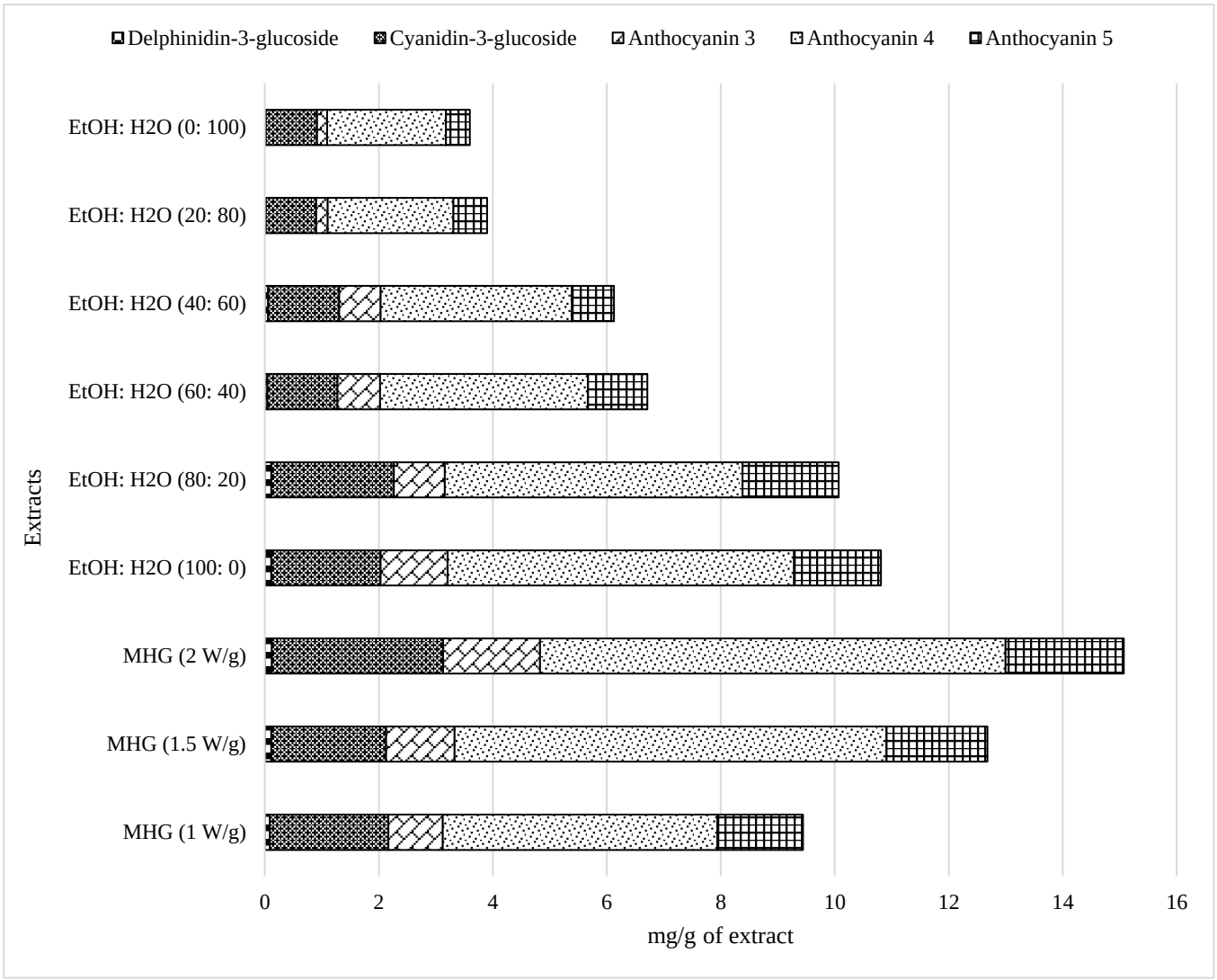


Figure 4.6. Anthocyanin quantitation by HPTLC

Total anthocyanin content was quantified by HPTLC and all MHG and UAE extracts were subjected to analysis. In total 5 anthocyanins were identified and individual concentration of delphinidin-3-glucoside and cyanidin-3-glucoside were quantified with respective standards. Other anthocyanins (anthocyanin 3, anthocyanin 4 and anthocyanin 5) were quantified as cyanidin-3-glucoside equivalents (Fig. 4.6.).

