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The extraction, identification, and encapsulation of phenolic compounds from *Prunus domestica* subsp. *Institia* towards their incorporation into a functional milk product

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Food technology

at Massey University, Manawatū,

New Zealand

Pu Xia

2022

Abstract

Damson plums are rich in bioactive compounds such as polyphenols, flavonoids, and anthocyanins, which are potent antioxidants with proven health-promoting properties. However, to date, there is no systematic publication/report about the type and concentration of various bioactive compounds in damson plums or using this type of plum as a food ingredient in the food industry. This study aimed to: 1) optimise the extraction of the bioactive compounds from *Prunus domestica* subsp. *Institia* (damson plums) grown in New Zealand using efficient extraction methods such as accelerated solvent extraction (ASE), ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), and their combined (E+UAE) extraction, in water or ethanol as the solvents; 2) analyse the total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and total antioxidant activities of the damson plums extract; 3) encapsulate the above-mentioned extract to protect the bioefficacy of its phenolic compounds; 4) compare the physical and chemical stabilities of the manufactured encapsulated ingredient in freeze-dried or liquid form during storage; and 5) assess the behaviour of the encapsulated plum extract after its incorporation into a functional milk product.

Fresh New Zealand damson plums were freeze-dried and ground into a powder and extracted with different methods explained above. The freeze-dried damson plum powder (FDDPP) was then encapsulated in liposomes made of soy lecithin granules

using high-shear homogenisation and/or microfluidisation. The encapsulation efficiency (EE) was assessed by the determination of various phenolic compounds using high-performance liquid chromatography (HPLC) before and after the application of Sephadex filtration to separate free phenolics and encapsulated phenolics. Finally, the encapsulants containing damson plum powder were incorporated into milk (whole/full-fat) as a functional beverage, and the effect on the physiochemical properties of the milk product was assessed.

The results showed that the plum samples extracted using the ASE with water as the solvent for 40 min showed the highest phenolic, anthocyanin, and antioxidant properties. The EAE method appeared to improve the extraction of anthocyanins, possibly due to the retardation of anthocyanin hydrolysis as the consequence of inhibition of polyphenol oxidase (PPO). However, the UAE was likely to suppress the extraction of TAC because of the degradation of anthocyanin glucosides, resulting from the action of PPO induced by ultrasound. The E+UAE method demonstrated the maximum extraction efficacy for the TFC as the extraction time was increased to 60 min. Different extraction methods obtained various extraction efficacies for the seven phenolic compounds (neochlorogenic acid, gallic acid, rosmarinic acid, catechin, epicatechin, rutin, and naringenin) found in the FDDPP. For example, neochlorogenic acid, the predominant phenolic compound of New Zealand damson plums was significantly ($p<0.05$) higher in the samples extracted using the ASE method (with ethanol as the solvent) for 40 min (1393.2±29.7) than the other samples. On the other

hand, rutin, one of the major flavonoids in damson plums, showed a significantly higher content (67.51±1.52) in the samples extracted using EAE for 40 min than the other extraction methods ($p<0.05$).

Neochlorogenic acid achieved the highest EE (98.86%) with the additional microfluidisation step in liquid liposomal encapsulants. In comparison, EE was around 81.40% in the liquid liposomes produced by microfluidisation; whereas, high-shear homogenisation alone produced liposomes with a much lower recovery rate (about 75.52%) for this important phenolic component of damson plum extract. Thus, the additional microfluidisation step resulted in the manufacture of liposomes with higher physical stability and with a smaller average particle size (73.2±1.5 nm), and the highest zeta potential (-35.39±0.97 mV) for the empty liposomes in liquid form. This confirms the stability of the liposomal system manufactured for the current experiment.

Milk was chosen as a suitable delivery vehicle (functional food) for the incorporation of the manufactured liposomes containing plum extract, due to its availability, convenience, and potential nutritional benefits. The physical and chemical stabilities of the phenolic compounds in the functional milk containing free and encapsulated plum extract were assessed using a pH meter, rheometer, and HPLC analysis. No significant differences were seen in the viscosity of different milk samples containing free extract, encapsulated extract, and freeze-dried encapsulants. The encapsulants achieved by various homogenisation techniques showed different recovery rates for rutin, catechin, epicatechin, neochlorogenic acid, and rosmarinic acid from the milk containing the

extract of New Zealand damson plum. However, further investigation is required to determine the effect of the extraction technique (i.e., ASE) and the liposomal encapsulation on the extraction and encapsulation efficiencies of New Zealand damson plums, respectively. Further study is also required to investigate the behaviour of encapsulated damson plum extract in different functional foods, the effect on their sensorial properties, and the bioaccessibility and bioavailability of its phenolic compounds after food consumption.

Acknowledgments

My master's thesis was completed with support from a great number of people who I would like to thank for their dedication.

First of all, I would like to express my sincere gratitude to my supervisor, Dr Ali Rashidinejad, for his advice and support during this project. He not only gave me the chance to advance my academic knowledge and abilities, but he also encouraged and supported me when I encountered academic challenges. Without his excellent guidance, I would not have been able to complete the research and write this thesis. The most impressive guide from him is that I have learned how to manage the whole project, and establish the connection with other academic researchers so that I would get more assistance from them.

I am very appreciative of Foot Steps Ltd. (Karamu, Hastings, New Zealand) who donated damson plums for my project. Indeed, this project would not have been completed without their sincere support.

I greatly appreciate the help I received from the staff at Massey University's School of Food and Advanced Technology. They gave me access to academic resources and lab resources along the way, enabling me to earn my Master's degree. I also want to express my gratitude to the employees and technicians at the Riddet Complex and the Riddet Institute laboratories (both at Massey University), including Ms Maggie Zou, Ms Jacinda Aplin, Mr Sihan Ma, Mr Rory Besaans, and Mr Peter Zhu who taught me how

to utilise the necessary lab apparatus. I also want to express my gratitude to the team at Massey University's Microscopy and Imaging Centre (Palmerston North, New Zealand). I want to thank Ms Yanyu He in particular for helping me with the microscopy imaging.

Finally, I want to thank my parents for their unwavering support, encouragement, and financial assistance during my study. They constantly reassured me and helped me release my pressure. Without their help, I would not be able to come to New Zealand and complete this Master's programme.

Table of content

Abstract I

Acknowledgments V

Table of content VII

List of Figures X

List of Tables XII

Abbreviations XIV

1 Introduction 1

2 Literature review 7

2.1 Background 7

2.2 General review of damson plums 7

2.2.1 Nutrients and nutritional properties 7

2.2.2 Health-promoting properties of damson plums 18

2.2.3 Safety concerns related to plums' consumption 29

2.3 Drying methods for plums 30

2.3.1 Convection drying 30

2.3.2 Vacuum drying 31

2.3.3 Sublimation/lyophilisation/freeze drying 32

2.4 Extraction methods for phenolic compounds from plums 33

2.4.1 Conventional extraction methods for phenolic compounds 33

2.4.2 Unconventional extraction methods for phenolic compounds 37

2.5 Identification and determination methods for phenolic compounds from plums 48

2.5.1 High-performance liquid chromatography (HPLC) 48

2.5.2 Mass spectrometry (MS) 50

2.5.3 Gas chromatography (GC) 51

2.5.4 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay 52

2.5.5 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay 53

2.5.6 Ferric reducing antioxidant powder (FRAP) assay 53

2.5.7 Oxygen radical absorbance capacity (ORAC) assay 54

2.5.8 Aluminium colourimetric assay with spectrophotometry 55
2.5.9 Folin-Ciocâlteu method with UV spectrophotometer 56
2.6 Encapsulation methods for the delivery of phenolic compounds from plums 56
2.6.1 Physical methods 57
2.6.2 Chemical methods.. 62
2.6.3 Physicochemical methods 65
2.6.4 Methods based on ionic interactions.. 67
2.6.5 Methods based on hydrophobic interactions 70
2.6.6 Other methods.. 74
2.7 Possible food applications for bioactive/phenolic compounds from damson plums 76
2.8 Review summary 79
3 Materials and Methods 80
3.1 Materials... 80
3.2 Methods... 81
3.2.1 Extraction of phenolics from FDDPP 81
3.2.2 Identification... 85
3.2.3 Liposomal encapsulation 90
3.2.4 Freeze drying 95
3.2.5 Incorporation of encapsulated FDDPP into the food model 95
3.2.6 HPLC analysis 96
3.2.7 Proximate analysis 97
3.2.8 Physical properties 97
3.2.9 Statistical analysis 99
4 Results and discussion..100
4.1 Extraction of phenolics from FDDPP 100
4.1.1 Total phenolic content (TPC) 100
4.1.2 Total flavonoid content (TFC)..106
4.1.3 Total anthocyanins (TAC) 111

4.1.4 Antioxidant capacity and radical-scavenging activity (DPPH and ABTS) 114

4.1.5 Quantification of phenolic compounds in FDDPP using HPLC analysis 120

4.2 Liposomal Encapsulation of FDDPP 127

4.2.1 Encapsulation efficiency (EE) and encapsulation yield (EY) determined with HPLC analysis 128

4.2.2 Loading capacity 133

4.2.3 The particle size of liposomes 134

4.2.4 Zeta potential of liposomes 137

4.2.5 Morphology of liposomes..142

4.3 Physical properties of liposomes before and after incorporation into milk .144

5 Conclusions, recommendations, and future directions....................................155

5.1 Conclusions 155

5.2 Recommendations and future directions..157

References..160

Appendices..176

List of Figures

Figure 2. 1. Pictorial representation of microwave-assisted extraction. Adapted from Alara et al. (2021). 40
Figure 2. 2. Pictorial representation of ultrasound-assisted extraction. Adapted from Alara et al. (2021). 42
Figure 2. 3. Schematic diagram of a spray drying apparatus. Adapted from Vehring (2008). 58
Figure 2. 4. Schematic representation of top-spray fluidized bed coating. Adapted from Dewettinck (1999). 59
Figure 2. 5. Schematic diagram of hot-melt extruder (adapted from Patil et al. (2016)). 61
Figure 2. 6. The principle of microencapsulation by interfacial polymerisation. A: The oligomer is soluble in the droplet; B: The oligomer is insoluble in the droplet (adapted from Munin and Edwards-Lévy (2011)). 65
Figure 2. 7. Encapsulation by emulsion/extraction (A) and emulsion/evaporation (B) methods (adapted from Munin and Edwards-Lévy (2011)). 67
Figure 2. 8. A schematic representation of a liposome. 71

Figure 3. 1. An overall overview of the experimental design. 83

Figure 4. 1. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total phenolic content (TPC) from the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-i) are significantly different (*p*<0.05) using the Tukey method. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction. 102
Figure 4. 2. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total flavonoid content (TFC) from the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-j) are significantly different (*p*<0.05) using the Tukey method. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction. 108
Figure 4. 3. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total anthocyanin content (TAC) from the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-j) are significantly different (p<0.05) using the Tukey method.

AAE: ascorbic acid equivalent. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction. 113

Figure 4. 4. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total antioxidant properties (measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay presented as ascorbic acid equivalence (AAE)) of the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-l) are significantly different ($p<0.05$). Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction..115

Figure 4. 5. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total antioxidant properties (measured using 2,2'-Azinobis-3-ethylbenzothiazoline-6-Sulfonic acid (ABTS) assay and presented as ascorbic acid equivalence (AAE)) of the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-k) are significantly different ($p<0.05$) using the Tukey method. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction. 118

Figure 4. 6. The chromatogram of standards (blue line) and the damson plum sample treated by ultrasound-assisted extraction for 20 min (green line). The peaks on standards were detected by diode-array detection (DAD) with a wavelength of 280 nm and paired with the sample and numbered as 1: gallic acid monohydrate, 2: neochlorogenic acid, 3: catechin hydrate, 4: epicatechin, 5: rutin hydrate, 6: rosmarinic acid, 7: quercetin, and 8: naringenin.........122

Figure 4. 7. Transmission electron micrographs of liposomes containing freeze-dried damson plum extract. A: Liposomes containing plum extract prepared using high-shear mixer, B: Liposomes containing plum extract prepared using high-shear homogeniser and microfluidiser, C: Control/empty liposomes prepared using high-shear mixer, and D: Control/empty liposomes prepared using high-shear mixer and microfluidiser under same magnification of 16500×. 143

Figure 4. 8. Transmission electron micrographs of empty nanoliposomes prepared with high-shear homogeniser viewed under different magnifications. A: 16,500×, B: 82,500×, C: 165,000×, and D: 285,000×. 144

Figure 4. 9. The viscosity of the control milk (Sample A) and the milks containing free damson plum extract (Sample B), non-freeze-dried (Sample C), and

freeze-dried (Sample D) liposomes loaded with the damson plum extract. 150

List of Tables

Table 2. 1. Nutritional composition of plums (Hussain et al., 2021). 8
Table 2. 2. Mean chemical characteristics of 11 varieties of damson plum (Nergiz & Yıldız, 1997)**.** 9
Table 2. 3. Amino acid composition of plums (Hussain et al., 2021). 11
Table 2. 4. Chemical structure of selected phenolic compounds in plums (Michaelis, 2020). 14
Table 2. 5. Some examples of a variety of liposome techniques that have been employed for the encapsulation of polyphenols (adapted from Munin and Edwards-Lévy (2011)). 72

Table 4. 1. Phenolic acids identified and quantified in the freeze-dried damson plum powder treated using different extraction methods/conditions.........124
Table 4. 2. Flavonoids identified and quantified in the freeze-dried damson plum powder treated using different extraction methods/conditions.................127
Table 4. 3. The encapsulation efficiency of the liposomes manufactured with different methods for the encapsulation of different phenolic compounds from the freeze-dried damson plum power...129
Table 4. 4. The encapsulation yield of the liposomes manufactured with different methods for encapsulation of the extract from damson plums. 129
Table 4. 5. The loading capacity of the liposomes manufactured with different methods for the encapsulation of different phenolic compounds from the freeze-dried damson plum power..133
Table 4. 6. Particle sizes of empty liposomes and liposomes containing freeze-dried damson plum powder extract during 28 days of storage at 4°C and in the dark. 135
Table 4. 7. Zeta potential of empty liposomes and liposomes containing freeze-dried damson plum extract during the storage for 28 days at 4°C and in the dark...139
Table 4. 8. The appearance of empty liposomes and liposomes containing freeze-dried damson plum extract during 28 days of storage at 4°C and in the dark. 141
Table 4. 9. The pH value, average particle size, and zeta potential of the control milk and the milk containing liposomes loaded with the damson plum extract. 145
Table 4. 10. Proximate analysis of control milk and the milk containing liposomes loaded with the damson plum extract. 149
Table 4. 11. The contents of seven phenolic compounds in control milk and the

milk fortified with different liposomes containing plum extract after the storage at 4°C for 28 days. 152

Table 4. 12. The recovery rates of five phenolic compounds in control milk and the milk fortified with different liposomes containing plum extracts after they were stored at 4°C for 28 days..152

Abbreviations

ASE	Accelerated solvent extraction
ABTS	2,2'-Azinobis-3-ethylbenzothiazoline-6-Sulfonic acid assay
AAE	Ascorbic acid equivalent
AD	Alzheimer's Disease
CVDs	Cardiovascular diseases
cyn-3-glu	Cyanidin 3-glucoside equivalent
DPPH	2,2-diphenyl-1-picryl-hydrazyl assay
DW	Dry weight
EL	Empty liposome
EAE	Enzyme-assisted extraction
E+UAE	Enzyme combined ultrasound-assisted extraction
EY	Encapsulation yield
EE	Encapsulation efficiency
FW	Fresh weight
FRAP	Ferric reducing antioxidant power
FDDPP	Freeze-dried damson plum powder
FDDPPE	Freeze-dried damson plum powder extract

GAE	Gallic acid equivalent
GC-MS	Gas chromatography-Mass spectrometry
HPLC	High-performance liquid chromatography
HSH	High-shear homogenisation
LC-MS	Liquid chromatography-Mass spectrometry
LC	Loading capacity
LL	Loaded liposome
MS	Mass spectrometry
MF	Microfluidisation
ORAC	Oxygen radical absorbance capacity
RE	Rutin equivalent
TEM	Transmission electron microscopy
TE	Trolox equivalent
TAC	Total anthocyanin content
TFC	Total flavonoid content
TPC	Total phenolic content
UAE	Ultrasound-assisted extraction

1 Introduction

The plum is a drupe fruit belonging to the *Prunus* subgenus (Family *Rosaceae*). Although there exist between 19 and 40 different plum species worldwide, based on a report from Topp et al. (2012), only two of these are commercially used. These include European plum (*Prunus domestica*) and Japanese plum (*Prunus salicina*), the nutritional makeup of which is thought to be comparable (Igwe & Charlton, 2016).

While plums contain various nutrients and phytochemical compounds, Stadlmayr et al. (2011) claimed that flavonoid content was an effective method for their categorisation, when it came to distinguishing the natural foods based on their different nutritional compositions. Since the 1990s, there has been substantial interest in plum-based research because of the high quantities of phenolic compounds (particularly flavonoids and the anthocyanin subclass) found in plum fruits (Walle et al., 2003). The nutritional values of plum fruit are linked to some health-promoting properties, including increased bone health, memory and cognition, antioxidant and anti-inflammatory actions, and constipation relief. Pawlowski et al. (2014) suggested that the plum's antioxidant capacity, which originated from its high phenolic content, was credited for these health-promoting characteristics.

Damson plums are a type of plum discovered approximately 2,000 years ago, according to Igwe and Charlton (2016). This species of plums has different varieties such as Reine Claude, and Stanley, with the colour ranging from purple to black. The antioxidant

capacity of the damson plum reported by Kim et al. (2003) was relatively high compared to the other plum cultivars such as Stanley, Yugoslavian Elite T101, and Long John. Additionally, damson plums also showed a higher total phenolic content (TPC) of about 375 per 100 g of fresh fruit as mg gallic acid equivalent (GAE), compared to about 174 in Stanley, 332 in Beltsville, 319 in Cacak Bes, and 217 in Yugoslavian Elite T101. However, there is currently only a few publications about the specific composition of phenolic compounds among various plum cultivars, with no systematic report being available for damson plums.

The extraction technique could be one of the significant parameters for the optimisation of the extraction efficacies of phenolic compounds from damson plums. According to the literature review, the extraction methods that could be possibly applied for the extraction of phenolic compounds from plums include ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), a combination of both UAE and EAE methods, and accelerated solvent extraction (ASE). Olawuyi et al. (2021) recently used the E+UAE approach to extract polyphenols from plum juice. They optimised the extraction timing while maintaining a constant enzyme concentration and ultrasound frequency. These two methods were also summarised as two of the most eco-friendly and phenolic-friendly methods, because of using water as the solvent, while the extraction condition did not require high-pressure or high-temperature that could damage or degrade the phenolic compounds. ASE was one of the novel extraction techniques previously reported by Yang et al. (2017), which could achieve a high

extraction efficiency for phenolic compounds from Mulberry. Therefore, this study could demonstrate the extraction efficiencies of phenolic compounds from damson plums grown in New Zealand using ASE with various extraction solvents (water or ethanol). The extraction efficacies of phenolic compounds and antioxidants can also be compared among these extraction methods and the method that obtains the highest phenolic compounds from damson plums can be optimised.

Phenolic compounds like those found in plums are generally unstable under environmental conditions (e.g., during the processing and storage of food products) (Mohd Zainol et al., 2009). As a delivery strategy, encapsulation technology can preserve such valuable substances as core materials by trapping them in the coating materials (also called encapsulant or shell) (Madene et al., 2006). Many encapsulation methods have been already used for the entrapment of phenolic compounds or plant extracts; e.g., spray drying, fluidised bed coating, extrusion, supercritical fluidic, and liposomes. The detailed mechanisms of these systems are addressed in the following literature review (Section 2.6). Different encapsulation methods are suitable for various core materials. In terms of phenolic extracts from damson plums, liposomes are one of the most suitable methods due to their flexibility, amphiphilic properties, biodegradability, biocompatibility, and non-toxicity. Especially, the conditions for the manufacture of liposomes are mild – e.g., room temperature or lower atmosphere pressure using a piece of simple processing equipment such as a high-shear homogeniser/mixer. The coating materials that are used for liposome manufacture are

also flexible and from natural sources such as soybeans (Rashidinejad et al., 2016). Nonetheless, one of the most important aspects to consider when using liposomes for the encapsulation of phenolic compounds is their stability. Controlling internal elements like phospholipid composition and concentration, and external factors such as temperature, pH, and ionic strength, would maximise the potential of this system (Taylor et al., 2005). Zeta potential is an accurate parameter to assess the ionic strength of liposomes, as liposomes will be more stable if their zeta potential values are higher; i.e., the aqueous phase with a higher zeta potential value enhances the repulsion between liposome particles so it increases the stability of the colloidal dispersion (Mady et al., 2009; Rashidinejad et al., 2014). Ionic strength and pH may also have a major impact on liposome stability with only minor adjustments. For example, a high pH value could significantly increase liposome stability, but a high ionic strength in the aqueous phase would noticeably decrease it. Rodriguez-Nogales and Delgadillo (2005) suggested that high temperature (80°C) could disrupt or destabilise the liposomal structure.

Ahn-Jarvis et al. (2019) suggested that flavonoid consumption between 50 and 400 mg per day could effectively achieve their corresponding health-promoting benefits; however, this seemed like a high concentration that was challenging to be added to regular diets. Therefore, the creation of functional foods or beverages is important to provide the body with the necessary dosage of these bioactive materials most practically and regularly (i.e., functional food innovation). In this regard, milk can be one of the

suitable candidates for the delivery of bioactive compounds from New Zealand damson plums, as it is one of the most popular and acceptable foods consumed globally. Therefore, the aims of the current study were to: 1) optimise the extraction of the bioactive compounds from *Prunus domestica* subsp. *Institia* (damson plums) grown in New Zealand using accelerated solvent extraction (ASE), ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), and combined (E+UAE) extraction, in water or ethanol as the solvents; 2) analyse the total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and total antioxidant activities of the damson plums extract; 3) encapsulate the above-mentioned extract to protect the bioefficacy of its phenolic compounds; 4) compare the physical and chemical stabilities of the manufactured encapsulated ingredient in freeze-dried or liquid form during storage; and, 5) assess the behaviour of the encapsulated plum extract after its incorporation into a functional milk product.

Based on these research aims, it was hypothesised that: 1) the novel ASE extraction method could achieve a high extraction efficacy for phenolic compounds in New Zealand damson plums; 2) a combination of ultrasound and enzyme-assisted extractions may perform better than any of those two extraction methods alone; 3) liposomal encapsulation technology would be efficient in protecting and delivery of damson plum extract; 4) an additional microfluidisation step might enhance the physicochemical stability of the manufactured liposomes containing damson plum extract; and, 5) individual phenolic compounds could be successfully

recovered/quantified after the incorporation of liposomes in a functional milk product.

2 Literature review

2.1 Background

This systematic review aimed to introduce the general nutrition value and bioactive properties of damson plums, demonstrate the beneficial health effects of these bioactive compounds, and present the potential methods for the extraction, identification, and encapsulation of phenolic compounds from New Zealand damson plums for the current study, and investigate possible functional foods for the application of such a plum extract. In this review, where no published material/evidence for the nutrient composition of damson plums was found, the published information for plums, in general, is provided.

2.2 General review of damson plums

2.2.1 Nutrients and nutritional properties

Plums, in general, are a high-efficient source of nutrients including vitamins, minerals, and phytochemicals. The mineral composition in plums was reported to increase as fruits ripen (Birwal et al., 2017). Additionally, there are also abundant bioactive compounds in plums such as phenolic compounds. Caffeic acid, neochlorogenic acid, chlorogenic acid, and crypto-chlorogenic acid are the main phenolic compounds that have been identified in plum samples (Hussain et al., 2021). Table 2.1 presents the nutrient composition of plums summarised from the review carried out by Hussain et al. (2021).

Table 2. 1. Nutritional composition of plums (Hussain et al., 2021).

Nutrients	Value per 100 g of fresh plums
Water	87.23 g
Carbohydrate	11.42 g
Energy	192 kcal
Fats	0.28 g
Protein	0.70 g
Dietary fibre	2.2 g
Potassium	157 mg
Phosphorus	16 mg
Magnesium	7 mg
Calcium	6 mg
Iron	0.17 mg
Vitamin c	9.5 mg
Vitamin b-3	0.417 mg
Vitamin a	0.017 mg

In the case of damson plums, one study by Nergiz and Yıldız (1997) investigated the chemical composition of some cultivars of this type of plum (*Prunus domestica*) obtained from the Aegean district of Turkey (Table 2.2). The water content of damson plum varieties was lower than that of the general plum species. However, the contents of the protein, carbohydrate, and soluble solids of the damson plum were slightly higher than that of the general plum species (Nergiz et al., 1997).

Table 2. 2. Mean chemical characteristics of 11 varieties of damson plum (Nergiz & Yıldız, 1997).

Chemical characteristics	g/kg damson plums
Moisture	837.4
Soluble solids	155.5
Titratable acidity	15.1
Protein	7.5
Ascorbic acid	157.9
Total sugar	96.5
Reducing sugar	51.9
Sucrose	42.4

2.2.1.1 *Carbohydrates and soluble sugars*

Damson plums supply a relatively great amount of carbohydrates, and it has been suggested that on average, 15 g of carbohydrates could be found in 100 g of these plums, which contributes to 30-60 kcal/100 g of the available source of energy (Hussain et al., 2021). Monosaccharides such as glucose and fructose and disaccharides (sucrose) make up a large proportion of carbohydrates in plums, and the latter also contributes to the sweetness of plums. The carbohydrate content of plums also depends on the plum cultivars and maturity. Sorbitol is a sugar alcohol that is present in most plums (2 g/100 g) and is responsible for their known laxative effects (Hussain et al., 2021).

2.2.1.2 *Fats*

Averagely, each plum sample (41.6 g) contains 0.3 g of healthy fats without cholesterol, one-third of them being polyunsaturated fats, and two-thirds monosaturated fats

(Hussain et al., 2021). The polyunsaturated fatty acids (PUFAs) in these fruits are linolenic, linoleic, oleic, and butyric, while the latter two respectively are the major monounsaturated and saturated fatty acids (Hussain et al., 2021).

2.2.1.3 Dietary fibre

We are all well aware of the beneficial effects of dietary fibre. A high level of fibre intake was suggested by Anderson et al. (2009) to present health-protective effects and disease-reversal benefits. People who have minimal fibre intake are at higher risk for getting a stroke, diabetes, obesity, hypertension, coronary heart disease, and certain gastrointestinal diseases than people who consume generously 14 g/1000 kcal or higher amounts of dietary fibre. Currently, there is no published evidence specifically provided for damson plums in terms of their dietary fibre content. Hussain et al. (2021) suggested that a total of 2.8 g of dietary fibre could be averagely found in 100 g of plums. Plums contain 1% of pectin, which is also a source of dietary fibre that could be found in most stone fruits.

2.2.1.4 Vitamins and minerals

Vitamin C is the main vitamin present in plums with an approximate content of 9.5 mg/100 g. Thiamine (vitamin B1) and riboflavin (vitamin B2), which are found in plums, are a source of β-complex vitamins that help in the breakdown of nutrients such as fats, carbohydrates, and proteins in plums (Hussain et al., 2021).

2.2.1.5 Proteins

Approximately, every 100 g of plums contains 0.7 g of proteins. 17 amino acids have been identified from plums, as presented in Table 2.3 (Hussain et al., 2021).

Table 2. 3. Amino acid composition of plums (Hussain et al., 2021).

Amino acid	mg/100g plums
Lysine	16
Histidine	9
Arginine	9
Aspartic acid	352
Threonine	10
Serine	23
Glutamic acid	35
Proline	27
Glycine	9
Alanine	28
Cystine	2
Methionine	8
Isoleucine	14
Leucine	15
Tyrosine	8
Phenylalanine	14
Valine	16

2.2.1.6 Organic acids

García-Mariño et al. (2008) demonstrated four major organic acids in seed, epicarp, and mesocarp of damson plums. These include quinic, malic, citric, and fumaric acids. They also illustrated the content of these four organic acids at three development stages for the seed (i.e., late green, maturation, and ripening) and two stages (i.e., maturation and ripening) for epicarp and mesocarp. The level of malic acid (about 7-17 mg/g fresh weight) peaked at the beginning of the maturation, while the content of quinic acid (about 5-7 mg/g) and citric acid (about 100-200 μg/g) peaked in the middle of maturation in the epicarp and mesocarp of damson plums. However, the fumaric acid (about 0.25-2.5 μg/g) was accumulated from the middle of the maturation to the late-ripening in the epicarp and mesocarp (García-Mariño et al., 2008).

*2.2.1.7 **Phenolic compounds***

The chemical structure of some of the selected phenolic compounds in plums (Michaelis, 2020) is presented in table 2.4.

1) Phenolic acids

Phenolic acids are divided into two categories as benzoic acid derivates and cinnamic acid derivates (Michaelis, 2020). Gallic acid (Table 2.4) can be commonly found in damson plums as a benzoic acid derivate (Michaelis, 2020). These derivates, also known as hydroxybenzoate, have a C6-C1 structure and are made up of a benzoic ring and an attached carboxyl group (Crozier, 2003). Gallic acid and its derivatives play a significant role in the development of galls in plants as a result of parasitic insect infestations and are also the key components of ellagic acid, as well as the polymers such as pentagalloylglucose and gallotannins (Crozier, 2003).

Cinnamic acid derivatives, also known as hydroxycinnamates, have a C6-C3 structure and are derived from cinnamic acid with various hydroxylations and methylations of the aromatic ring (Michaelis, 2020). The selected examples of cinnamic acid derivatives found in damson plums are trans-caffeic acid and neochlorogenic acid (Table 2.4) (Michaelis, 2020). Vicente et al. (2009) suggested that caffeic acid was the most abundant phenolic acid in fruits, followed by coumaric acid. Recently, it was also suggested by Michaelis (2020) that the esterification of hydroxycinnamic acids with other organic compounds is very common (e.g., esterification of caffeic acid with quinic acid).

Table 2. 4. Chemical structure of selected phenolic compounds in plums (Michaelis, 2020).

a. Gallic acid		h. Coumestan	
b. Trans-caffeic acid		i. (+)-catechin	
c. Neochlorogenic acid		j. Cyanidin 3-O-glucoside	
d. Rutin		k. Aureusidin	
e. Apigenin		l. Trans-resveratrol	
f. Naringenin		m. Mecoisolariciresinol diglucoside	
g. Naringin			

Note: Phenolic acids; benzoic acid derivative: a. cinnamic acid derivates: b and c. Flavonoids: flavonol: d, flavone: e, flavanones: f and g, isoflavone: h, anthocyanin: I, flavan-3-ol: j, aurone: k, stilbene: l, lignan: m. Source: PubChem (https://pubchem.ncbi.nlm.nih.gov). software MarvinSketch© 18.28, ChemAxon Ltd (http://ww.chemaxon.com).

2) Flavonoids

Flavonoids have several subgroups including flavones, chalcones, flavonols, flavan-3-ol, isoflavones, and anthocyanins. Flavylium, a group of flavonoids derived from anthocyanins, has been used as a food additive to utilise its colour and antioxidant properties (Pina et al., 2012). Flavonols are mostly present as O-glucosides with a hydroxyl group at the 3-position (Crozier, 2003). Since flavonols are predominantly synthetised in reaction to light, they are mostly found in the epidermis of fruits (Vicente et al., 2009). Macheix et al. (1990) suggested that quercetin, kaempferol, and their 3-O-monoglycosides (e.g., rutin) are mostly found in fruits such as apples, cherry, and cranberry.

Flavan-3-ols or flavanols are structurally similar to flavonols, but they do not have the double bond between Positions 2 and 3 in the carbon ring and lack a carbonyl group in Position C4 (Vicente et al., 2009). Catechin, epicatechin, gallocatechin, and epigallocatechin are the most typical flavanols in fruits (Michaelis, 2020). Flavanols are common in some species of the *Prunus* genus (Macheix et al., 1990). Lim (2012) reported that (+)-catechin was abundantly presented in damson plums (57-81% of all terminal units of procyanidins existed in the skin and 54-77% in the flesh).

Isoflavones have also been previously reported (Kuhnle et al., 2009) to be found at low concentrations in *Prunus* species such as European plum, peach, cherry, and apricot. They differ from most other flavonoids in that the B-ring is attached in the C3 position rather than the C2 position. Hydroxylations, methylations, and prenylations are the

common modifications of this flavonoid category, which result in the formation of other isoflavonoids such as coumestans (Figure 2.1). The function of these flavonoids was previously demonstrated by Crozier (2003) as phytoestrogens, due to the likelihood of blockage of ovulation, as they mimic the effects of the steroidal hormone estradiol. Such phytoestrogens are claimed by Crozier (2003) to lower the incidence of breast and prostate cancers.

The flavylium cation is the fundamental structure of anthocyanidins (Pervaiz et al., 2017). Pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin are the most common anthocyanidins. Anthocyanins, known to be natural antioxidants responsible for blue, purple, and red colours in plant tissues (Michaelis, 2020) – compounds such as cyanidin 3-O-glucoside are substances found in nature in conjugation with some sugars.

Proanthocyanidins (PACs) are oligomers composed of flavan-3-ol monomers that can be found in a range of plants including fruits, seeds, flowers, nuts, and bark (Sheng et al., 2020). Based on the findings reported by Sheng et al. (2020), the PACs extracts from grape seed could significantly ameliorate dextran sulfate sodium-induced colitis including inflammatory factor modulation. Procyanidin B1, B2, and A-type dimers are the PACs that were identified in plums (Nunes et al., 2008), but their presence in damson plums has not been confirmed yet.

There is currently no evidence that damson plums contain some other flavonoids such as flavones and flavanones, because not enough research has been done in this area.

Flavones have a similar ring structure as flavonols, but they mostly have modifications such as hydroxylation, methylation, O- and C-alkylation, or O- and C- glycosylation in positions 7, 5, and 4’ instead of a hydroxyl group at the 3-position (Michaelis, 2020). However, fruits except for citrous fruits, do not contain flavones (Crozier, 2003; Macheix et al., 1990; Ooghe et al., 1994).

Flavanones such as naringin and flavanone glycosides are typically presented in citrus fruits (Michaelis, 2020). Flavanones are very reactive and are known to undergo glycosylation, hydroxylation, and omethylation alterations (Michaelis, 2020). However, there is no current evidence that they could be found in damson plums.

3) Other phenolics

There are some other polyphenolics, which belong to the class of neither phenolic acids nor flavonoids, including lignin, coumarins, diarylheptanoids, hydrolysable tannins, and proanthocyanidins (Table 2.4) (Vicente et al., 2009). For example, lignin was reported by Famiani et al. (2012) to be abundantly found in plum endocarp, but the specific content was not reported. Additionally, hydrolysable tannins are generated by polymerisation of gallic acid and have been reported to be found in black plums by Chaudhary et al. (2016), which achieved considerably beneficial properties of this fruit for diabetics. Proanthocyanidins (e.g., catechin and epicatechin), were the major phenolic compounds in damson plums in the study carried out by Selvaraj (2014) in

concentrations ranging from 0.03 to 38.03 µg/mL. Epicatechin was found to be in a concentration between 0.02 and 10.62 µg/mL in the sample extracts of various damson plum cultivars such as 'Topend Plus', 'Angelina Burdette', and 'Goldzwetsche'.

2.2.2 Health-promoting properties of damson plums

2.2.2.1 Antioxidant and anti-inflammatory properties

It was suggested by Lea et al. (2008) that the high concentration of phenolic compounds such as anthocyanin from damson plums could be mostly responsible for their antioxidant property. Ripe plum fruit and its products have been studied for this health effect. Generally speaking, natural components are now extensively studied for their antioxidant activity correlated with total phenolic content, because of the immense potential of phenolic compounds in lowering the incidence rate of various diseases. Milala et al. (2013) reported that the oxygen radical scavenging capacities of plum cultivars like Promis, Cacanska Najbolja, and Dąbrowicka were around 6259 µmol TE (Trolox equivalent)/100 g. Additionally, Ko et al. (2005) stated that lutein, zeaxanthin, and cryptoxanthin in plums acted as scavenging agents by quenching reactive oxygen species (ROS) thus inhibiting the prevalence of chronic diseases.

Vitamin C in plums has antioxidative properties, which not only maintain a strong immune system for the human body but also protect the body against some symptoms such as cancer, arthritis, and asthma. Furthermore, based on a recent report by Patterson et al. (2021), high intravenous vitamin C therapy has effectiveness ranging from

moderate to high in preventing and inhibiting the duration of viral infection from COVID-19. It was also suggested that the consumption of plums helped to prevent the growth of hepatic cancer due to the antioxidant and anti-inflammatory properties that could prevent lipid peroxidation and reduce serum aminotransferase (Hussain et al., 2021).

A recent study on phenolic profiles, bio-accessibility, and antioxidant activity of *Prunus Salicina* Lindl used an *in vitro* gastrointestinal digestion model (Yu et al., 2021). These researchers reported that a total of 13 free and 11 bound phenolic compounds were identified and quantified by ultra-performance liquid chromatography (UPLC)-Q-Exactive Orbitrap Mass Spectrometry from Sanhua plums. They also suggested that the phenolic content and antioxidant activities of this type of plum changed in different digestion stages. It was also hypothesised that these changes may relate to the different stability profiles of phenolics and the liberation of bound phenolic compounds during simulating in *vitro* digestion. Epicatechin (183.90±1.21 mg/100 g dry weight (DW)), neochlorogenic acid (163±4.48 mg/100g DW), and procyanidin B2 (100.96±1.29 mg/100g DW) were reported as three main phenolic compounds identified from undigested plums. The correlation analysis between phenolic compounds and antioxidant activity was conducted based on the antioxidant activity of plums being determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-Azinobis-3-ethylbenzothiazoline-6-Sulfonic acid (ABTS), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC) assays, and the Pearson

correlation analysis. It was suggested that the undigested and digested total phenolics were both positively related to antioxidant activity (Yu et al., 2021). Such evidence was also provided by other studies (Hussain et al., 2021; Kristl et al., 2011; Traore et al., 2021). However, undigested catechin, digested rosmarinic acid, and digested naringenin-7-O-β-D-glucoside did not show a significant correlation with antioxidant activity for the DPPH and ABTS assays. The first one was even negatively strongly correlated with antioxidant activity for FRAP and ORAC. The second one was significantly positively correlated with antioxidant activity for ORAC but not for FRAP, and the third one showed a significant correlation with antioxidant activity for both FRAP and ORAP (Yu et al., 2021).

2.2.2.2 Prevention of cardiovascular diseases (CVDs)

In Noratto et al. (2015)'s study, the obese Zucker rats were randomly assigned to three groups (n=10) control, peach, and plum. The control group was fed with glucose sugary water in the same concentration as the average concentration of reducing sugars in plum and peach juices. The experiment was conducted for 11 weeks, and the consumption volumes of sugary water, peach, and plum juices by obese Zucker rats were on an average 50.6±8.65, 47.5±9.0, and 45.2±11.8 mL. A significant difference between the control and plum groups was shown after three weeks of the experiment. However, the body weight gain was not prevented in the group of rats consuming peach juice, which could be resulted from the lower concentration of polyphenols in peach juice

(approximately one-third of plum juice) (Noratto et al., 2015). It was also suggested that the polyphenolics including anthocyanins and catechins in plums could modulate the physiological disorders associated with obesity and reduce liver and body weights (Meydani & Hasan, 2010). Additionally, a high concentration of anthocyanins in plum and plum juice might help with preventing platelet aggregation induced by adenosine diphosphate and collagen (Chai et al., 2012).