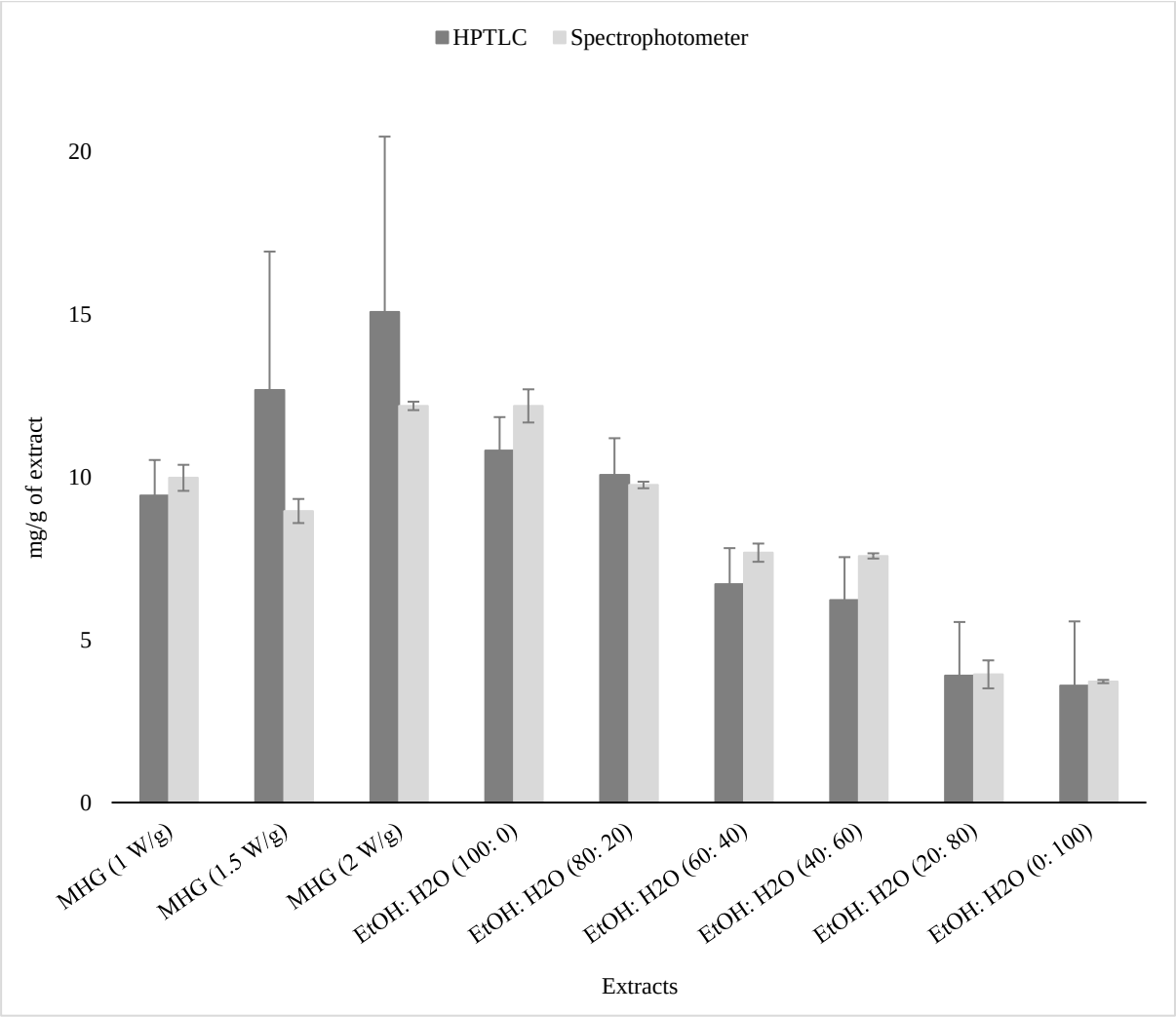


Figure 4.7. Anthocyanin concentration by UV-Vis Spectrophotometer vs. HPTLC

The total anthocyanin concentration in all extracts followed a trait similar to that of total monomeric anthocyanin content elucidated by spectrophotometric method. The concentration of individual anthocyanin followed a specific order Anthocyanin 4 > Cyanidin-3-glucoside > Anthocyanin 5 > Anthocyanin 3 > Delphinidin-3-glucoside. Surprisingly, distribution of individual anthocyanin in both MHG and UAE ethanol/water extracts were similar. It can be speculated that the low concentration of delphinidin-3-glucoside in both MHG and UAE extracts might be because the extraction was performed from pomace and not the whole berry.

The results (Fig. 4.7.) obtained by spectrophotometric and chromatographic method had good correlation. Relatively, the total anthocyanin concentration in UAE ethanol/water extracts determined by UV-Vis spectrophotometry was in good agreement with the data obtained by High-performance thin layer chromatography. This correlation validates the accuracy of total monomeric anthocyanin content quantitation by a pH differential method with delphinidin-3-glucoside as standard.