Hussain et al. (2021) recorded that the consumption of plums could suppress systolic blood pressure as they possessed anti-inflammatory and anti-adipogenic effects, and plum juice could also act as a diuretic. The accumulation of cholesterol could result in the development of some heart-related diseases such as atherosclerosis. Plums contain vitamin C, which could prevent the oxidation of fatty acids and cholesterol, and accordingly, reduce the incidence of heart diseases and the risk of stroke (Hussain et al., 2021). Furthermore, the presence of propionic acid in plums was also recorded by Hussain et al. (2021), which might inhibit HMG-CoA reductase, leading to lower production of cholesterol in the liver (Zaelani et al., 2021). Moreover, the soluble fibre in prunes was also suggested to enhance bile acid secretion from the liver, thus, improving the digestion of fats (Hussain et al., 2021).

2.2.2.3 Prevention of bone diseases such as osteoporosis and osteoclastogenesis

Ferdous et al. (2016) demonstrated that osteoporosis is a bone disorder manifested by loss in bone strength and mineral density, which could lead to an increased risk of

fractures. It was recently reported by National Osteoporosis Foundation (2021) that there were more than 300,000 people over age 65 hospitalised with a hip fracture each year, and three-quarters of them were women. Plums contain vitamin K, vitamin D, and calcium, which are essential for building strong and dense bones when people are young and to maintain their health as they age. These nutrients have been claimed by National Osteoporosis Foundation (2021) to be the three most important nutrients for bone health. Vitamin D is required by the body to absorb calcium, and calcium is critically necessary to bone health as 99% of the calcium in the human body is in the bones and teeth. Hussain et al. (2021) also claimed that eating plums could promote the mineral density of bone for postmenopausal women because the high content of vitamin K in dried plums helps to restore calcium balance.

Additionally, it was suggested by Smith et al. (2014a) that plums and prunes were rich in bioactive compounds that attribute to healthier bones by increasing bone mass. This could then inhibit osteoclastogenesis through the enhancement of cell signalling intermediates that improved osteoblast differentiation. Moreover, Pawlowski et al. (2014) studied and reported that consuming plum extract was likely to increase calcium retention in bones by 20%. Chlorogenic acid and its derivatives such as cryptochlorogenic acid and neochlorogenic acid in plums were reported to improve bone formation and enhance bone-specific alkaline phosphate (Smith et al., 2014b).

2.2.2.4 Cognitive improvement

Anthocyanins in plums could help control systolic blood pressure and reduce the incidence of diseases such as Alzheimer's disease (AD) and even memory loss for aged people (Hussain et al., 2021). There is no current evidence that eating plums can improve the cognition of humans, and most of the available evidence was found in the studies conducted on animals. Bouayed et al. (2007) tested the relationship between the effect of chlorogenic acid from plums and anxiety-related behaviour of mice using the light/dark test. The results showed a decline in anxiety-related behaviours or anxiolytic-like effects and protection of granular leukocytes (a type of white blood cell) or granulocyte from oxidative stress. Additionally, Shahidi et al. (2013) used plum extract for the supplementation of the diets of three treated groups of mice with different doses (75, 100, and 150 mg/kg), and the control group. The results showed there were statistically significant ($p<0.05$) differences in evaluating the learning and memory of mice between the control and plum extract-contained groups. Results were also in line with other studies conducted by Sharma and Sisodia (2013) and Kao-Ting et al. (2013), as they also verified and substantiated the health effect of plums on the enhancement of cognition.

Another report (Kao-Ting et al., 2013) stated that the mice that consumed lyophilized and ground plum powder combined (at 2% w/w) with the basal diet took less time to reach the coloured platform in the circular water tank apparatus, which indicated the spatial learning of mice and the effect of plum consumption. A further study conducted

by Kao-Ting et al. (2013) also demonstrated that the diet consumed with plums could significantly improve the spatial learning and memory of mice by assessing them with a Morris water maze. Additionally, the expressions of cerebral beta-amyloid involved with the incidence of AD, insulin resistance, hyperglycaemia, and oxidative stress were also significantly reduced in the treated mice (Kao-Ting et al., 2013). A later study conducted by Kuo et al. (2015) claimed that plums, which contained abundant potassium, could stimulate kallikrein (a hormone produced by kidneys), and thus, reduce sodium levels in the body. This could help to regulate the levels of blood pressure, which could consequently not only improve cognitive functioning but also prevent kidney disease, diabetes, and CVDs.

A recent study conducted by Chellammal et al. (2020) showed that *Prunus domestica* contained a potential anti-amnesic effect by regulating oxidative stress and acetylcholinesterase (AChE) inhibition. The former was the main reason for cognitive dysfunction and neurodegenerative disease, so it might eventually result in the progression of amnesia or disruption of memory and learning (Chellammal et al., 2020). The latter resulted from the generation of free radicals caused by the accumulation of amyloid-beta (Aβ) peptides, as it was demonstrated by Madav et al. (2019). Postu et al. (2019) suggested that aChE could consequently impair the learning and memory of organisms by initially converting Aβ peptides into a highly neurotoxic compound (neurofibrils), as AD type of memory and learning impairment was caused by the deposition of that neurotoxic compound.

2.2.2.5 Anti-cancer and anti-tumour activity

Bioactive compounds in plums such as beta carotene had an anti-cancerous effect (Igwe & Charlton, 2016). It has been recorded by Hussain et al. (2021) that the polyphenolic compounds in plums exerted anti-cancer function against cancer cells in the breast and colon. They recommended that vitamin C from this fruit could also prevent the progression of malignant cells in the human body. Additionally, Bender and Atalay (2021) suggested that chlorogenic acid and neochlorogenic acid could potentially prevent the proliferation of breast cancer cells by apoptosis. They added that antineoplastic characteristics of chlorogenic acid might inhibit the cancer phenotype in breast cancer in many molecular ways. Additionally, it also has chemo-preventive properties for different types of cancer. For example, chlorogenic acid from plums could inhibit the proliferation of kidney cancer cells, and apoptosis of cancer cells was induced via caspase, Bax, and Bcl-2 signalling arrangements (Wang et al., 2019). This could also reduce the ability of the migration and colonization of lung cancer cells (Yamagata et al., 2018), as well as suppress the growth of colon cancer cells by ROS induction (Hou et al., 2017). Therefore, based on the evidence from these studies, the anti-cancer efficacy of chlorogenic acid was supported/verified, which could not only affect different cancer-associated signalling pathways and apoptosis programs in cells but also presented selective function on the proliferation of cells.

2.2.2.6 ***Anti-allergy and antimicrobial properties***

Based on the literature research carried out in this study, I could only find one piece of evidence provided by Karasawa et al. (2012) who experimented on five-week-old BALB/c mice. These mice were on a prune extract-added or extract-free diet and injected with mite solutions between 5 to 11 weeks of their age. Two different doses with various solvents were used in the first three weeks and the last three weeks for the mite solution. Results showed that the supplementation of the plum extract significantly lowered the total sneezing count of mice and mite allergen-specific immunoglobulin E levels in serum, though the anti-allergic phytochemical compounds in prune extracts were not identified in their study.

Cevallos-Casals et al. (2006) in the early days found that plums have antimicrobial activity properties, and it was shown that phenolics including anthocyanins, chlorogenic acid, caffeic acid, and several hydroxycinnamic acid derivatives had antimicrobial effects against several microorganisms such as *E. coli*. Additionally, it was also proved by Yaqeen et al. (2013), who did a test on five different gram-positive bacteria, that crude ethanol extracts along with ethyl acetate and chloroform fraction of *P. domestica* exerted a varying degree of antibacterial effect against these microorganisms.

In a recent study conducted by Silvan et al. (2020), the researchers tested the antibacterial activity of plum exact powder (PEP) in response to five of the most relevant foodborne pathogen bacteria (*C. jejuni, S. Typhimurium, E. coli, S.aureus, and*

L.monocytogenes). There was only a single concentration of PEP (1 mg/mL) in triplicate as the experimental group for the powder being processed with different drying methods such as freeze drying, spray drying, and vacuum drying at 40, 60, and 80 °C, and the PEP was replaced by the same volume of sterile water for control. From the results, the addition of plum extract powder in different drying methods had varying levels of growth inhibition against foodborne pathogens. Additionally, freeze drying was the most active bactericidal extract compared to the other drying methods, as a considerably higher concentration of quercetin-3-*O*-galactoside or hyperoside was found in the freeze-dried samples (Silvan et al., 2020). Ku et al. (2014) found that a flavonol glycoside was responsible for different biological activities such as antibacterial, anti-inflammatory, and antioxidant activities. Orhan et al. (2007) showed that the total phenolic content of the plant species (3 Crataegus species) was not directly related to the antimicrobial activity, but the presence of certain specific polyphenolic compounds such as quercetin-3-*O*-galactoside (hyperoside).

2.2.2.7 Prevention of muscle wasting[1] in certain chronic diseases

Alsolmei et al. (2019) reported that a polyphenol-enriched plum extract could have potent health benefits on muscles. For instance, it might promote myotubule formation and anabolism while reducing colon cancer's effects on C2C12 cells' cellular damage,

[1] A weakening, shrining and loss of muscle caused by disease or lack of use (National Cancer Institute).

which could stop muscle atrophy in some chronic illnesses like cancer. This was presumably due to its demonstrated capacity to stimulate myogenesis, increase protein synthesis in the muscles, prevent protein degradation, and protect muscle cells from cytotoxicity induced by tumours (Alsolmei et al., 2019). These authors (Alsolmei et al., 2019) also reported that the concentration of plum extract positively affected the C2C12 myoblast cell size, with no cytotoxic effect on myoblast even when it was used at a high concentration of 250 µg/mL.

2.2.2.8 ***Laxative effect***

Constipation is nowadays a serious problem in human communities, because of the consumption of protein-rich foods. In this regard, plums may become useful and they possess a laxative effect, which is attributed to their high fibre content. A study conducted by Piirainen et al. (2007) observed that the consumption of prune juice reduced the difficulty in defecation among 54 volunteers (13 men and 41 women) who were otherwise healthy but had certain mild gastrointestinal symptoms. The positive effects of prune juice on gastrointestinal function were shown only after two weeks of its regular consumption. However, it was reported that the consumption of prune juice could increase flatulence, because of the indigestible carbohydrates giving rise to fermentation in the colon (Cummings et al., 2001; Piirainen et al., 2007). Cheskin et al. (2009) added that in the case of adults with chronic constipation, drinking a daily portion of plum juice before a meal could have their stool softened. Their results of the

consistency of daily bowel movements from 36 of their enrolled participants showed that the treatment with plum juice resulted in a statistical trend (p=0.1) of a lower average hardness of bowel movements as 0.74±0.41.

2.2.3 Safety concerns related to plums' consumption

2.2.3.1 Risk of kidney stones compared to other fruits

Besides all health benefits reported for plums and their products, it appears that there is a concern about the risk of kidney stones, which is attributed to oxalic acid. However, no systematic study could be found in this regard and the current evidence is contradictory. Kessler et al. (2002) reported that plum juice contained the highest amount of oxalic acid (297 µmol/L) compared to cranberry juice (167 µmol/L) and blackcurrant juice (204 µmol/L). Holmes and Assimos (2004) demonstrated that oxalate-rich food is a potentially critical factor in determining the impact of ingested oxalate on kidney stones. Additionally, the significant increase in the risk of uric acid stone formation reported by Kessler et al. (2002) was a consequence of the decreased pH value. In their study, consuming plum juice did not alter the urinary composition though it contained the highest amount of oxalic acid. However, the relative supersaturation for uric acid and the excretion of oxalic acid increased after drinking cranberry juice, as cranberry juice decreased the urinary pH. Consumption of blackcurrant juice increased the urinary pH and excretion of citric acid, which accompanied with the increased excretion of oxalic acid (Kessler et al., 2002).

2.3 Drying methods for plums

2.3.1 **Convection drying**

A convection dryer is basically an oven system that can remove the solvent from the product/solid using heat. In this process, heat transfer heats the solvent, and mass transfer removes the solvent; therefore, either higher temperature or higher mass transfer means a faster drying process. There are some advantages of convection heating such as the temperature can be controlled accurately according to the product's physical and chemical profile; the product can be heated evenly irrespective of product shape or size; and the heating process can be conducted without contact. However, there are also some drawbacks to this method. For example, the efficacy of heat transfer of this method is generally lower than other heating methods; it requires more product exposure time; the rate of drying depends on the thermal conductivity of the product to heat the interior of thick products, and airflow is the most important factor that may contaminate or oxidase the product. It was suggested by Gościnna et al. (2021) that the convection drying method was mostly applied on an industrial scale due to the low cost compared to lyophilization or freeze drying. In their study, the convection drying was conducted at 60°C for 48 h at an airflow of 2 m/s for 150g of plums. However, they also reported that using the convection drying process obtained a considerable reduction in both the anthocyanin content by approximately 82% and the polyphenolic compound content by around 41%.

2.3.2 Vacuum drying

This drying method utilises vacuum pumps in an air-tight vessel. By reducing the atmospheric pressure inside the tank, materials inside the vessel quickly dried, as they contact with indirectly heated walls. Using this method for product drying was more energy-efficient and controlled than convection drying (Gościnna et al., 2021). Additionally, this method is a highly efficient technique without safety issues for drying large volumes of heat-sensitive granules or powders at a lower temperature than heat-involved drying methods. The temperature of the products does not need to reach the boiling points of their solvents to remove the solvents. For example, the vacuum dryer can reduce the boiling point from 100 to just 35°C. Therefore, the application of this method can achieve less product degradation during the drying process than the utilization of heat-involved traditional drying methods such as convection drying. In a study conducted by Gościnna et al. (2021), 150 g of plum was vacuum dried at 60°C under a pressure of 133 Pa for 24 h. However, this method significantly recovered more total anthocyanins (about 1-1.3 g/kg) and total phenolic acids (about 1.1-1.4 g/kg) compared to the total anthocyanin (201.7-286.2 mg/kg) and total phenolic acid (743.6-1004.3 mg/kg) recovered by convection drying for all three extraction methods (basic extraction, ultrasound-assisted extraction, and microwave-assisted extraction).

2.3.3 Sublimation/lyophilisation/freeze drying

This drying method was a slow process conducted at cooler temperatures and requires heat energy to drive the phase change process from solid to gas (Barley, 2009). This method was recently reported by Gościnna et al. (2021) as the best drying method for a better result of bioactive compounds from plums. However, the lyophilisation or sublimation drying method costs a lot more than the other two drying methods. In comparison, the convection drying method is commonly used on an industrial scale but the high temperature of this process involved with long exposure to oxygen results in considerable losses of bioactive compounds or antioxidants in the plums or materials being dried. Gościnna et al. (2021) reported a significantly low recovery rate of anthocyanin and polyphenols by using convection drying referred to in Section 2.3.1. Additionally, it was previously also reported by Michalska et al. (2016) that there was a significant disappearance of catechin under vacuum drying. It was also proven by Mohd Zainol et al. (2009) that the concentration of catechin could decrease by almost two times when vacuum drying method was used vs freeze drying method, which was associated with the effect of high temperature used during vacuum drying. Therefore, based on the finding of the previous research, freeze drying method seems to be more favourable compared to other methods, especially in terms of the preservation of bioactive compounds. Accordingly, sublimation/freeze drying will be used for drying fresh samples of New Zealand damson plums in the current study.

2.4 Extraction methods for phenolic compounds from plums

Extraction is one of the most significant processes in this study, as different extraction methods cooperated with various extraction solvents could present distinct extraction efficacies on various phenolic compounds. This section reviews four conventional and eight unconventional extraction methods. The suitable extraction methods, solvents, and conditions for damson plums are also discussed in this section.

2.4.1 Conventional extraction methods for phenolic compounds

2.4.1.1 Solid-liquid extraction (SLE)

This method was a separation process that could transfer solutes from a solid matrix to a solvent with a fluid involvement (Tzia & Liadakis, 2003). This has been widely used to extract and recover several important food components; e.g., recovering sucrose in cane or beets, oil from oilseeds, proteins from oilseed meals, functional hydrocolloids from algae, and phytochemicals from various plants (Tzia & Liadakis, 2003). Additionally, it was also considered to be used to remove undesirable contaminants and toxins present in foods and solid feeds.

This method has not been previously used for extracting phenolic compounds from damson plums, but there was a report about its use for the extraction of phenolic compounds from milled grape cane (*Vitis vinifera*) (Karacabey & Mazza, 2008). In this study (Karacabey & Mazza, 2008), the ethanol concentration and temperature both affected the extraction rate of phenolics from the milled grape cane. For example, increasing ethanol concentration appeared to result in a linear increase in the yield of

resveratrol at low-temperature levels, but not at higher temperature levels (Karacabey & Mazza, 2008). Different phenolics required various temperatures and ethanol concentrations to achieve the maximum yield. For instance, 58% of ethanol solution at 83.6°C was predicted to achieve the highest yield of *trans*-resveratrol of 4.25 mg/g dry weight. (Karacabey & Mazza, 2008).

2.4.1.2 Liquid-liquid extraction (LLE)

This method is to separate compounds or metal complexes based on their different solubilities in two various immiscible liquids such as polar and non-polar solvents (water and organic solvent). This method can be used to extract plums' phenolics, but they should be in a liquid form for the extraction.

Mocan et al. (2018) used modified liquid-liquid extraction such as dispersive liquid-liquid microextraction (DLLME) and sugaring-out liquid-liquid extraction (SULLE) for 14 cultivars of damson plum dry leaves. The extraction procedures involved the addition of 1 mL of extraction medium (could be water or organic solvent), 100 μL of acetonitrile, and 200 μL of ethyl acetate in the Eppendorf tube containing 10 mg of dry leaves for DLLME before the sample solution was centrifuged for 5 min at 12000 × *g*. The dry residue containing damson plum leaf extract was then redissolved in 50% of mobile phase solution and injected into the HPLC system after 5 min of ultrasonication. For SULLE, the solvent composition was altered to 200 μL of water, 400 μL of acetonitrile, and 200 μL of corresponding sugar solution after 30 s of gentle shaking.

However, the top layer contained the leaf extract of the damson plum after the centrifugation at the same settings. Compared to SULLE and microwave-assisted extraction (MAE), the DLLME could extract some phenolics (Syringic acid, p-Coumaric acid, and Naringin) that could not be extracted by the other two. Additionally, catechin, a major phenolic compound of damson plums, was doubly or triply extracted by the DLLME compared to the SULLE and MAE. However, the extraction efficacy of MAE was similar to the DLLME for the extraction of epicatechin but doubled or higher than what was extracted using SULLE. Hydrolytic maceration distillation could be used to extract certain plant materials such as leaves of wintergreen (Singh, 2008). This plant material contains the precursor gaultherin and the enzyme primeverosidase. The enzyme could act on the gaultherin and enhance the liberation of free methyl salicylate (oil of wintergreen) when the leaves were macerated in warm water (Singh, 2008). A similar function of maceration also applies to sinigrin from brown mustard, amygdalin from bitter almonds, and alliin from garlic.

2.4.1.3 Maceration extraction and percolation method

Olejar et al. (2015) demonstrated that the maceration extraction method was a convenient process of soaking a pulverised sample in a suitable solvent in a closed system, followed by a constant or sporadic agitation at room temperature. A separation process such as filtration, clarification, or decantation would be applied to separate the solids from the solvent after the exaction process. However, the operation of this

method required abundant time and a large volume of solvents (Alara et al., 2018a).