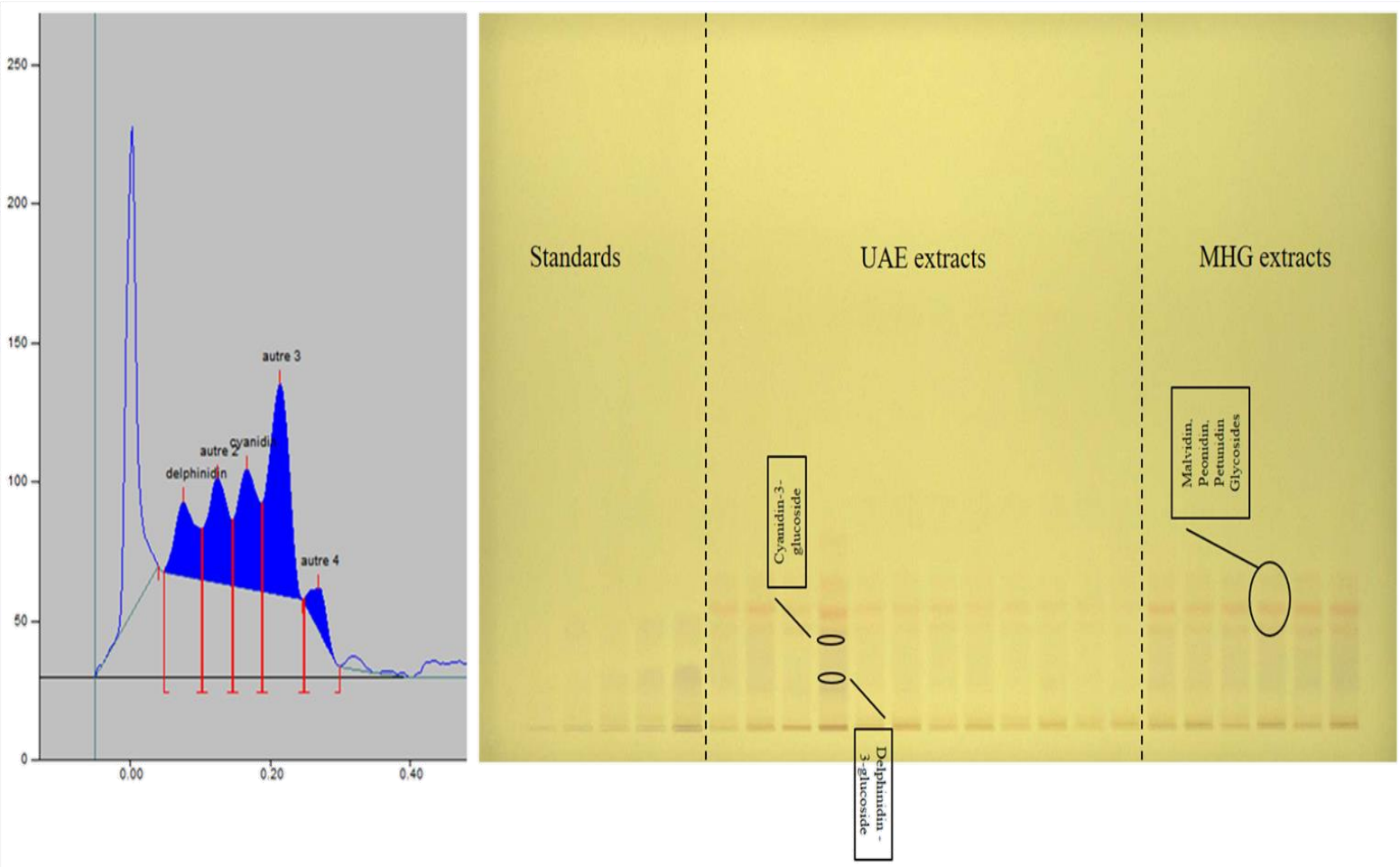


Figure 4.8. HPTLC plate with ultrasound and microwave extracts

Figure 4.8. is the screenshot of HPTLC plate which clearly indicates the presence of five anthocyanins, only two anthocyanin standards cyanidin-3-glucoside and delphinidin-3-glucoside were used for comparison, two concentration levels of each extract were placed on the plate and the quantitation were done in triplicate.

4.10. Computational prediction and correlation: COSMO-RS

The simulation predicts the solubility index of different solute in the solvent system. The solute (target polyphenols) were selected after exhaustive literature review and each solute represents a polyphenol class. The relative solubility log10(x_RS) values are given in Table. 3.2. and the best solvent for extraction of all solutes was found to be ethanol (100%), thus making it the reference solvent. The values highlighted in green indicates that these solvents possess higher solubility index (0 to -1) when compared to the other proportions of ethanol/water ratio in the solvent system. The values highlighted with orange and red stipulates that these particular values

have relatively medium (-1 to -2) and lower solubility index (> -2) than that of the reference solvent. For, anthocyanins (delphinidin-3-glucoside) solvent ethanol: water (100:0; 80:20) had better solubility index. Similarly, flavonols (hyperoside, epicatechin) and stilbenes (trans-resveratrol) had good solubility index in ethanol: water (100:0; 80:20 and 60:40) as the solvent system. Ironically, ascorbic acid being a water-soluble vitamin had good solubility index in almost all ethanol/water concentrations with the best at ethanol (100 %) and lowest in water (100%). Though only select colorimetry assays were performed to quantify the polyphenol, flavonoid and anthocyanin content, these results can be used for correlation and comparison with the theoretical prediction since each solute represents a particular class of polyphenol.

4.11. Lipophilic fraction analysis

The lipid fraction was extracted using bead milling, which is an innovative method to extract lipids from a limited quantity of raw material. Authors (Meulemiestre et al., 2016) have already demonstrated in their study that bead milling is more efficient than the conventional method for extraction of lipids. All samples (Bilberry, pomace, MHG and UAE residues) had similar fatty acid profiles. As shown in Fig. 4.9. lipid extracts from all samples comprised of 6 individual fatty acids primarily Palmitic acid (C16), Stearic acid (C18), Vaccenic acid (C18:1n7), Linoleic acid (C18:2n6), α-Linolenic acid (C18:3n3) and cis-11-eicosenoic acid (C20:1). Linoleic acid (MUFA) had the highest relative percentage around 40%, followed by α-Linolenic acid (PUFA) with 30% cumulatively taking the total of unsaturated fatty acids to 70%. These results were in good agreement with Bunea et al (2012), where the authors used a modified Folch method to extract lipids from two varieties of bilberries (Romania). Yet, the variation in fatty acid profile can be attributed to the geographical location, genotype, harvesting period, extraction condition etc., Unsaturated fatty acids, particularly linoleic and linolenic acids are widely known for their preventive action against cardiovascular disorders and diabetes (Angerer et al., 2000 & Riserus et al., 2009).