The percolation method was close to maceration because the pulverised sample was also placed in a closed system and the solvent drops gradually from the top to the bottom. However, extra filtration was not required in this method because the filters were installed on percolator devices that only allowed solvent-containing extract to pass through. Apart from the problem of abundant time and solvent volume requirements, other issues such as solubility of polyphenols, sample size, and extraction time should also be concerned for this method (Alara et al., 2021). There was no evidence of two extraction methods on plum in general or damson plums. However, they were used to extract medicinal and aromatic plants by Singh (2008). They suggested that the cold percolation was not efficient because the mass transfer rate was slow, but a higher temperature of the solvent increased the solubility of the active principle, and it enhanced the mass transfer of active compounds from solid material to the solvent, but it was not suggested for heat-sensitive active compounds.

2.4.1.4 Soxhlet extraction

With the application of this method, the pulverised samples were placed in cellulose-made timbles and positioned in the extraction chamber over the collecting flask, beneath a condenser (Azwanida, 2015). The solvent in the heating bottle was then heated to produce vapour, which would condense and fall back into the timbles (Azwanida, 2015). As reflux was maintained severally, the aqueous extract was gained

back from the heating flask. Soxhlet extraction was advantageous due to less time and solvent requirements and its convenience compared to the maceration and percolation method (Azwanida, 2015). Nevertheless, this process should be carefully operated, since the excess heat might have a negative impact on the thermolabile phenolics (Seidel, 2012). Additionally, Alara et al. (2018a) stated that the highest yields of antioxidant extracts from *Venonia cinerea* leaves could be obtained using an extraction time of 2 h with the feed: solvent ratio of 1:20 g/mL and concentration of ethanol of 60% v/v.

2.4.2 Unconventional extraction methods for phenolic compounds

Mostly, the conventional extraction methods were used in laboratories because of their low cost and they were easy to follow. However, those studies above suggested that using conventional extraction methods such as maceration, percolation, and Soxhlet extraction methods in the industry might cause environmental problems due to their requirement of large volumes of organic solvent. For example, Widdowson and McCance (1935) extracted sugar and starch content from different fruits including damson plums, blackberries, cranberries, grapes, grapefruit, orange juice, and pears using Soxhlet extraction with hot 80% alcohol for 16 h. The sum of glucose, fructose, sucrose, and starch was 9.6% of the edible portion of fresh fruit which ranked 2 among these fruits after grape (15.5%). Below, some of the unconventional methods that could be used for the extraction of polyphenolic compounds in the products such as damson

plums are explained.

2.4.2.1 Supercritical CO_2 extraction (SC-CO_2)

Stengel (2019) demonstrated this extraction method. Its processes started with the placement of plant material into an extraction vessel, and the supercritical CO_2 gas (under high temperature and pressure) was pumped into the extraction vessel and dissolved the plant material as it broke the trichomes. The material was then allowed to flow into a different vessel by a pressure release valve, where pressure and temperature were controlled by an internal compressor and heater. Certain molecules could bond to CO_2 and separate from the plant, as the temperature, pressure, and flowrate were changed. In some cases, CO_2 could be recycled into the tank and used for the next batch. Finally, the extract flowed into a collection flask (Stengel, 2019).

CO_2 was required as a supercritical fluid in this method. The advantages of using this fluid were its non-toxicity, non-flammable, cost-effectiveness, and inert properties (Morgan, 2013). Additionally, it had a higher extraction capacity because of the higher mass transfer between the phases occasioned by lowered viscosity and increased diffusion coefficient of CO_2 compared to the solvents. Furthermore, different pressure-temperature combinations were possible to achieve better extraction conditions adaptability. Moreover, it was suggested that recyclable CO_2 might reduce the emission of greenhouse gas (De Zordi et al., 2014; Morgan, 2013). However, it took longer for the SC-CO_2 extraction method to complete than other methods because of the

continuous fluctuation in temperature and pressure. For instance, it took 4 to 6 h to complete the extraction of 9 kg of plant material (Stengel, 2019). However, there are no evidence of application of this extraction method for plums or damson plums.

2.4.2.2 Microwave-assisted extraction (MAE)

The application of microwave radiation energy was involved in this extraction method to heat the solute-solvent mixture (Alara & Abdurahman, 2019). The diffusivity of the solvents in the sample was facilitated by heat to improve the diffusion of the target phenolics and polyphenols out of the sample (Alara et al., 2018b). The diffusion of the solvent could assist in disrupting hydrogen bonds that hold the sample, so it allowed the target phytochemicals to dissolve into the extraction fluid (Alara et al., 2021). The MAE was found to be useful in the extraction of short-chain polyphenols such as phenolic acids and flavonoids, but it was not suggested to be used when considering polymeric polyphenols such as anthocyanins and tannins because of the likelihood that MAE destroyed polyphenols with several heat-sensitive and hydroxyl-type substituents like anthocyanins (Alara et al., 2021). A pictorial representation of MAE is shown in Figure 2.1 (Alara et al., 2021). Unlike most conventional extraction methods, the advantages of using the MAE method included less time consumption and the use of a low volume of organic solvent. It was been suggested that solvent diffusion and extraction kinetics of this method could be promoted under higher temperatures and with smaller sample volumes (Pinela et al., 2016).

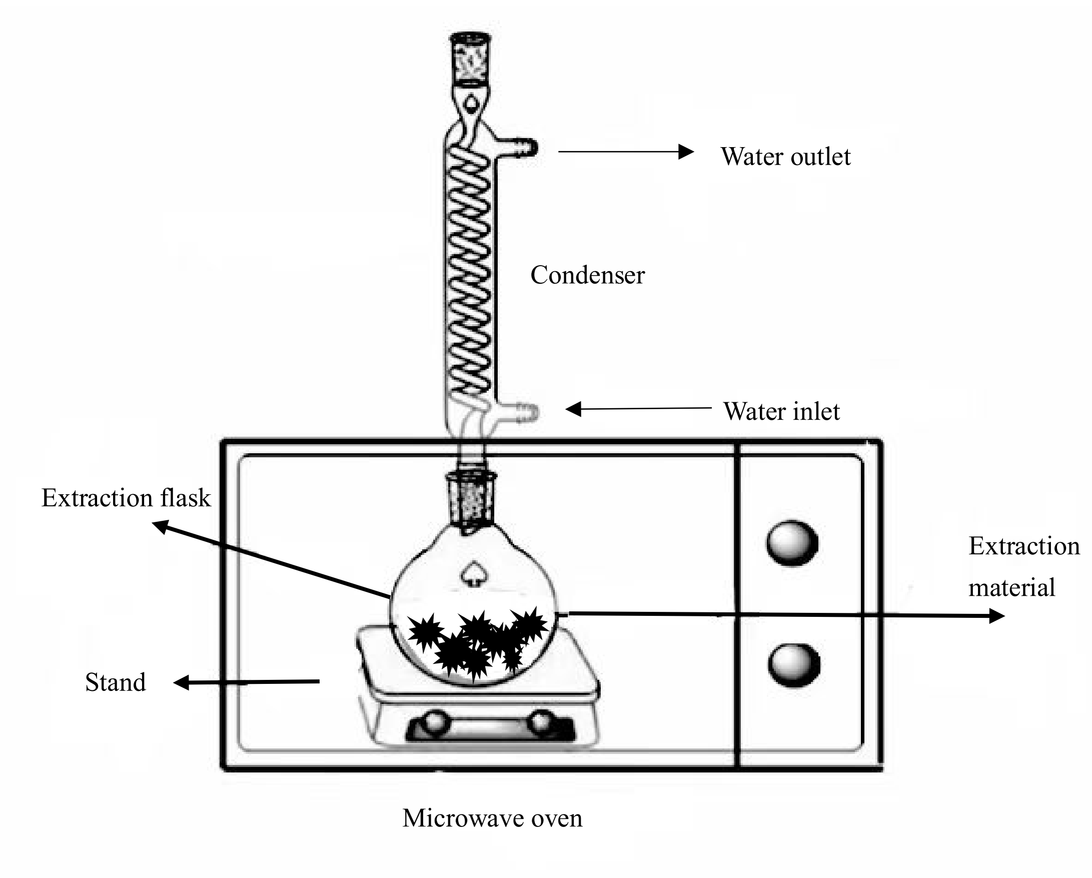


Figure 2. 1. Pictorial representation of microwave-assisted extraction. Adapted from Alara et al. (2021).

2.4.2.3 Enzyme-assisted extraction (EAE)

This technique exploited the capability of the enzymes to weaken and break down the cell wall compartment to expose their cellular or cytoplasmic content to the extraction fluids (Swer et al., 2016). Therefore, an enzymatic pre-treatment could facilitate the effectiveness of the release of bonded phytochemicals. Some of these enzymes were used to accelerate the extraction of phytochemicals from plant materials such as hemicelluloses, cellulases, and pectinases (Alara et al., 2021). Additionally, Aires (2017)

suggested that the EAE was better to enhance the content of polyphenol in the extracts than other methods such as UAE and solid-liquid extraction. Furthermore, this method was considered eco-friendly, because the water was used as the extraction solvent instead of organic solvents (Alara et al., 2021). This method now is usually used in a combination with other methods. For example, Mushtaq et al. (2015) combined the EAE and SFE methods (enzyme-assisted supercritical fluid extraction) to extract phenolic antioxidants from pomegranate peel. Wu et al. (2015) used a combined method of EAE and UAE (E+UAE) to extract polyphenols from broccoli, and they found that the combined method achieved higher content of polyphenol and high antioxidant activity.

2.4.2.4 Ultrasound-assisted extraction (UAE)

According to Vinatoru (2001), the UAE method utilised the induced mechanical influence through the explosion of a micro-sized bubble to rapidly disorganise the tissues and facilitate the diffusion of the target phenolics and polyphenols from the sample into the solvent. This method was used for the extraction of phenolic antioxidants from olive leaves and the authors recognised it as a simple and low-cost method for both small and large-scale extraction settings (Shirzad et al., 2017). An ultrasound device with a frequency range between 20 and 2000 kHz was required to increase the permeability of the cell wall and produce cavitation (Alara et al., 2021). A schematic representation of the UAE can be seen in Figure 2.2 (Alara et al., 2021).

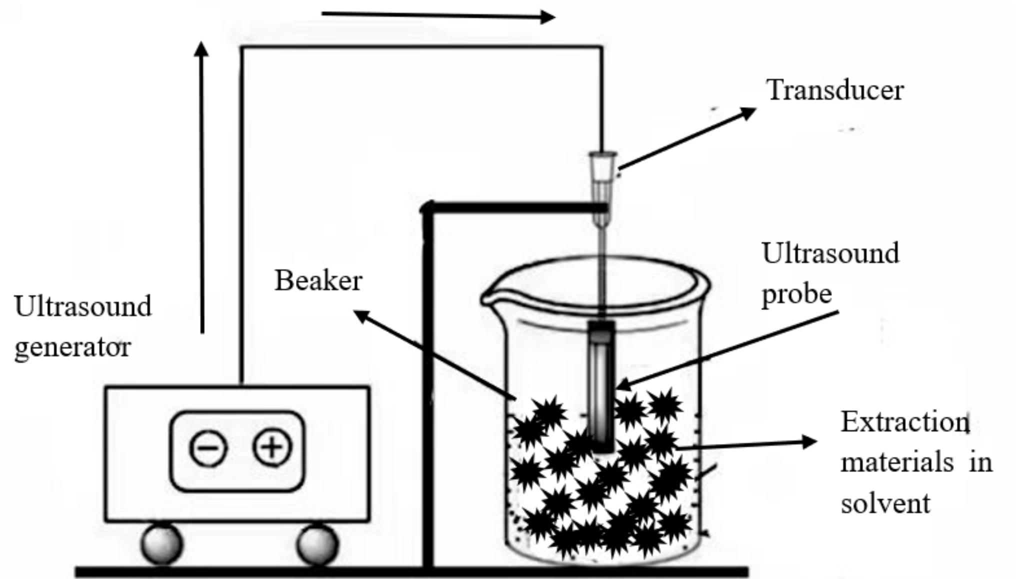


Figure 2. 2. Pictorial representation of ultrasound-assisted extraction. Adapted from Alara et al. (2021).

Compared to other techniques of extraction, using the UAE method appeared to ensure higher efficient extraction of polyphenols with minimum size breakdown of the extraction materials (Vinatoru, 2001). For example, Zu et al. (2012) reported that the UAE technique was more effective for the extraction of rosmarinic and carnosic acids from *Rosmarinus officinalis* than the conventional extraction methods such as maceration and Soxhlet extraction. Additionally, Cai et al. (2016) suggested that the application of the UAE method achieved a higher extraction efficacy of anthocyanin from purple sweet potato compared to the bath stirring extraction method. However, accelerated solvent extraction (ASE) in their study was performed as the more suitable method to achieve more stable anthocyanins, as the ASE method can extract more diacyl anthocyanins and less nonacyl and monoacyl anthocyanins (Cai et al., 2016).

The disadvantage of the UAE method was that the extracted phytochemicals could be severely affected by a period of sonication longer than 40 mins at a high energy level that was above 20 kHz, which resulted in an increased diffusion distance but lowered rate of diffusion area or rate in the study of Wang et al. (2008).

Nevertheless, a recent study conducted by Olawuyi et al. (2021) used an ultrasonic bath (38kHz) equipped with a cooling system maintaining the temperature at and the E+UAE treatment to extract antioxidant compounds from plum (*Prunus salicina* L.) juice. The optimal extraction timing was 30 min for the individual UAE, as the TPC considerably increased as the treatment time was reaching 30 min and slightly decreased when the time was extended to 75 min from 35 min for sonication of plum juice (Olawuyi et al., 2021).

2.4.2.5 Pressurized liquid extraction (PLE) or accelerated solvent extraction *(ASE)*

This method involved the desorption of solutes from the various active sites in the sample matrix under high pressure and high-temperature conditions followed by the diffusion of extraction fluid in the matrix. The solutes might then partition themselves from the sample matrix into the extraction fluid and would eventually be chromatographically eluted out of the extraction cell or unit to the collection vial or flask.

Zhang et al. (2018) suggested that high temperatures ranging from 40 to 200°C could be applied in combination with high pressure ranging from 3.3 to 20.3 MPa to facilitate

the desorption and solubility of molecules into solvents. The advantage of this method was also explored by Nieto et al. (2010), who demonstrated that the application of the PLE or ASE method ensured a rapid extraction process with considerably less solvent than that of the conventional methods. Several solvents such as methanol, ethanol, and their combination with water and their mixture were utilized for extracting phenolic components through several sources in a typical PLE or ASE process (Alara et al., 2021). However, the decision on the combination of these solvents had to be systematically taken because it was demonstrated that sufficient selection of solvent could affect the level of extraction of various phenolics from the same sample due to the significant distinction in the chemical composition of those components (Alara et al., 2021). For example, Maqsood et al. (2014) provided evidence that it was hard to find a common solvent that could extract all the phenolic compounds in parsley.

2.4.2.6 Subcritical water extraction (SWE) or pressurized hot water extraction

The PLE or ASE method also encouraged a better usage of water as the extraction solvent, which was referred to the subcritical water extraction (SWE) or pressurized hot water extraction (PHWE) with a high-temperature condition between 100 (boiling point of water) and 374°C (critical point of water) (Teo et al., 2010). The process of this method is the same as the PLE or ASE method, but there are some differences like more critical temperature and pressure requirements and the organic extraction solvent being replaced by water. The solvent replacement was explained by Plaza and Turner (2017)

that the dielectric properties of water could be changed at higher temperatures of around 200°C, as they made water act as a standard organic solvent so that improved the capacity of extraction.

This extraction method was used by Budrat and Shotipruk (2008) to obtain the phenolic compounds from bitter melon (*Momordica charantia*). The SWE at 200°C (0.6462 mg/g DW) showed a significantly higher extraction efficacy of antioxidative phenolic compounds from bitter melon than solvent extraction (methanol extraction for 2 h obtained 0.0271 mg/g DW) and Soxhlet extraction (methanol extraction for 4 h obtained 0.012 mg/g DW). However, the evidence of application of this method for extraction of plums or damson plums was not found.

2.4.2.7 ***Solid-phase extraction***

This extraction method was initially developed as a complement or replacement for a conventional extraction method LLE, and then was a widely used sample preparation technique in many areas of chemistry such as environmental, pharmaceutical, clinical, and food (Poole, 2003). It was not only used to extract target organic compounds from samples but also to eliminate the components that interfere from the complex matrices, in order to obtain a purer extract containing the target analytes (Żwir-Ferenc & Biziuk, 2006). The principle of this method was achieved through the interaction of the sorbent, the solvent, and the analyte (Żwir-Ferenc & Biziuk, 2006). The processes were usually conducted to isolate the selected or target analytes from a mobile phase like gas, fluid,

or liquid, and the analytes were transferred to the solid phase for the sampling process. Then, the solid phase was isolated from the sample and the analytes were recovered by thermal desorption into the gas phase or elution using a liquid or fluid (Poole, 2003). The applicability of SPE was mainly determined by the sorbent used in the extraction column, as a more selective extraction could be achieved using higher-energy interactions when electrostatic interaction energy was much stronger than hydrophobic and hydrogen bonding energy (Żwir-Ferenc & Biziuk, 2006). Silica-based or organic resin-based sorbents were usually used for the SPE method due to their suitable physical characteristics and chemical properties (Żwir-Ferenc & Biziuk, 2006).

2.4.2.8 High hydrostatic pressure extraction (HHPE)

This is a novel technique used for the high-pressure processing of food in the extraction of active ingredients from plants and food. Compared to high-pressure homogenisation or SFE, this technique requires cold isostatic superhigh hydraulic pressure ranging from 100 to 800 MPa or even higher (Shouqin et al., 2005). In foods with fragile structures, this process could result in structural changes, such as protein denaturation, cell deformation, and cell membrane damage (Food, 2000; Xi et al., 2009). According to Shouqin et al. (2005), the extraction yield with the application of the HHPE method for extracting flavonoids from propolis was higher than what was extracted by two conventional methods; i.e., heat reflux extraction and extraction at room temperature. Additionally, the application of the HHPE method was demonstrated by Briones-

Labarca et al. (2015) that could obtain a high efficiency of antioxidant extraction with moderate concentrations of ethanol at room temperature within a short period from the papaya seed waste.

2.4.2.9 The final decision on extraction methods for further studies

Unconventional extraction methods appeared to be more efficient for the extraction of bioactive compounds from plant materials, while they also require less time and extraction solvent than conventional extraction methods. However, for the extraction of phenolic compounds on the laboratory scale, the application of conventional extraction is comparatively much cheaper than the unconventional extraction methods, in consideration of the equipment required for the extraction. To achieve a high content of bioactive compounds such as phenolics, vitamins, and organic acids from the New Zealand damson plums, stable extraction conditions should be considered. High temperature and pressurized conditions may destroy polyphenols with several hydroxyl-type substituents (Alara et al., 2021) and contribute to the loss of polyphenolic compounds (Gościnna et al., 2021). Nevertheless, there are soluble and insoluble phenolic acids and polyphenols in plum powder. Correspondingly, based on these, in the case of the current research, unconventional methods under comparatively low temperatures such as UAE, EAE, or their combined method can be used to extract soluble phenolics, because other aforementioned six unconventional extraction methods require high temperatures.

The E+UAE method was recently conducted by Olawuyi et al. (2021) to extract polyphenols from plum juice. They optimised the timing of extraction under a fixed concentration of enzyme and fixed frequency of ultrasound. The concentration of the enzyme was previously optimised by Mieszczakowska-Frąc et al. (2012) and Siddiq et al. (2018), as 0.2% (vol/vol) of pectinase enzyme yield high antioxidant composition. However, there was no evidence provided that 38 kHz frequency could achieve a higher yield of antioxidant composition from a plum in the same amount of time. Therefore, in future studies, the frequency of the ultrasound should be optimised in the first stage extraction of the E+UAE, to obtain the soluble phenolic acids and polyphenols from plum powder solutions under a comparatively low temperature of 45°C.

2.5 Identification and determination methods for phenolic compounds from plums

2.5.1 High-performance liquid chromatography (HPLC)

This method was recently used by Olawuyi et al. (2021) to determine the content of ascorbic acid and phenolic compounds in plum juice samples. In their study, samples were diluted five times and filtered through a 0.45 μm syringe. 20 μL of filtered juice of each sample was injected into the HPLC system. The HPLC was equipped with an Athena C18 reversed-phase column (250 mm × 4.6 mm, 5 μm) a quaternary gradient pump, and a UV/VIS detector (UV-2075 plus, Jasco International Co., Ltd). HPLC techniques could be specified by different types of mobile phase or eluent, where the

normal phase (NP) and reverse phase are the two major retention mechanisms that have been widely used in the food industry as a modern chromatographic practice (Nollet & Toldrá, 2012).

In the trial done by Olawuyi et al. (2021) for the determination of phenolic compounds of plum (*Prunus salicina L.*), the mobile phase consisted of 1% aqueous acetic acid (A solution) and acetonitrile (B solution) with a flowrate of 0.7 mL/min and column temperature of 25°C, which was adapted from Olawuyi et al. (2020). The gradient elution was conducted by varying the proportion of solution B to A, as it was changed from 10% to 40% of B solution in a linear fashion for 28 min, from 40 to 60% B in linear fashion for 39 min, and then from 60% to 90% in linear fashion for 50 min. Each compound was then identified by pairing the retention times and spectra of samples with those of standards with the UV detector setting at 272 nm. Specifically, the solvent must be degassed before and during the analysis, to avoid the problem with the air bubbles that affect the stability of flow and separation ability, that in turn, might affect the results. Finally, the results could be quantified using a calibration curve of the corresponding standard solution.

Currently, few pieces of the literature are available on the phenolic compounds of plum species. For example, a recent study conducted by Moscatello et al. (2019) identified and determined eight phenolic compounds including catechin, chlorogenic acid, neochlorogenic acid, chlorogenic acid derivates, quercetin, quercetin-3-*O*-glucosides, kaempferol, and kaempferol-3-*O*-glucoside from three plum species including *Prunus*

domestica. Neochlorogenic acid, chlorogenic acid, and catechin were predominantly found in those three plum species. Especially, the former two compounds were greatly found in damson plum species (Moscatello et al., 2019).

2.5.2 Mass spectrometry (MS)

The basic principle of this analytical technique is to generate ions from either organic or inorganic compounds by an appropriate method that could separate these ions by the *mass-to-charge ratio (m/z)* of each compound and detected and identified them qualitatively and quantitatively by the respective *m/z* and their abundance in the sample (Gross, 2017). The analyte could be thermally ionised by an electric field or by impacting energetic electrons, photons, or ions. Ions could be single ionized atoms, clusters, molecules, or their fragments or association. Their separation by *m/z* is associated with dynamic or static electric or magnetic fields. The separation could also be impacted by the field-free regions as it was explained by Gross (2017) that the ions had kinetic energy at the entrance of the flight path. Additionally, apart from electrons, other factors could affect the ionisation of the analyte such as photons, ions, energetic neutral/electronically excited atoms, and even electrostatically charged microdroplets (Gross, 2017).

There are several techniques for ionisation such as electron ionisation, chemical ionisation/desorption, field ionisation, matrix-assisted laser ionisation/desorption, electrospray ionisation, and ambient ionisation or desorption. These various ionisation

techniques and their applications were roughly classified by Andrews (2009) according to the hardness or softness of the ionisation and molecular mass of the analytes.

Liquid chromatography-Mass spectrometry (LC-MS) was used by Jaiswal et al. (2013) to identify and analyse the phenolic compounds in both *Prunus salicina L.* and *Prunus domestica L*. The operation conditions of MS were optimized using 5-caffeoylquinic acid and proanthocyanidin B1 with a capillary temperature of 365°C. The flow rate of dry gas was set at 10 L/min with a nebulizer pressure of 10 psi (Jaiswal et al., 2013).

2.5.3 Gas chromatography (GC)

GC is a technique to separate components from the mixture samples and obtain information about their molecular compositions and amounts. The results of the test would be shown in a chromatogram (a graphical image of a detector output), where the heights and the areas of the resolved peaks, and the retention time for each identified compound were presented (Poole, 2012). Each peak represents a chemical compound in the sample, as it takes different times for the various solute to pass through a chromatography column, while the retention time of each peak represents the time required from injection to detection of the solute that passes through a chromatography column, and the area of the peak represents the amount of specific chemical compound. Standard solutes can be run through the chromatography column under the same analysis condition as a reference to the specific chemical compounds by knowing their retention time. More detailed content such as the ideal gas and the analysis condition

(static or dynamic), temperature, and/or pressure program of this method have been discussed and demonstrated by Poole (2012) and Karasek and Clement (2012). However, this technique is for volatile compounds mainly, so it will not be applied to this current study, as phenolic compounds from damson plums are temperature and pressure sensitive, and no volatile compounds are to be quantified.

2.5.4 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

The principle of the DPPH free radical method assay involves the reduction of this free radical in ethanol solution by the antioxidant molecules from the test material. DHHP itself is the indicator in ethanol as a violet solution, and it will turn colourless when it is reduced by the active compounds (i.e., antioxidants) in the sample. A recent study conducted by Olawuyi et al. (2021) used a DPPH assay for the determination of the antioxidant capacity or radical-scavenging activity of plum juice. They initially prepared DPPH solution using 0.1 mM DPPH in ethanol with equilibrating for two h. Then, 100 μL of diluted juice was mixed with 900 μL of DPPH solution and the sample was set to react at room temperature in the dark for 30 min before it was finally measured by UV spectrophotometer at 517 nm. The results were expressed as mg ascorbic acid equivalent mg ascorbic acid equivalence (AAE）/100 mL of plum juice while they used ascorbic acid as standard (Olawuyi et al., 2021).

2.5.5 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay

The principle of the ABTS assay is to measure the antioxidant capacity in the sample to scavenge the generated ABTS cation in the aqueous phase compared with a Trolox standard (a water-soluble analogue of vitamin E) (Ratnavathi et al., 2016). This assay was recently used by Olawuyi et al. (2021) to determine the radical-scavenging activity of the antioxidant activity of plum juice. A diluted juice sample was initially mixed with 950 μL of ABTS cation solution and reacted in the dark for 30 min (Olawuyi et al., 2021). The ABTS cation solution was previously demonstrated by Olawuyi et al. (2020), which was prepared by the reaction of 7mM of ABTS (Sigma-Aldrich Co., St. Louis, USA) and 2.45 mM aqueous potassium persulfate with 10 mL of distilled water in the dark condition at room temperature for 16 h. Finally, the absorbance was measured at 734 nm using a UV spectrophotometer (Olawuyi et al., 2021). The results were expressed as mg AAE/100 mL of plum juice.

2.5.6 Ferric reducing antioxidant powder (FRAP) assay

The principle of FRAP assay was defined as the involvement of an electron reaction between an electron donor ArOH and an electron acceptor $FE(TPTZ)_2(III)$ to produce $Fe(TOTZ)_2(II)$ and $ArOH^{+\cdot}$ (Ou et al., 2002). This assay was previously done by Ou et al. (2002) on a COBAS FARA II spectrofluorometric centrifugal analyser (Roche) to analyse the antioxidant activities of common vegetables such as white cabbages, carrots, snap beans, and cauliflower. The temperature was controlled at 37°C and pH was

manipulated at 3.6 acid condition. In this assay, antioxidants in the sample reduce Fe (III) or tripyridyltriazine complex to produce the blue ferrous form that could show an increase in absorbance at 593 nm. Finally, the results are presented as micromole Trolox equivalents (TE) per gram on dried basis.

Benzie and Strain (1999) considered the antioxidant as any species that reduced the oxidising species that would otherwise damage the substrate, so they further treated the 'total antioxidant power' as the 'total reducing power'. The antioxidant activity was presented as the reducing capability of the antioxidant. There were three compulsory conditions: 1) only and all antioxidants could reduce Fe $(TPTZ)_2$(III) under thermodynamic conditions; 2) the reaction time should be controlled within four min in the actual FRAP assay, thus the reaction rate must be sufficiently fast enough; and, 3) the subsequent reaction products of the oxidised antioxidant $ArOH^+$ and their absorbance at 593 nm should be zero.

2.5.7 Oxygen radical absorbance capacity (ORAC) assay

The principle of ORAC assay is to compare the area under the fluorescence decay curve of the sample to that of the blank, which contains no antioxidant, to determine the protective effect of the antioxidant. A mole of 2,2′-azobis (2-amidino-propane) dihydrochloride (AAPH) lost dinitrogen to generate two moles of AAPH radical at a constant rate under the reaction condition provided by Ou et al. (2002). The generated AAPH radical rapidly reacted with oxygen in an air-saturated solution to generate

peroxyl radical ROO^- with higher stability. When fluorescein reacts with peroxyl radical, it loses its fluorescence, and this indicates the extent of damage from the reaction. However, antioxidants would donate a hydrogen atom to ROO^- to make hydroperoxide (ROOH) and a stable antioxidant radical (ArO^-) that could inhibit the damage that peroxyl radicals caused to fluorescein.

Ou et al. (2002) also applied the ORAC assay to determine the antioxidant activity of common vegetables. It was carried out on a COBAS FARA IIspectrofluorometric centrifugal analyser (Roche Diagnostic System Inc., Branchburg, NJ). Trolox was used as a control standard, and the environmental condition was controlled at 37°C under pH 7.4 conditions (Ou et al., 2002). The analyser was programmed to record the fluorescence of fluorescein disodium every minute after the addition of AAPH. Finally, the results were calculated based on the differences in areas under the fluorescein disodium decay curve between the samples and a blank and were presented as μmol Trolox equivalents (TE)/g. The detailed procedure also referred to the study conducted by Ou et al. (2001) who significantly improved the ORAC assay compared to its initial version developed by Cao and Prior (1999).

2.5.8 Aluminium colourimetric assay with spectrophotometry

This assay has recently been utilised by Olawuyi et al. (2021) and Teng et al. (2009) to analyse the total flavonoid content (TFC) in the samples of Chinese Quince. Firstly, 70 μL of diluted plum juice was mixed with 430 μL of distilled water, 50 μL of 10%

$Al(NO_3)_3 9H_2O$, and 50 µL of 5% of $NaNO_2$, and kept at room temperature for six min. Secondly, the absorbance was measured by spectrophotometry at 510 nm after adding 500 µL of 1 N NaOH. Finally, the results were presented as mg rutin (mg RE)/100 mL of plum juice (Olawuyi et al., 2021).

2.5.9 Folin-Ciocâlteu method with UV spectrophotometer

Total phenolic content (TPC) can be determined using this method. 100 µL of diluted sample juice is initially mixed with 300 µL of 2% Na_2CO_3 and 50 µL of Folin-Ciocâlteu reagent, a mixture of phosphomolybdate and phosphotungstate, and the whole mixture is stored for 15 min. Next, the absorbance is measured by a UV spectrophotometer at 725 nm (Olawuyi et al., 2021; Olawuyi & Lee, 2019) after adding 1 mL of distilled water to the mixture. Finally, the results are presented as mg gallic acid equivalent (mg GAE)/100 mL of the sample.

2.6 Encapsulation methods for the delivery of phenolic compounds from plums

The application of encapsulation is to protect the encapsulated materials from moisture, heat, or other extreme conditions, to enhance their stability and maintain their viability (Gibbs, 1999). This technique can also mask the odours or tastes of the materials in the food industry. Many techniques have been developed for this purpose, some of which include spray drying, spray chilling or spray cooling, extrusion coating, liposome entrapment, fluidised bed coating, centrifugal extrusion, rotational suspension

separation, coacervation, and inclusion complexation. Different coating agents might be used to encapsulate different materials with any specific method, as the coating could be mechanically ruptured such as by the act of chewing (physical release), melted when exposed to heat (thermal release), or dissolved when placed in solvents (Hegenbart, 1993). This review will classify these encapsulation methods into physical, chemical, and physicochemical methods.

2.6.1 Physical methods

2.6.1.1 Spray drying

In the food industry, spray drying was the most common encapsulating technique because of its rapid drying capabilities, as a result of the large surface area created by atomizing the liquid feed (Jafari & Rashidinejad, 2021; Sharma et al., 2022). Recently, using this technique demonstrated by Munin and Edwards-Lévy (2011), high-quality and stable particles could be produced at a relatively low cost and with a great deal of flexibility.

The principle of this technique involves a specific apparatus that allows the formation of particles from a dispersion of the active compound in a solution of a coating agent or wall material (Figure 2.3) (Vehring, 2008). First of all, a liquid mixture containing the active ingredient and the coating agent in a solvent was atomized into droplets by either a rotary atomizer using a wheel rotating at high speed or a nozzle using compressed gas to atomize the liquid feed (Munin & Edwards-Lévy, 2011). The coating

agent or wall material can be protein (gelatin or sodium caseinate), hydrocolloids (gum arabic), and hydrolysed starch (lactose, starch, or maltodextrin). Secondly, a heated gas (air or nitrogen) is dispersed into contact with the atomized feed fluid with a gas disperser, thus evaporating the solvent. Then, the particle formed as the fluid rapidly evaporates from the droplets and droppes to the bottom of the chamber. Finally, the powder is collected in a bag filter or cyclone from the exhaust gases (Munin & Edwards-Lévy, 2011). Spray drying can also be used as a stabilising dehydrating method without the application of wall materials, apart from its encapsulation function.

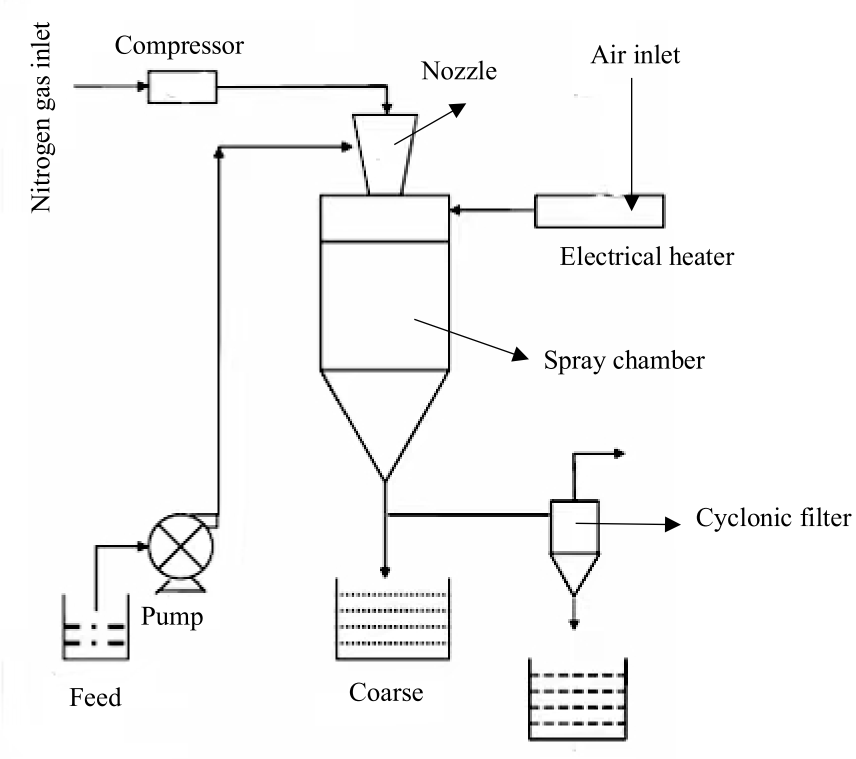


Figure 2. 3. Schematic diagram of a spray drying apparatus. Adapted from Vehring (2008).

2.6.1.2 Fluidised bed coating

Fluidized bed coating was developed by Wurster in the 1950s, and functional

ingredients and additives could be tuned or enhanced using this technology, such as natural and synthetic flavours and spices, vitamins and minerals, acids, and salts preservatives, and processing aids such as leavening agents and enzymes (Dewettinck & Huyghebaert, 1999). A schematic process of this technique is presented in Figure 2.4. In this method, the particles are suspended in a high-velocity air chamber with controlled temperature and humidity, where the coating material is atomized and sprayed counter-currently onto the randomly fluidised particles (Dewettinck & Huyghebaert, 1999). A high-velocity airflow transports the coated particles through the coating zone into the expansion chamber, where they fall back into the container and continue cycling until the entire cycle is completed (Jones, 1988).

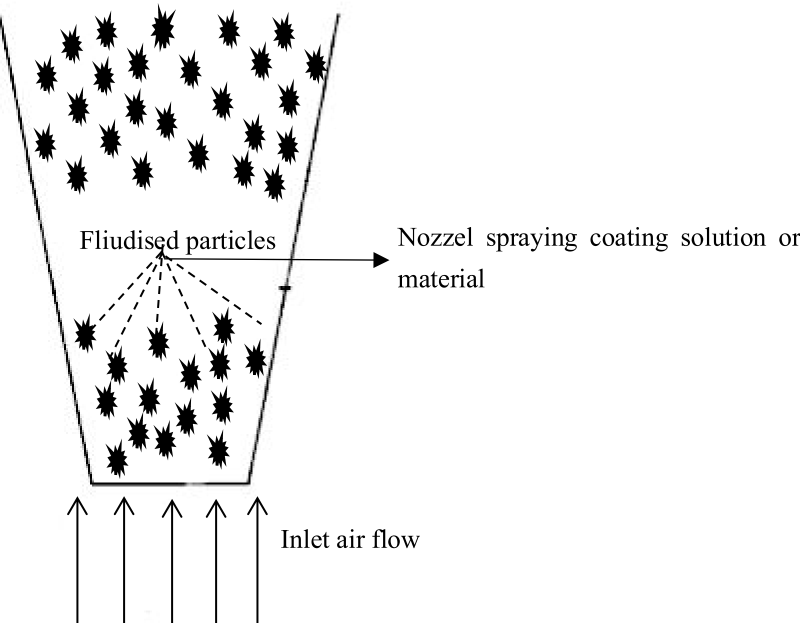


Figure 2. 4. Schematic representation of top-spray fluidized bed coating. Adapted from Dewettinck (1999).

This technique is applicable for not only hot-melt coatings such as stearines, fatty acids,

hydrogenated vegetable oil, and emulsifiers, but also solvent-based coatings like gums, starches, and maltodextrins (Gibbs, 1999). Cool air is used to harden the carrier for hot melt, but hot air is used to evaporate the solvent for solvent-based coating. Water-soluble coatings release their content when they are dissolved in water, whereas hot-melt ingredients release their contents by physical breakage or when the temperature is increased (Gibbs, 1999).

2.6.1.3 Extrusion

This method was initially mainly used for the protection and delivery of colour, vitamin C, and visible flavour pieces, with a shell-life of up to at least two years for the final product in the early stage (Gibbs, 1999). The advantage of using this method is that the material is completely isolated from the wall material. This method is widely used in food applications including cakes, gelatin dessert mixes, and cocktails since the encapsulated materials are soluble in cold or hot water (Gibbs, 1999). Figure 2.5 is a schematic diagram of the hot-melt extruder (adapted from Patil et al. (2016)).