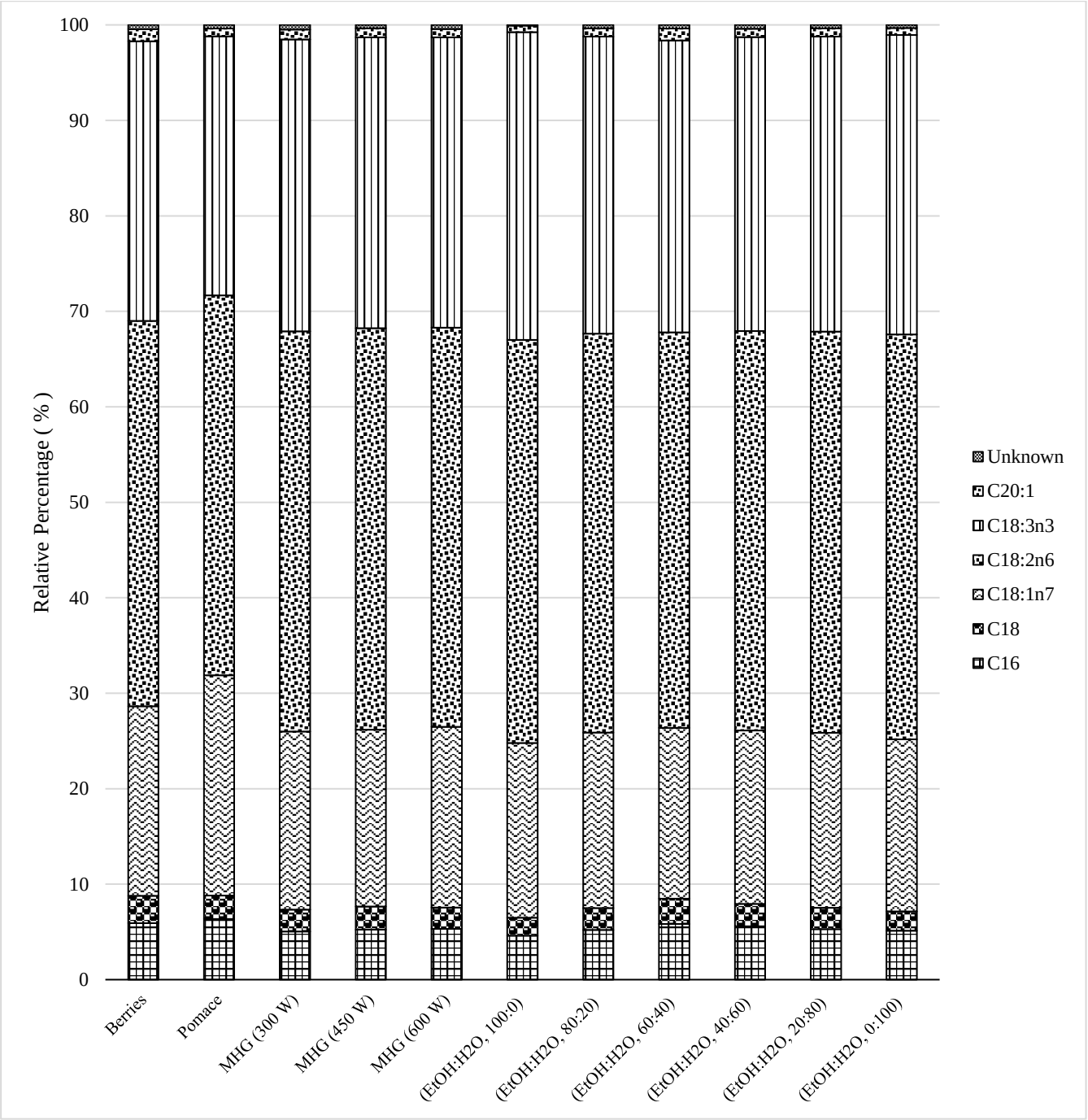


Figure 4.9. Fatty acid distribution in berry, pomace, and residues

The HPTLC analysis was carried to determine the lipid composition of all samples as stated above. Only neutral lipids monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG) and free fatty acids (FFA) were present in the samples. A standard mixture with all (MAG, DAG, TAG and FFA) individual lipid class was used as a reference for quantification. Triacylglycerols were the predominant lipid class (Fig. 4.10.) present in the lipophilic fraction of bilberries. The lipid class distribution was TAG (87%), DAG (8.7%),

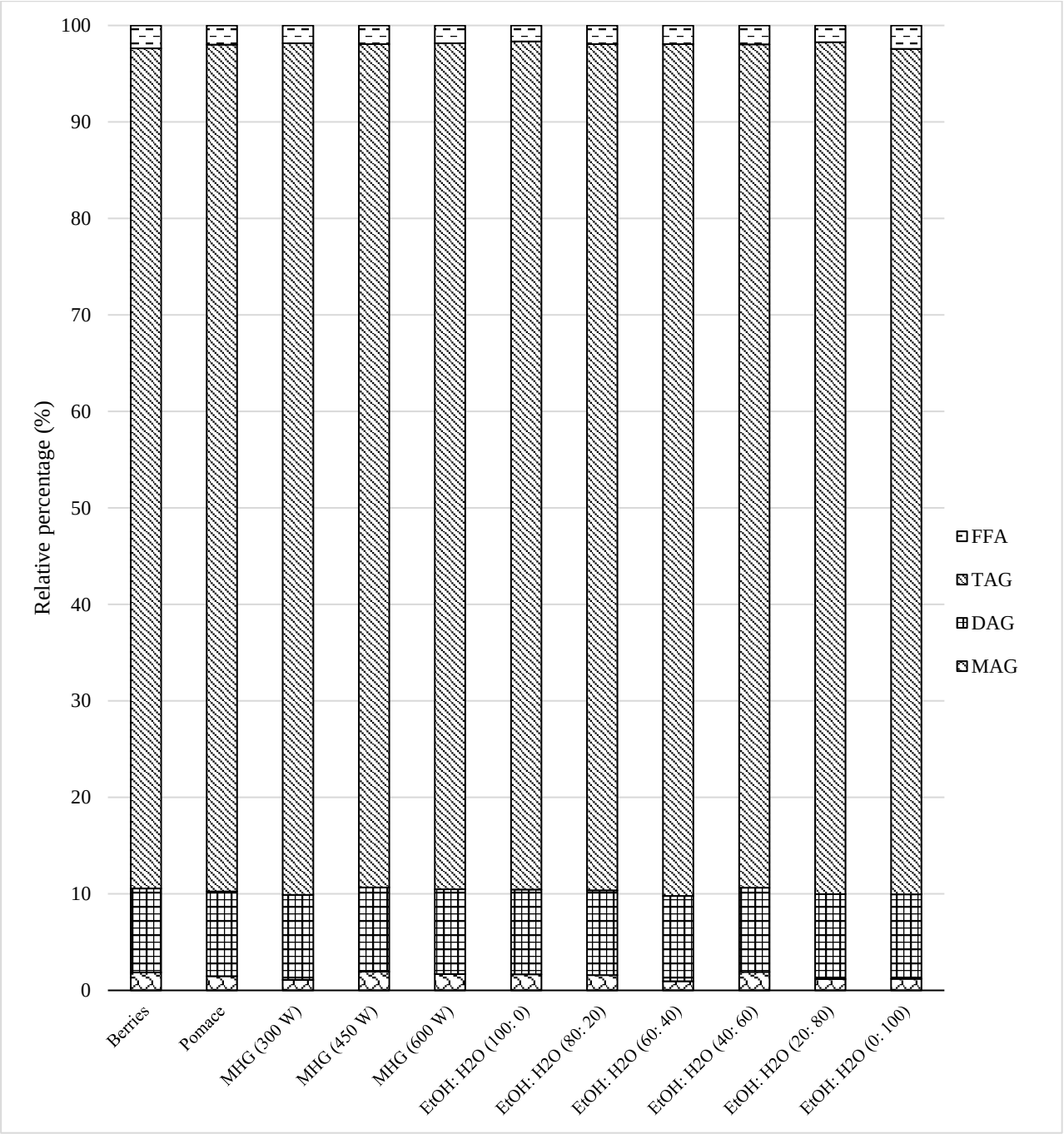


Figure 4.10. Lipid composition in berry, pomace, and residues

FFA (2%) and MAG (1%). So, it is safe to assume that neither microwave nor ultrasound had a degradative effect on the lipophilic fraction of bilberry.

5. CONCLUSION

Phytochemical investigation and antioxidant activity of *E. Henryi* and *E. Sessiliflorus*, and bilberry pomace were elucidated in this thesis.

1. Supercritical fluid extraction with CO_2 as a solvent was performed to remove the lipophilic compounds and further sequential extraction was carried with an accelerated solvent system.

2. The fatty acid profile of the lipophilic fraction from both fruits was similar, where unsaturated fatty acids (oleic and linoleic) accounted for 87 % of the lipid fraction.

3. The tocopherol composition in the lipid fraction was also studied and *E. Henryi* fruit had a relatively higher concentration (3.0573 mg T/g of extract) of tocopherol than *E. Sessiliflorus* (2.4859 mg T/g of extract) fruit.

4. Screening of the bioactivity rich fraction resulted in the identification of two phenolic acids (quinic acid derivative, chlorogenic acid), one flavonol glycoside (hyperoside), and one anthocyanin (cyanidin-3-sambubioside) which contributed to the radical scavenging capacity of the extracts.

5. Cumulative data on the antioxidant properties, the phytochemical composition of *E. Henryi* and *E. Sessiliflorus* fruits makes it a compelling source for pharmaceutical and nutraceutical applications.