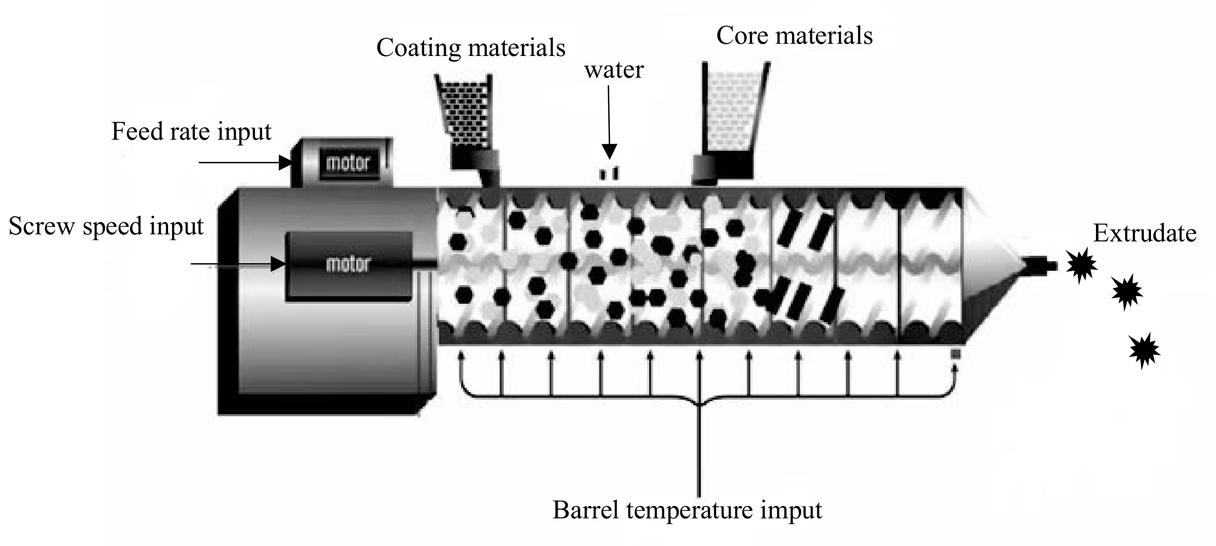


Figure 2. 5. Schematic diagram of hot-melt extruder (adapted from Patil et al. (2016)).

The principle of extrusion technology was defined by Alam et al. (2016) as a process that involves forcing the material to flow under different conditions such as low and high temperature, moisture, and speed through the orifice with various diameters at a predetermined rate. This is to obtain various types of products based on producer specifications or consumer needs. A recent review conducted by Bamidele and Emmambux (2021) categorised the extrusion technology into five types: hot-melt extrusion, melt injection, centrifugal or co-extrusion, electrostatic or electrospinning, and Particle from Gas Saturated Solution (PGSS). The operations of all extruders are similar as they include raw materials (encapsulant and bioactive compounds) feeding through a feeder into the barrel and screws and pushing the material toward die and cutter.

2.6.1.4 Encapsulation processes using supercritical fluids

This encapsulation technique is a comparatively new technology. With differences in pressure and temperature, supercritical fluids could easily change their properties, so they were described as intermediate between liquid and gas (Munin & Edwards-Lévy, 2011). CO_2 is reported as the most widely used supercritical fluid by Munin and Edwards-Lévy (2011), due to its relatively low critical temperature (T_c=304.2 K, or 31.0°C) and pressure (7.38 MPa). Especially, its low T_c supports its high suitability for

processing heat-sensitive materials (such as those present in plums), while CO_2 is used as a non-flammable, non-toxic, and inexpensive material with GRAS status (Munin & Edwards-Lévy, 2011).

The processes of this technique have been divided into three categories, depending on the role of the supercritical fluid that is played in the process. It could be used as a solvent in Rapid Expansion of Supercritical Solution (RESS) and derived processes, or as a solute in Particles from Gas Saturated Solutions (PGSS) and derived processes, or as an anti-solvent in Supercritical Anti Solvent (SAS) and derived processes (Munin & Edwards-Lévy, 2011). The latter two processes could be applied to polyphenol encapsulation, and the processing was demonstrated by Munin and Edwards-Lévy (2011) in detail.

2.6.2 Chemical methods

2.6.2.1 In situ polymerisation

The main process of *in situ* polymerisation is emulsifying the monomer component in an aqueous phase added with a suitable surfactant, and the components are mostly acrylic and vinylic compounds such as methyl methacrylate or styrene (Munin & Edwards-Lévy, 2011). This encapsulation method is mainly used to synthesise nanocomposites. A study conducted by Bernardy et al. (2010) demonstrated the possibility of encapsulating quercetin with the *in situ* polymerisation method. This study revealed the interference induced by the quercetin in the methyl methacrylate

solution on the reaction speed and quality of the polymerisation. Additionally, ascorbic acid facilitated the polymerisation reaction and mitigated the oxidation of immobilised quercetin (Bernardy et al., 2010).

2.6.2.2 Interfacial poly-condensation

Interfacial polycondensation can result in a polymer membrane being formed around emulsion droplets as a chemical reaction (Janssen & Te Nijenhuis, 1992). The continuous phase reacts with the dispersed phase at the interface between them. Each phase of the emulsion has a different monomer type (Figure 2.6). This technique can be used for the encapsulation of both aqueous or organic active ingredients (Munin & Edwards-Lévy, 2011). For active ingredients that are soluble, the procedure is as follows: distilled water is used to make a solution that contains the active ingredient and a water-soluble monomer A. In the organic external phase, the aqueous phase is emulsified, followed by the addition of the organosoluble monomer B. Finally, the O/W interface goes under an interfacial polycondensation process between the two monomers. An organic-active substance in an organic solution can also be used in this approach. The interfacial polycondensation reaction is carried out in a water-in-oil (O/W) emulsion, in this case, using the same technique (Munin & Edwards-Lévy, 2011). There are two possibilities suggested by Munin and Edwards-Lévy (2011). If the oligomer is dissolved in the droplets (Figure 2.6A), a polymeric matrix is formed inside the droplets, resulting in the formation of microspheres. When the oligomer becomes

insoluble in the droplets (Figure 2.6B), a polymeric membrane forms around them, encapsulating the droplets individually. Reservoir microcapsules are formed as a result of this process (Munin & Edwards-Lévy, 2011).

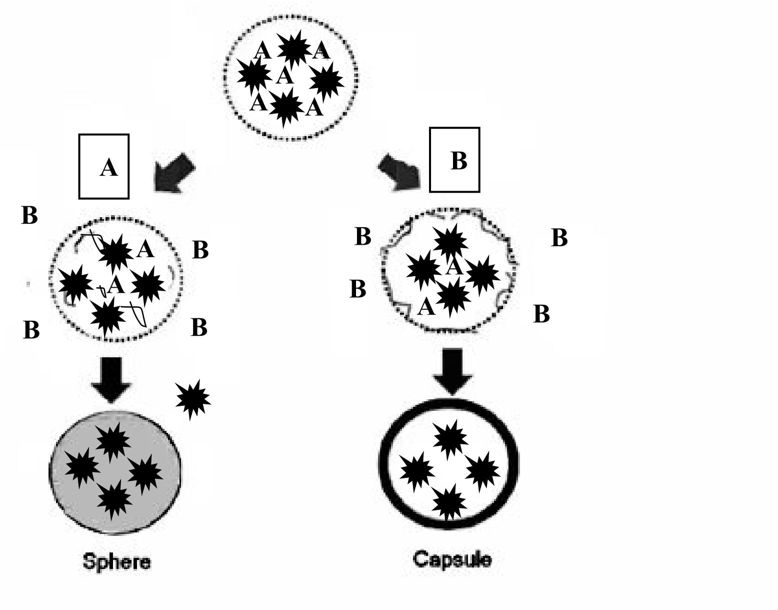


Figure 2. 6. The principle of microencapsulation by interfacial polymerisation. A: The oligomer is soluble in the droplet; B: The oligomer is insoluble in the droplet (adapted from Munin and Edwards-Lévy (2011)).

2.6.3 Physicochemical methods

2.6.3.1 Emulsification-solvent removal methods

The principle of these processes involves the extraction or evaporation of the internal phase of an emulsion inducing the precipitation of the polymer coating in the form of particles (Munin & Edwards-Lévy, 2011).

2.6.3.2 Solvent extraction method or nanoprecipitation

In a solvent extraction method, the solvent must be miscible with water in all proportions. Under agitation, a polymer solution containing an active compound is introduced into an aqueous phase containing a surfactant. As the solvent spontaneously

diffuses in the aqueous phase, nanoparticles are formed by trapping the active ingredient by the insoluble polymer precipitates in the mixture of water and solvent (Munin & Edwards-Lévy, 2011).

2.6.3.3 Solvent evaporation method

In the solvent evaporation process, the polymer is initially dissolved in a volatile solvent that had very low miscibility with water (Figure 2.7) (Munin & Edwards-Lévy, 2011). Secondly, the active ingredient is dispersed or dissolved in the polymer solution. The mixture is then emulsified finely with various methods such as homogeniser or ultrasounds in a large amount of water with surfactants to achieve the oil-in-water (O/W) emulsion (Munin & Edwards-Lévy, 2011). Evaporation of the solvent is achieved by heating and/or under a vacuum and with gentle stirring, but it is not suggested to encapsulate volatile ingredients or compounds that have a higher affinity for the continuous phase. After the solvent removal, the encapsulated particles containing both encapsulants and core materials are washed and collected by centrifugation or filtration, then dried or freeze-dried for the water removal.

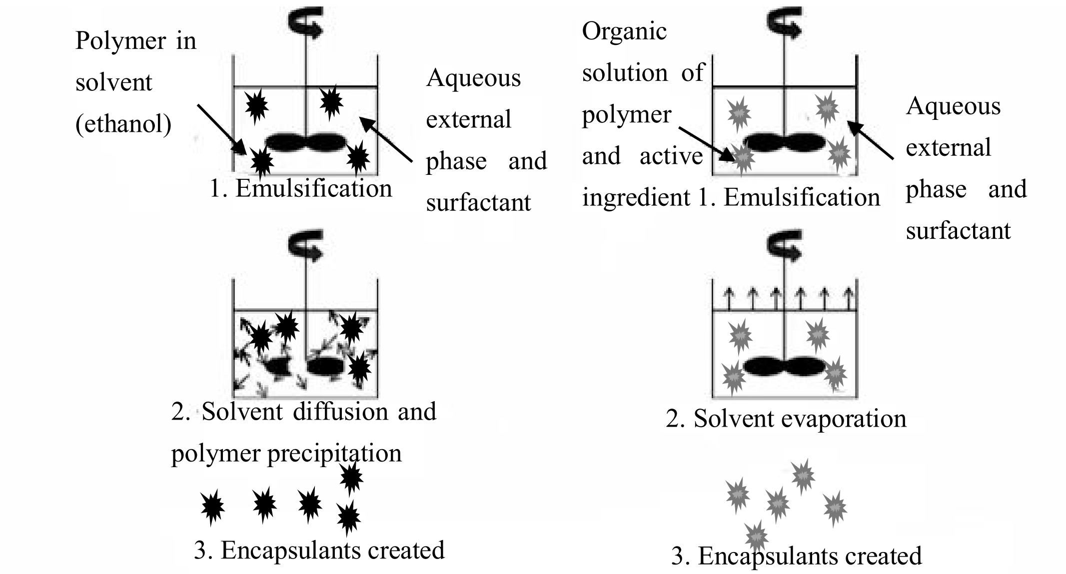


Figure 2. 7. Encapsulation by emulsion/extraction (A) and emulsion/evaporation (B) methods (adapted from Munin and Edwards-Lévy (2011)).

In comparison, the operation speed of solvent extraction is faster than that of solvent evaporation. It has been suggested by Munin and Edwards-Lévy (2011) that the operation speed could directly impact the characteristics of the particles, for example, the solvent extraction method could form the porous microspheres in the encapsulated particles. There is scientific literature that correlates the encapsulation methods with different solvents used for various active ingredients (i.e., polyphenols from plants) (Munin & Edwards-Lévy, 2011).

2.6.4 Methods based on ionic interactions

2.6.4.1 Ionic gelation

The principle of ionic gelation is the formation of alginate gels by ionic cross-linking

with multivalent cations. The process of this method is to extrude an aqueous solution of the polymer through a nozzle or a syringe needle, where the active material was dispersed (Munin & Edwards-Lévy, 2011). Tzatsi and Goula (2021) suggested that ionic gelation was an effective way to prevent phenolic extracts from deterioration. They encapsulated the extract from chokeberries with spray drying, co-crystallisation, and ionic gelation. The encapsulation efficiency was optimised up to 94.2% with the concentration of 20.0% w/w extract, 2.0% w/w sodium alginate solution, and a calcium chloride solution of 2.5% w/v.

2.6.4.2 Complex coacervation

The principle of this method is to create a neutral, liquid, polymer-rich phase named coacervate based on the capability to interact between cationic and anionic polymers, which are water-soluble (Munin & Edwards-Lévy, 2011). The process of this method was to separate two miscible liquid phases, one a dilute equilibrium phase and the other a dense coacervate phase, from an aqueous polymeric solution (Munin & Edwards-Lévy, 2011). Complex coacervation can spontaneously start as the mixing of polyelectrolytes with opposite charges in an aqueous solution (Timilsena et al., 2019); when the charges are strong enough to induce interaction but not excessively strong to form precipitation (Munin & Edwards-Lévy, 2011).

According to a study by Deladino et al. (2008), two processes included ionic gelation

of calcium alginate and complicated coacervation between chitosan and calcium alginate, were used to successfully encapsulate a yerba mate (*Ilex paraguariensis*) freeze-dried extract.

2.6.4.3 Acidic precipitation

The principle of this method is to encapsulate the active ingredient in calcium or sodium caseinate beads by acid precipitation of the casein (Munin & Edwards-Lévy, 2011). A study conducted by Dehkharghanian et al. (2009) optimised the most efficient material to form the beads that preserved the highest antioxidant properties among sodium caseinate bead, sodium caseinate bead solution with green tea polyphenol extracts (GTPE), calcium caseinate bead solution, and calcium caseinate bead solution with GTPE. The precipitation process involved the dissolution of the bead solutions in 1 mL of deionised and distilled water at 80°C in an Eppendorf tube for 6 h and the addition of 500 µL of trichloroacetic solutions in 500 µL of bead solutions. Solutions were centrifuged at 4°C and 15,000 rpm for 15 min after they were incubated in an ice bath for 15 min. Supernatants were collected for measuring the antioxidant activity (Dehkharghanian et al., 2009). It was suggested that polyphenolic extract loaded with calcium caseinate beads made encapsulated presented better antioxidative properties than others (Dehkharghanian et al., 2009).

2.6.5 Methods based on hydrophobic interactions

2.6.5.1 Micelles

The principle of the formation of micelles is the self-assembly of amphiphilic molecules (Joseph et al., 2017). The most common methods for micelle preparation so far include solid dispersion, O/W emulsion, solvent evaporation, and dialysis (Joseph et al., 2017). Amphiphilic polymers, in an aqueous solution, could self-organise into supramolecular arrangements possessing a hydrophilic crown and hydrophobic central core (Xu et al., 2013). Micelles were recommended by Joseph et al. (2017) to deliver both hydrophilic and hydrophobic bioactive materials. As for surfactant micelles, they could be achieved if the polymer concentration was higher than the critical micellar concentration in the solution (Xu et al., 2013). For example, Ray et al. (2011) used a micelle-forming polymer to encapsulate curcumin (the primary bioactive substance in turmeric) and showed the possibility to treat Alzheimer's disease (AD) (Mishra & Palanivelu, 2008). This polymer was used in its hydrophobic core (NanoCurc®) using nanotechnology to protect and preserve isolated curcumin in tiny particles to be more bioavailable. The authors (Mishra & Palanivelu, 2008) also suggested that the encapsulated curcumin in NanoCurc® micelles could potentially be delivered to the brain in considerably higher concentrations than free curcumin.

2.6.5.2 Liposomes

This technique was initially demonstrated and synthesised in 1965, and the principle of liposomes is artificial encapsulants formed by one or more concentric lipid bilayers containing hydrophilic and hydrophobic tails separated by water compartments (Figure 2.8). The function of liposomes was claimed to target, protect, immobilise, release, or isolate lipophilic, hydrophilic, or amphiphilic substances because of their structure (Munin & Edwards-Lévy, 2011; Wen et al., 2018).

Munin and Edwards-Lévy (2011) suggested that liposomes were distinguished based on their size and number of lamellae, with small or large unilamellar (SUV or LUV) and multivesicular vesicles (MVV) or large multilamellar vesicles (MLV) being the main classes. Table 2.5 is a review of different liposomal techniques that have been used for the encapsulation of phenolic compounds.

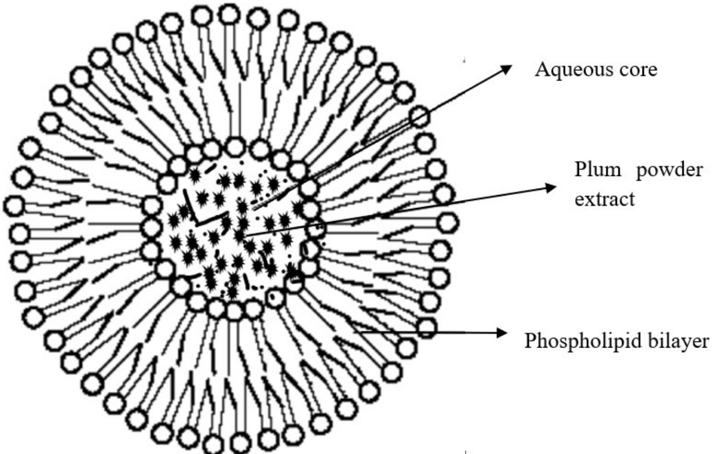


Figure 2. 8. A schematic representation of a liposome.

Table 2. 5. Some examples of a variety of liposome techniques that have been employed for the encapsulation of polyphenols (adapted from Munin and Edwards-Lévy (2011)).

Polyphenol(s)	Application(s)	Biological activitie(s)	Loading capacity (if known)	Route of administration
Curcumin	Photo-ageing attenuation (demonstration in mice)	Antioxidant, anti-inflammatory, and photo-protector	-	Oral
Resveratrol	Improvement of the cellular answer to oxidative stress via rapid and potent cellular internalisation;	Antioxidant and photo-protector	>70%	*In vitro*
	nano-sized vesicles; the inclusion of resveratrol retarded drug release in vitro; this system was associated with no or poor liver and kidney toxicity *in vivo*.	Cardiovascular protector	≈70%	*In vitro* and *in vivo* Intraperitoneal injection
Quercetin	Reduced anxiety and cognitive functions; the dose administered decrease; increase in circulation time; vectorisation; increase in brain penetration efficiency.	Antioxidant and anti-cancer	≈60%	Nasal
	Biodisponibility increased; vectorisation; hepatic membrane penetration efficiency greatly improved.	Hepato-protector	-	Transdermie
Myrtle Theme (*Thymus sp.*) extracts	Antioxidant and antimicrobial activities are superior to free forms.	Antioxidant and antimicrobial	-	*In vitro*
Silymarin	Biodisponibility increase	Hepato-protector	>69%	Oral

Catechin	Skin penetration efficiency improved.	Chemo-protector and antioxidant	>90%	Transdermie
Catechin, (-)-epicatechin and EGCG	Biodisponibility; EGCG encapsulated has tissue penetration ability improved versus 2 other catechins.	Antioxidant and anti-cancer		Intratumora
	Liposomes may influence drug deposition in tumour tissues	Antioxidant and anti-cancer		Topical application and intratumoral
Tea extract	Stability at 4°C increased.	Feasibility evaluation		*In vitro*

There are several methods for the manufacture of liposomes, which have been reviewed in detail by Meure et al. (2008). The most classical method recommended by Munin and Edwards-Lévy (2011) was realised when dried phospholipid films were hydrated, and the Bangham method was invented and named after Alec Bangham (Bangham et al., 1965).

The application of liposomes can enhance the bioavailability and bioactivity of polyphenols (Munin & Edwards-Lévy, 2011). For example, Takahashi et al. (2009) used lecithin liposomes to encapsulate curcumin, a polyphenolic pigment with very powerful antioxidant properties, to achieve better bioavailability and functionality. The encapsulation efficiency was 68%, and the manufactured liposomes were SUV of 263 nm in size.

Although damson plum extract has not been reported to be encapsulated using this method yet, Kanlayavattanakul et al. (2013) encapsulated Salak plum peel extract containing caffeic, gallic acid, and chlorogenic acids (which can also be found in

damson plums) in liposome for cosmetics purposes. They optimised the entrapment efficacy of the liposomes by altering the ratio of lecithin to hydrophobically modified hydroxyethylcellulose to cholesterol. Results showed that the highest entrapment efficacy of the liposomes (89.235±1.499%) could be achieved with the referred mole ratio at $7:2\times10^{-9}:0$, and the liposomes were LUV of 105.33±1.75 nm in size. However, liposomes also have several restrictive limitations in terms of their application in the food industry (Lemercier & Huille, 2001; Wen et al., 2018). For example, they can be quite unstable in biological fluids, as well as under the manufacturing, processing, and storage conditions of the product they were added to. Furthermore, the efficiency of loading the active ingredients is comparatively low. Nevertheless, the most significant disadvantage of the application of liposomes on the industrial scale has been their low reproducibility (Munin & Edwards-Lévy, 2011).

2.6.6 Other methods

2.6.6.1 Encapsulation in yeasts

Yeast cells (e.g., *Saccharomyces cerevisiae*) have been used for the encapsulation of essential oils and aromas in the early years, as the encapsulant material was cheap and also effective with a high loading capacity (Bishop et al., 1998). The advantage of using this method was that the permeable cell membrane guaranteed an active diffusion, and it could strongly protect the enclosed material from evaporation or oxidation phenomena. Nowadays, this method is suitable for the encapsulation of water-soluble

polyphenols. For example, baker's yeast cells have been used to encapsulate chlorogenic acid in the trials conducted by Shi et al. (2007). A plasmolysing agent (NaCl 5%) was applied to induce the autolysis of cells and empty the cells' content. The prepared empty cells were then dispersed or diffused in an aqueous phase solution containing chlorogenic acid and loaded by re-swelling in the solution. Encapsulation efficiency of up to 12.6% could be achieved when chlorogenic acid with a purity of 98% was used (Shi et al., 2007). Although this encapsulation method improved the stability of the core materials under hydric and thermal stress, it did not hinder the *in vitro* release (Shi et al., 2007).

2.6.6.2 Co-crystallisation

The principle of this process is to introduce the aromatic or active ingredients into a saturated sucrose solution. The crystallisation of this sucrose solution can be spontaneously achieved at a high temperature (above 120°C) with low humidity (Munin & Edwards-Lévy, 2011). As the sucrose crystal structure is modified, small crystal aggregates (<30 μm) trap the active ingredient molecule (Munin & Edwards-Lévy, 2011). It was also suggested by Bhandari and Hartel (2002) that this technique offered an economic and flexible alternative due to its simplicity. It can also enhance the physical properties (e.g., solubility, flowability, stability, wettability, anti-dusting, and anticaking) of the active ingredient/compound and even can mask its bitter taste.

The granular product obtained from this technique possessed good stability and fluidity,

but low hygroscopicity (Munin & Edwards-Lévy, 2011). Additionally, this technique is flexible, simple, and cheap to implement. It was successfully used to encapsulate a yerba mate extract, which contains caffeic acid derivatives and flavonoids, in a saturated sucrose solution (Deladino et al., 2007). The size of crystals achieved was in the range of 2 and 30 µm. This technique considerably lowered the hygroscopicity of the yerba mate extract without altering its high solubility. Munin and Edwards-Lévy (2011) suggested that this approach seemed to be a promising alternative to preserving phenolic compounds in future industrial practice and application. Nevertheless, there is no available study about the encapsulation of plum extract by this process.

2.7 Possible food applications for bioactive/phenolic compounds from damson plums

The extracted bioactive compounds from damson plums may be incorporated into different food products so that they can widely be integrated into daily food consumption that satisfies the nutritional requirements of different people. Such food products might include various types of snacks (e.g., a nut bar, nutrition bar, cheese, yoghurt) or drinks/beverages (e.g., milk, drinking yoghurt, smoothie, or juice).

Currently, there is no evidence of the application of damson plum extracts in any food materials. However, the bioactive compounds from other ingredients have been applied to the food and the effect on the original food properties such as sensorial, nutritional, and functional profiles has been studied. For example, a study conducted by Ramírez-

Jiménez et al. (2018) suggested that the addition of bean flour to a snack bar improved its nutritional and bioactive profile and achieved a product with less calorie content than the oats-based snack bars. In another study conducted by Zoidou et al. (2014), oleuropein was introduced as a bioactive ingredient in milk and yoghurt to create novel functional foods with considerable health advantages. They also recommended that high-oleuropein olive extracts could be used to make similar dairy products, as the sensory panel described all the products as "very good" with no off flavours.

Additionally, Komes et al. (2013) investigated the impact of adding five dried fruits (dried prunes, papaya, apricots, raisins, and cranberries) to bitter or milk chocolate, to assess the impact on their polyphenolic content and antioxidant capacity. They (Komes et al., 2013) concluded that bitter chocolates had higher polyphenol content and radical scavenging activity than milk chocolates; however, adding dried cranberries and raisins to chocolate boosted its polyphenol content when compared to plain chocolate. Chocolates with dried cranberries and apricots were rated the best in terms of sensorial acceptability (Komes et al., 2013).

Green tea catechins were encapsulated with soy lecithin and applied in a novel functional full-fat hard cheese by Rashidinejad, Birch and Everett (2016). They studied the recovery rate of the individual catechins in liposomes after their incorporation into full-fat cheese by HPLC during a 90-day ripening period. The chemical composition and pH of the samples were also compared to indicate the effect of liposomal encapsulants containing different concentrations of catechin and green tea extract on

the nutrition composition of cheeses and their physical stability. They found the nanoliposomes obtained from soy lecithin as coating material protected the green tea catechins from degradation during cheese ripening. The encapsulated green tea extract containing a high concentration of antioxidants could be potentially delivered to the human body, according to the findings from the *in vitro* digestion study (Rashidinejad, Birch, & Everett, 2016).

O'connell and Fox (2001) suggested that the addition of exogenous phenolic compounds from plants such as hawthorn, raspberry, and strawberry could make milk and dairy products more suitable candidates as functional ingredients. Such a practice could enhance the product quality and its health effects, due to the greater chemical reactivity of the flavonoids and tannins. Servili et al. (2011) utilised phenolic compounds extracted from olive vegetation water to make a functional milk beverage fermented with lactic acid bacteria. In their study, the milk beverages fortified with phenolic compounds with 100 and 200 mg/L were not significantly changed during fermentation and further storage. The phenolic composition was not altered after the fermentation compared to the phenol extract from olive vegetable water. This indicated that the physiochemical reaction in functional milk during fermentation and storage did not significantly affect the phenolic compounds extracted from olive vegetation water. In other words, the exogenous phenolic compounds were stable in the functional milk product. However, there no study was found on the effect of phenolic compounds extracted from damson plum on the properties of milk or other food/beverage products.

2.8 Review summary

In conclusion, this systematic review has introduced the general nutritional value and bioactive/phenolic compounds of damson plums, as well as the corresponding health-promoting effects of these bioactive compounds. This review also presented the possible methods for extraction, identification, and encapsulation of the phenolics from these plums. The possible functional food products that could be used for the incorporation of damson plum extract or its isolated bioactive compounds have also been covered. No systematic study was found on the assessment of various properties of damson plums or the extraction, isolation, and encapsulation of their phenolic compounds, or the application of damson plum extract (or its isolated phenolic compounds) in any functional food product. However, some studies have recommended the application of extracts from the fruit in milk and dairy products. Damson plums could be one of the most suitable candidates for this purpose, due to their high antioxidant activity. Therefore, the current study will be a novel investigation to fill the existing gap in the literature about damson plums, their phenolic/antioxidant properties, protection of their bioactivity using encapsulation technology, and their incorporation into functional food/beverage products.

3 Materials and Methods

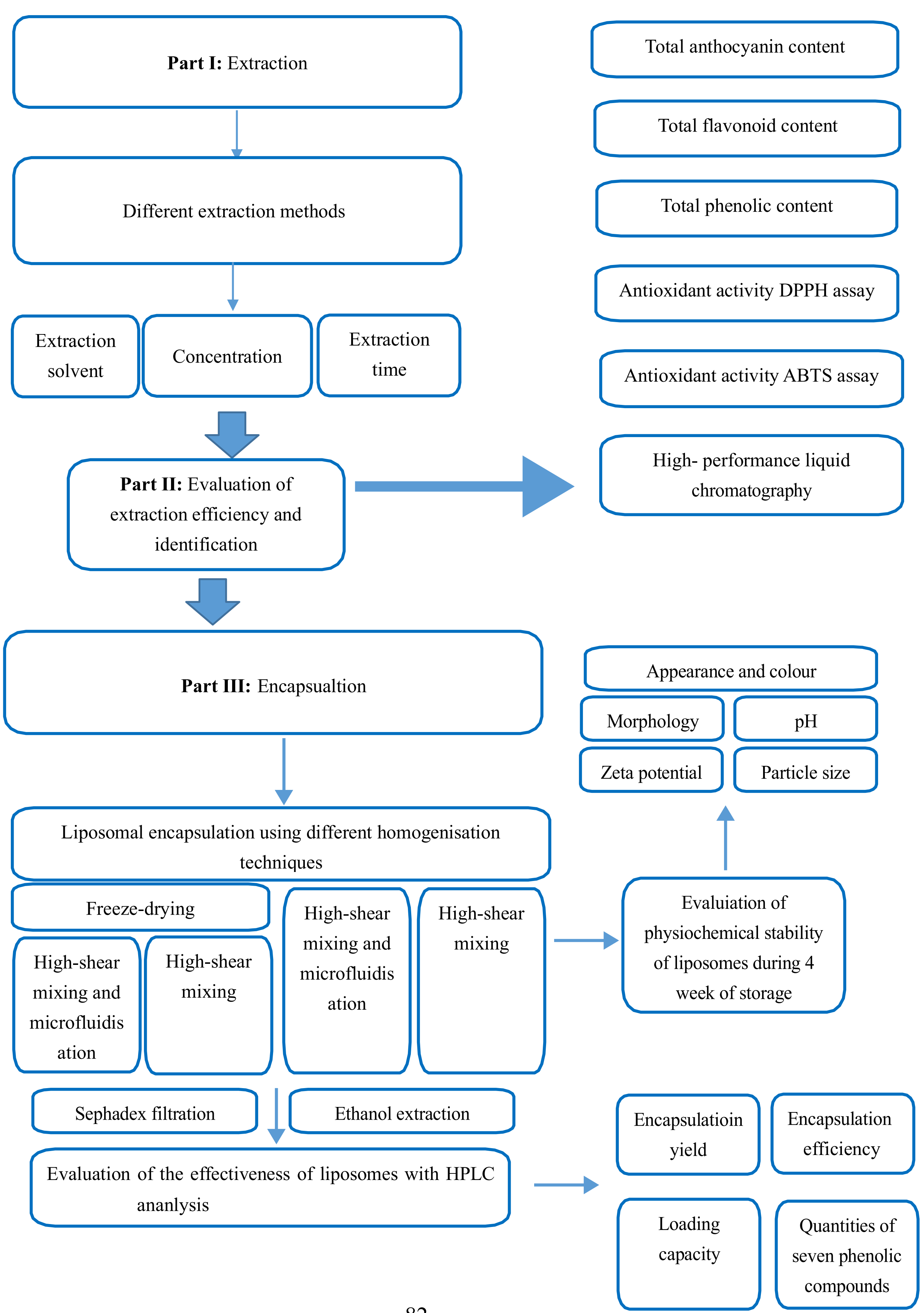
3.1 Materials

Fresh New Zealand damson plums were donated by Foot Steps Ltd. (Karamu, Hastings, New Zealand) and stored at -20°C upon arrival. The fruit was then freeze-dried and ground to obtain a food ingredient that is named 'freeze-dried damson plum powder' (FDDPP) in this study. Catechin, epicatechin, naringenin, gallic acid monohydrate, rutin hydrate, quercetin, neochlorogenic acid, rosmarinic acid, and cyanidin 3-glucoside chloride (HPLC grade) standards were purchased from Sigma-Aldrich Co., Inc. (Darmstadt, Germany). 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) and Folin–Ciocâlteu's reagents were procured from Sigma-Aldrich Co., Inc. (Darmstadt, Germany) and Merck Co., Inc. (New Jersey, USA), respectively. Pectinex Ultra SPL from Aspergillus aculeatus (3800 units/mL) was obtained from Sigma-Aldrich Co., Inc. (Darmstadt, Germany). Lotus™ Lecithin Granules GMO-Free was purchased from My Natural Health Ltd. (Manukau, Nea Zealand). The instant milk powder (Pams™ whole milk) was purchased from a local supermarket (PAK'nSAVE, Palmerston North, New Zealand) and was stored at room temperature until used. All chemicals and reagents used in this study were of analytical grade.

3.2 Methods

3.2.1 Extraction of phenolics from FDDPP

The extraction of phenolic compounds from FDDPP was based on the methods developed by Olawuyi et al. (2021) with modifications, as presented in Figure 3.1. This resulted in an extract that is referred to as 'freeze-dried damson plum powder extract' (FDDPPE) hereafter. Four methods of extraction were applied: i.e., enzyme-assisted extraction (EAE), ultrasound-assisted extraction (UAE), the combination of enzyme and ultrasound-assisted extraction (E+UAE), and accelerated solvent extraction (ASE). Milli-Q® water was used as the solvent for EAE, UAE, and E+UAE, while ethanol and milli-Q water were applied for the ASE. The volume of extraction solvents was controlled at 20 mL, and the temperature was controlled at 45°C throughout the extraction process. The extraction time (0, 20, 40, and 60 min) was the variable for EAE, UAE, and ASE. All samples were centrifuged at 1370 × *g* for 10 min at room temperature after the extraction (Le & Le, 2012), and they were stored at -20°C under dark conditions until further processes.



Part I: Extraction
Different extraction methods
Extraction solvent
Concentration
Extraction time
Part II: Evaluation of extraction efficiency and identification
Total anthocyanin content
Total flavonoid content
Total phenolic content
Antioxidant activity DPPH assay
Antioxidant activity ABTS assay
High- performance liquid chromatography
Part III: Encapsualtion
Liposomal encapsulation using different homogenisation techniques
Freeze-drying
High-shear mixing and microfluidis ation
High-shear mixing
High-shear mixing and microfluidis ation
High-shear mixing
Evaluiation of physiochemical stability of liposomes during 4 week of storage
Appearance and colour
Morphology
pH
Zeta potential
Particle size
Sephadex filtration
Ethanol extraction
Evaluation of the effectiveness of liposomes with HPLC ananlysis
Encapsulatioin yield
Encapsulation efficiency
Loading capacity
Quantities of seven phenolic compounds

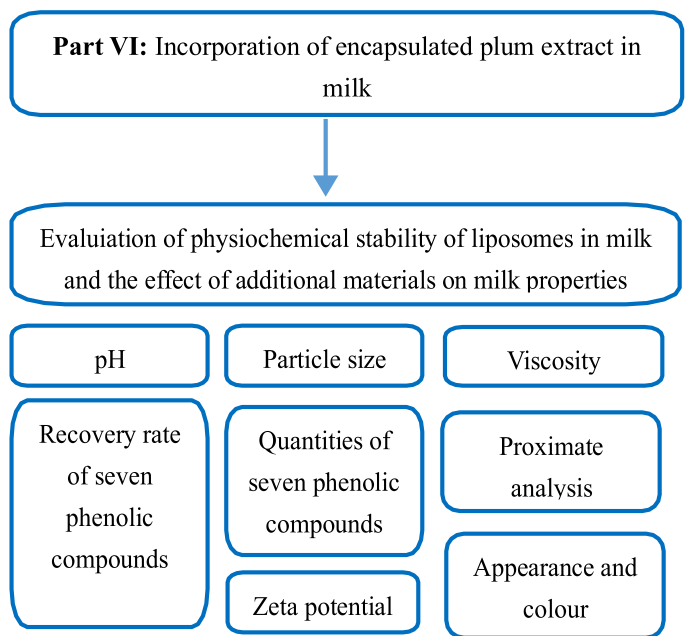


Figure 3. 1. An overall overview of the experimental design.

3.2.1.1 EAE

An aliquot of 40 µL Pectinex Ultra SPL from Aspergillus aculeatus (3800 units/mL) was added to 20 mL of sample solution (1g of FDDPP in 20 mL milli-Q water). The enzyme concentration for this extraction was 0.2% v/v (Olawuyi et al., 2021). The extraction was conducted in a 45°C incubator for 0, 20, 40, and 60 min. Samples were in a 90°C water bath for enzyme inactivation after the extraction and incubated at 45°C for 65, 45, 25, and 5 min before being centrifuged (Heraeus Multifuge X3R, ThermoFisher, Germany) at 1370 × *g* for 10 min at room temperature. The supernatants were collected and stored at -20°C under dark conditions for further analyses.

3.2.1.2 UAE

The temperature of both the ultrasound bath (Brandelin Sonorex Digitec, Germany) and the water bath was set at 45°C. Sample solutions were kept in a water bath for 0, 20, 40, and 60 min after the extraction in the ultrasound bath at 38 kHz for 0, 20, 40, and 60 min. Samples were centrifuged (Heraeus Multifuge X3R, ThermoFisher, Germany) at 1370 × *g* for 10 min at room temperature, and the supernatants were collected and stored in dark at -20°C until further processes.

3.2.1.3 E+UAE

Samples were prepared the same way as used for EAE; however, the extraction was conducted in an ultrasound bath at 45°C, and sample solutions were kept in a 90°C water bath for 2 min after the extraction in the ultrasound bath at 38 kHz for 0, 20, 40, 60 min before being kept in the 45°C water bath for 60, 40, 20, 0 min. Samples were centrifuged (Heraeus Multifuge X3R, ThermoFisher, Germany) at 1370 × *g* for 10 min at room temperature, and the supernatants were collected and stored in dark at -20°C for further analyses.

3.2.1.4 ASE

An Accelerated Solvent Extractor (350 DIONEX, Dionex Corp., Bannockburn, IL, USA) with 22 mL of extraction cell was selected for this type of extraction. One cycle

extraction, 90% of manual rinse volume, and 5 mL of automatic rinse volume (system rinse not collected) were set up. A cellulose filter pad (ASE Extraction Filters, Thermo Scientific, Australia) was installed at the bottom of the extraction cell. A layer of 1 cm depth of diatomaceous earth (DE)/MAP mixture was filled under the cellulose filter pad, and 1 cm depth of DE/MAP and 1 g of FDDPP sample mix was in the middle layer, while the top was filled up with white sand (ECP Ltd.). The temperature was maintained at 45°C through the extraction process for 20, 40, and 60 min. Samples were centrifuged (Heraeus Multifuge X3R, ThermoFisher, Germany) at 1370 × *g* for 10 min at room temperature, and the supernatants were collected and stored in dark at -20°C for further analyses.

3.2.2 Identification

3.2.2.1 Total phenolic content (TPC)

TPC was determined using the Folin–Ciocâlteu method developed by Singleton et al. (1999) with some modifications. A 96-well plate (ThermoFisher Scientific™, the USA) was used for loading samples and reading the absorbance. 50 µL of diluted sample solutions or gallic acid standard solutions were mixed with 25 µL of Folin–Ciocâlteu reagent, and 150 µL 2%Na_2CO_3 and kept in dark for 15 min (Olawuyi et al., 2021). The sample solution was diluted with 50% of ethanol and the absorbance was measured by the microplate reader of the UV spectrophotometer (GENESYS 10 Series, UV-vis,

USA) at 725 nm. The results were expressed as mg gallic acid equivalent (mg GAE)/g of FDDPP according to the absorbance of the gallic acid standard curve.