6. the utilization of new innovative extraction techniques such as microwave hydro diffusion gravity and ultrasound assisted extraction with different concentrations of ethanol/water, as efficient means to extract free and trapped polyphenols from bilberry pomace thereby, establishing a new biorefining scheme for the valorization of an industrial by-product like bilberry pomace.

7. The highest concentration of polyphenols, flavonoids, monomeric anthocyanin content and radical scavenging capacity were observed in MHG 2 W/g (600 W) extracts. In UAE, the highest concentration of flavonoid and anthocyanin were found in 100 % ethanol extract.

8. The highest polyphenol content was found in ethanol/water (80:20) extract and highest radical scavenging capacity in ethanol/water (60:40) extract. Therefore, solvents ethanol/water (100:0; 80:20) could be considered efficient for the extraction of target polyphenols, as their respective extracts possessed the highest bioactivity in the studied in vitro assays.

9. A conductor-like screening model for real solvents (COSMO-RS) was used for prediction of the solubility index of solute representing each class of polyphenol in the solvent system employed. COSMO-RS prediction was in good correlation with experimental results and supported the argument that ethanol/water (100:0; 80:20) was the suitable solvent for extraction.

10. Fatty acid profile, and lipid composition distribution after each treatment in residues were identified and quantified. Linoleic acid and α-linolenic acid were the major fatty acids (70 %) and triacylglycerols were the major lipid class (87 %) found in all bilberry residues.

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HARISH KARTHIKEYAN RAVI

KAUNAS, 2017

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