3.2.2.2 Total flavonoid content (TFC)

TFC was analysed using an aluminium colourimetric assay (Teng et al., 2009). A 96-well plate was used for loading samples. Approximately, 19 μL of sample solutions or rutin standard solutions were mixed with 116 μL of 50% ethanol, 14 μL of $NaNO_2$, and 14 μL of 10% $Al(NO_3) \cdot 9H_2O$. 135 μL of NaOH was added after the plate was kept in dark at room temperature (25°C) for 6 min (Olawuyi et al., 2021). The absorbance was measured by the microplate reader of the spectrophotometer (GENESYS 10 Series, UV-vis, USA) at 510 nm, and the results were expressed as mg rutin equivalent (mg RE)/g of FDDPP according to the absorbance of the rutin standard curve.

3.2.2.3 Total anthocyanins content (TAC)

TAC was identified using the AOAC pH-differential method (Lee et al., 2005). The pH 1.0 potassium chloride (0.025 M buffer) and pH 4.5 sodium acetate (0.4 M buffer) were prepared. Two 96-well plates were used for loading samples. In plate A, 20 μL of diluted sample solutions or cyanidin-3-glucoside standard solutions were mixed with 180 μL potassium chloride buffer for 15 min. In plate B, 20 μL of diluted sample solutions were mixed with 180 μL of sodium acetate buffer and incubated for 15 min. The absorbance

at both 520 nm and 700 nm was read using the microplate reader of the spectrophotometer (GENESYS 10 Series, UV-vis, USA) for both plates A and B. The TAC values were calculated using the following formula:

$$Cyanidin - 3 - glucoside\ equivalent\ (mg) = \frac{Ab \times MW \times DF \times 1000}{\varepsilon} \quad (1)$$

$$Ab = (Ab520nm - Ab700nm)(pH\ 1.0) - (Ab520nm - Ab700nm)(pH\ 4.5) \quad (2)$$

Where, MW stands for molecular weight = 449.2 g/mol for cyanidin-3-glucoside; DF is dilution factor studied according to the original extraction ratio (mL/mg); and, ε indicates the molar extinction coefficient = 26900/L/M/cm. Results were expressed as mg cyanidin-3-glucoside equivalent per g of FDDPP (Lee et al., 2005).

3.2.2.4 ***ABTS radical-scavenging activity***

ABTS radical-scavenging activity was determined using the method developed by Arnao et al. (2001) and modified by Olawuyi et al. (2020). In this trial, the ABTS cation was previously generated through a 1:1 volumetric reaction between a 2.45 mM potassium persulfate solution and a 7 mM ABTS solution. The mixture was kept in the dark at room temperature for more than 16 h before use. The mixture was diluted 50 times with 50% of ethanol to achieve an absorbance of 0.7±0.02 at 734 nm, read using a spectrophotometer (GENESYS 10 Series, UV-vis, USA). In the adopted method, a

96-well plate was used for loading samples. 20 μL of 50% ethanol (blank), sample solutions, or ascorbic acid standard solutions was added to 280 μL of ABTS cation solution in each well. The mixture was incubated in dark at room temperature for 6 min, and the absorbance was read at 734 nm. Results were expressed as mg ascorbic acid equivalent to mg AAE/g of FDDPP (Olawuyi et al., 2020).

3.2.2.5 DPPH radical-scavenging activity

DPPH radical-scavenging activity was measured using the method described by Blois (1958) with some modifications. A 0.1 mM DPPH stock solution was prepared in ethanol. DPPH was kept in dark for 2 h with stirring before being used. A 96-well plate was used for sample loading. 20 μL of the standard solutions (ascorbic acid), a blank sample (50% ethanol), and the sample solutions were mixed with 280 μL of DPPH solution and reacted in the dark at room temperature for 30 min. The absorbance was read using a spectrophotometer (GENESYS 10 Series, UV-vis, USA) at 517 nm. Results were calculated and presented as mg AAE/g of FDDPP.

3.2.2.6 High-performance liquid chromatography (HPLC) analysis

The concentrations of neochlorogenic acid, gallic acid, catechin, epicatechin, rutin, rosmarinic acid, naringenin, and quercetin in FDDPP sample extract were determined using the HPLC method adopted from Podse˛ dek et al. (2006). An Agilent 1200 series

HPLC machine equipped with a Kinetex® 2.6 µm XB-C18 (LC Column 100 X 4.6 mm) was employed for this experiment. The extracted sample solutions were diluted twice and ultra-centrifuged at 14,100 × *g* for 10 min before being injected into the HPLC system. The binary mobile phase consisted of 6% acetic acid in 2 mmol/L of sodium acetate (mobile phase A) and 100% acetonitrile (mobile phase B). A 20% of methanol mixture was used as a blank to wash the needle and column in between the injections. The injection volume was set as 5 µL, the flowrate was set as 1 mL/min, and the total run time was 70 min for each sample. A gradient programme was applied according to Podsędek et al. (2006) as follows: 100-85% A from 0-45 min, 85-70% A from 45-60 min, 70-50% A from 60-65 min, and 50-0% A from 65-70 min. The column temperature was set at 25°C. The UV detector was set at four wavelengths of 280, 320, 360, and 520 nm for the determination of eight phenolic compounds including neochlorogenic acid, gallic acid, rosmarinic acid, catechin, epicatechin, rutin, naringenin, quercetin, according to the retention times and spectra with those of standards (Podsędek et al., 2006). The calibration curves of corresponding standards (0-100 µg/mL) were made for calculation. However, quercetin was not found in any of samples using HPLC analysis in current study.

3.2.3 Liposomal encapsulation

3.2.3.1 Liposome manufacture

The preparation of liposomes was adapted from the approach developed by Rashidinejad et al. (2014) with slight modifications. Soy lecithin granule (1% w/v) was dispersed in the diluted solution of FDDPPE in acetate buffer (pH 3.8) or only acetate buffer (control). The whole dispersion process took 30 min with a magnetic stirrer at 500 rpm (Shaker et al., 2017). Two treatments were applied for the manufacture of the liposomes. For Treatment 1, a high-shear mixer (D500 series, Labserv, Germany) was used for both control and the samples containing the extract, based on the method from Rashidinejad et al. (2014). The rotary speed was set at 24,000 rpm for five rounds, and each round consists of a 1min burst and a 30-second stop cycle. For Treatment 2, a microfluidiser (M-110P Microfluidizer™, Westwood, MA 02090, USA) was also used after the high-shear mixing. Samples or control were homogenised at the pressure of 24,000 psi (one cycle).

3.2.3.2 Morphology of liposomes

Transmission Electron Microscopy (TEM) was used to assess the morphology of the obtained empty and FDDPPE-loaded liposomes made by microfluidisation and/or high-shear homogenisation. Samples were prepared in agarose tubes before being dyed. Samples were incubated with glutaraldehyde in sodium cacodylate buffer at room

temperature for 45 min twice (buffer was discarded in between) before being incubated with 1% osmium in cacodylate buffer for one hour. Samples with osmium were stored at 4°C overnight. The samples were washed three times with cacodylate buffer after their temperature returned to room temperature, followed by consecutive dehydration steps with 25%, 50%, 75%, 95%, and 100% acetone in triplicate on a rotator (45 min for each step). A 50:50 mixture of resin and acetone was added to the samples and incubated overnight on a rotator. Then they were incubated with 100% resin on a rotator for 8 h before being incubated with 100% resin with catalyst overnight on a rotator. The previous process was repeated three times, the samples were embedded in a silicone mould and incubated in an oven at 60°C for 48 h before they were cut into the semithin section.

A simple observation of samples was done to find the samples on the semithin section before the preparation of the ultrathin section. After this, the staining process was conducted with uranyl acetate and lead citrate as follows. The staining processes took 4 min for each colouring agent, and samples were washed three times with distilled water before, in between, and after the staining processes. Finally, the microstructure of liposomes was studied under TEM (FEI Tecnai G2 Spirit BioTWIN, Czech Republic) and micrographs were taken with a Veleta CCD camera (Olympus Soft Imaging Solutions, Germany).

In terms of sample preparation for TEM analysis in this experiment, the first part of the sample preparation was the fixation. Currently, two fixation methods (chemical fixation

and cryofixation) can be used for the primary fixation of such samples. Chemical fixation reported by Li et al. (2017) was conventionally employed for molecular biology applications such as clinical sample preservation, and nano cytometry. However, cryofixation was suggested by Ebersold et al. (1981) as the best method to preserve ultrastructure in biological samples because the samples prepared by cryofixation are free from the by-product produced by chemical fixation, dehydration, cryo-protectants or resin embedding and do not alter the milieu in the specimen as the molecules are immobilised within milli-seconds (Muller, 1984). In the case of the current research, chemical fixation was more achievable, though the other method could help obtain better results. 2.5% glutaraldehyde was used for the primary chemical fixation. This agent was suggested because it could rapidly react with proteins due to its dialdehyde structure, and it could stabilise the structure by cross-linking before the sample is extracted by the buffer (Li et al., 2017; Peracchia & Mittler, 1972). The purpose of osmium tetroxide was used for the secondary fixation which was also employed as a stain for fats or lipids of liposomes (Wigglesworth, 1957).

Uranyl acetate and lead citrate were used in the staining process. The formal one could enhance the contrast of TEM images, but it was reported by Rashidinejad et al. (2014) that could cause a negative effect on the structure of liposomes. The latter was used as stains to improve the electron-scattering properties of biological material components assessed in the electron microscope (Watson, 1958). However, the timing of staining must be controlled within 4 min, or it could damage and irreversibly excessive dye the

sample thus resulting in indistinguishable samples under TEM.

3.2.3.3 ***Sephadex gel filtration***

To achieve a purified liposomal system, Sephadex (G50) gel filtration was applied, following the protocol developed by Rashidinejad et al. (2014), to entrap most free phenolic compounds and small particles. 5% of Sephadex solution was prepared and kept at room temperature overnight for swelling. Sephadex columns were prepared with 5 mL syringe columns, and the narrow end of the column was filled up with 5-mm-depth glass wool. 4 mL of Sephadex gel was added to the column, and columns were put in test tubes and centrifuged at 1207 × g for 10 min after adding 1 mL of acetate buffer (pH 3.8). The samples at the bottom of the test tubes were collected for further HPLC analysis to quantify the concentration of the encapsulated phenolic extracts from FDDPP (see the following section).

3.2.3.4 ***Encapsulation efficiency (EE)***

The EE was calculated using the equation below (Rashidinejad et al., 2014):

$$EE(\%) = \frac{C_e}{C_t} \times 100 \qquad (3)$$

where, C_t stands for the total concentrations of phenolic extracts from FDDPP, and C_e indicates the concentration of encapsulated phenolic extracts from the FDDPP.

The total concentration was known, and the concentrations of encapsulated phenolics

were calculated based on the HPLC analysis on the filtered samples (Section 3.2.2.6).

3.2.3.5 Loading capacity (LC)

The LC represents the ratio of the weight of maximum phenolic compounds (mg) that were encapsulated to the weight of the total weight of soy lecithin used, based on the following equation (Rashidinejad et al., 2019):

$$LC(\%) = \frac{C_e \times V_t}{W_{sl}} \times 100 \qquad (4)$$

where, C_e indicates the concentration of encapsulated phenolic extracts from the FDDPP; V_t is the total volume of the sample mix; and, W_{sl} stands for the total weight of soy lecithin capsules.

3.2.3.6 Encapsulation yield (EY)

The EY is the ratio of the dry weight of liposomes that contain phenolics to the dry weight of the combination of the FDDPPE and soy lecithin before the encapsulation process (Rashidinejad et al., 2014). EY was calculated using the equation below (Rashidinejad et al., 2014):

$$EY(\%) = \frac{W_{fd}}{W_t} \qquad (5)$$

where, W_{fd} is the weight of the sample after freeze drying, and W_t is the total weight of soy lecithin and the FDDPPE.

3.2.4 Freeze drying

In this method, the encapsulated samples were frozen at -20°C before being freeze-dried using a freeze drier (Labconco™ Freeze Dryer with Stoppering Tray Dryer, Kansas City, the USA). The temperature was set at -10°C and the pressure of 20 Pa for the primary drying for 48 h, and the temperature was adjusted to 10°C for the secondary drying for another 24 h.

3.2.5 Incorporation of encapsulated FDDPP into the food model

Milk was chosen as the food model for this purpose, due to its availability, convenience, and potential nutritional benefits. Additionally, a liquid product like milk in this case was easier to be sampled and extracted for the following corresponding analyses. Compared to other liquid products such as fruit juices that already contain free abundant phenolic compounds, milk is a type of food that barely contains plant phytochemicals or phenolic compounds. Additionally, Rashidinejad et al. (2014) conducted a study on a dairy product (cheese) and found a high recovery rate of encapsulated green tea catechins in liposomes after being applied to cheese made from full-fat milk by comparing the total phenolic content, antioxidant activity, and catechin content of the cheeses made from full-fat milk on 0, 30, and 90 days of ripening periods.

Milk was made up of Pams™ whole milk instant milk powder following the manufacturer's instructions. Both freeze-dried and non-freeze-dried liposomal

encapsulants were stored in the dark at 4°C for 28 days before they were applied to milk to assess the chemical stability of encapsulated phenolic compounds during the processing and storage. A part of the freeze-dried sample was dispersed into an acetate buffer (pH 3.8) to obtain the same concentration of the extract in the dispersion. The original FDDPP extract before encapsulation (same concentration as the FDDPP extract in liposomal encapsulant), encapsulated sample (in dispersion), and freeze-dried encapsulated samples were added into milk in a ratio of 1:4 v/v. All these samples and control were also subsampled for the quantification using HPLC, as well as proximate and physical property analyses.

3.2.6 HPLC analysis

The prepared encapsulated samples made with microfluidisation and/or high-shear homogenisation (freeze-dried and non-freeze-dried), and milk samples fortified with liposomal encapsulants (stored for 28 days) went through an extraction process before HPLC analysis. The extraction method was adapted from Rashidinejad et al. (2014) with slight modifications. 1 mL of each sample was mixed with 2 mL of pure ethanol at 70°C and vortexed for 1 min (ethanol was heated in a water bath at 70°C). Samples were ultracentrifuged at 14,100 × *g* for 10 min before HPLC analysis (for the detailed setup of HPLC please see Section 3.2.2.6). The results could assess the chemical stability of phenolic compounds of the FDDPP in liposomes during 28 days of storage

in dark at 4°C, as well as the encapsulation efficiency of liposomes.

3.2.7 Proximate analysis

The proximate analysis was carried out at a certified labarotory (Nutrition Laboratory at Massey University, Palmerston North, New Zealand), and based on the methods adapted from the official methods 990.19 and 990.20 from official analytical chemists (AOAC) with slight modification. Ash was analysed using the method adapted from the AOAC 942.05 in a furnace at 550°C. Crude protein content was obtained using the Dumas method adapted from AOAC 968.06. Crude fat was analysed using the Mojonnier method adapted from AOAC 989.05, and carbohydrate values were obtained by calculation (calculated by difference) (Horowitz & Latimer, 2006).

3.2.8 Physical properties

The physical properties of the freeze-dried and non-freeze-dried encapsulated samples obtained by microfluidisation and/or high-shear homogenisation were analysed by the determination of their zeta potential and particle size weekly for 28 days. One part of the freeze-dried samples was redispersed in acetate buffer (pH 3.8), while the other part of freeze-dried and non-freeze-dried samples were stored at 4°C for 28 days before they were applied to functional milk. Similarly, the pH, zeta potential, particle size, and viscosity of milk samples fortified with four types of liposomal encapsulants (stated

above) were assessed to study the stability of the liposomes through 28 days of storage, as well as studying the effect of the encapsulated FDDPPE on the physical properties of the milk product.

3.2.8.1 Particle size and zeta potential

Particle size and zeta potential of the freeze-dried and non-freeze-dried encapsulated samples and the milk containing them were assessed with Malvern™ Zetasizer Pro (Malvern Panalytical Ltd., Australia). New disposable folded capillary cells 1070 were used to load the samples, which allow Zetasizer to test the particle size and zeta potential within the method. Software ZS Xplorer (Malvern Panalytical Ltd, UK) was used to set up the instrument and method, and to collect and analyse data. The encapsulated samples did not need dilution before the analysis, as the trials showed a polydispersity index (PDI) between 0.4514 and 0.4652. This range was regarded as suitable, because it was smaller than 0.6-0.7, which indicated a reliable measurement. Additionally, the particle size distribution of the trial measurements referred to in the appendix also showed a trend of normal distribution.

3.2.8.2 pH

The pH of standard milk, milk with freeze-dried or non-freeze-dried encapsulated samples, milk with non-encapsulated FDDPPE, and milk with the same concentration

of acetate buffer was measured using a calibrated pH probe (Rashidinejad, Birch, Sun-Waterhouse, et al., 2016).

3.2.8.3 Viscosity

The viscosity of standard milk, milk with freeze-dried or non-freeze-dried encapsulated samples, milk with non-encapsulated FDDPPE, and milk with the same concentration of acetate buffer was measured using a rheometer (AR-G2, TA Instruments, UK) equipped with a Din Rotor concentric cylinder geometry (Deshpande & Walsh, 2018). The running process was adapted from Herceg and Lelas (2005) with slight modifications. The viscosity of the milk mix and shear stress were measured from a ramp shear rate in the range of $0s^{-1}$ to $1290s^{-1}$. The software AR Instrument Control, Newcastle, UK was used as a setup method, and software TA Data Analysis, Newcastle, UK was used for data collection and analysis.

3.2.9 Statistical analysis

The data presented are the average of at least three replications. The statistical analysis was conducted using Minitab (Version 17.3.1) Statistical Software (Minitab Inc., State College, PA). For the mean comparison, the data were subjected to analysis of variance (ANOVA) for any significant differences ($p<0.05$). Microsoft Excel 2016 was used to create all the graphical presentations.

4 Results and discussion

4.1 Extraction of phenolics from FDDPP

The first purpose of this study was to optimise the extraction efficiency and assure that the physical condition of the extraction did not affect the stability of phenolics in the FDDPP. Because, high temperature and high pressure have the potential to destroy phenolic compounds (Alara et al., 2021). Therefore, the temperature was adjusted to 45°C for all extractions (UAE or/and EAE, and ASE), following the experiments conducted by Alara et al. (2021). Different solvents (water and ethanol) were also compared for the ASE method so that the insoluble phenolics from FDDPP could be determined by difference. Ethanol was avoided as much as possible, as the content of phenolic compounds in the extracts could decrease by higher concentrations of ethanol, and the proportion of certain phenolic compounds to the total phenolics could alter (Waszkowiak & Gliszczyńska-Świgło, 2016). This chapter will compare and discuss the results of the determined TPC, TFC, antioxidant activity (with DPPH and ABTS assays), and seven phenolic compounds identified and quantified by HPLC.

4.1.1 Total phenolic content (TPC)

Figure 4.1 shows the effect of the extraction method and time on extractable TPC from the FDDPP. There were statistically significant (p<0.05) differences between groups of means of TPC contents of the samples treated using different extraction methods. The

TPC of the samples extracted with ASE in water (1.63±0.01 mg/g of FDDPP) and ethanol (1.39±0.03 mg GAE/g) were significantly higher than those extracted with EAE (1.14±0.02 mg GAE/g), UAE (1.07±0.04 mg GAE/g), and the combination of EAE and UAE methods (1.31±0.04 mg GAE/g). Additionally, the FDDPP with ASE in water presented a higher TPC than that in ethanol, while the E+UAE method also showed a notedly higher TPC content than both UAE and EAE methods.

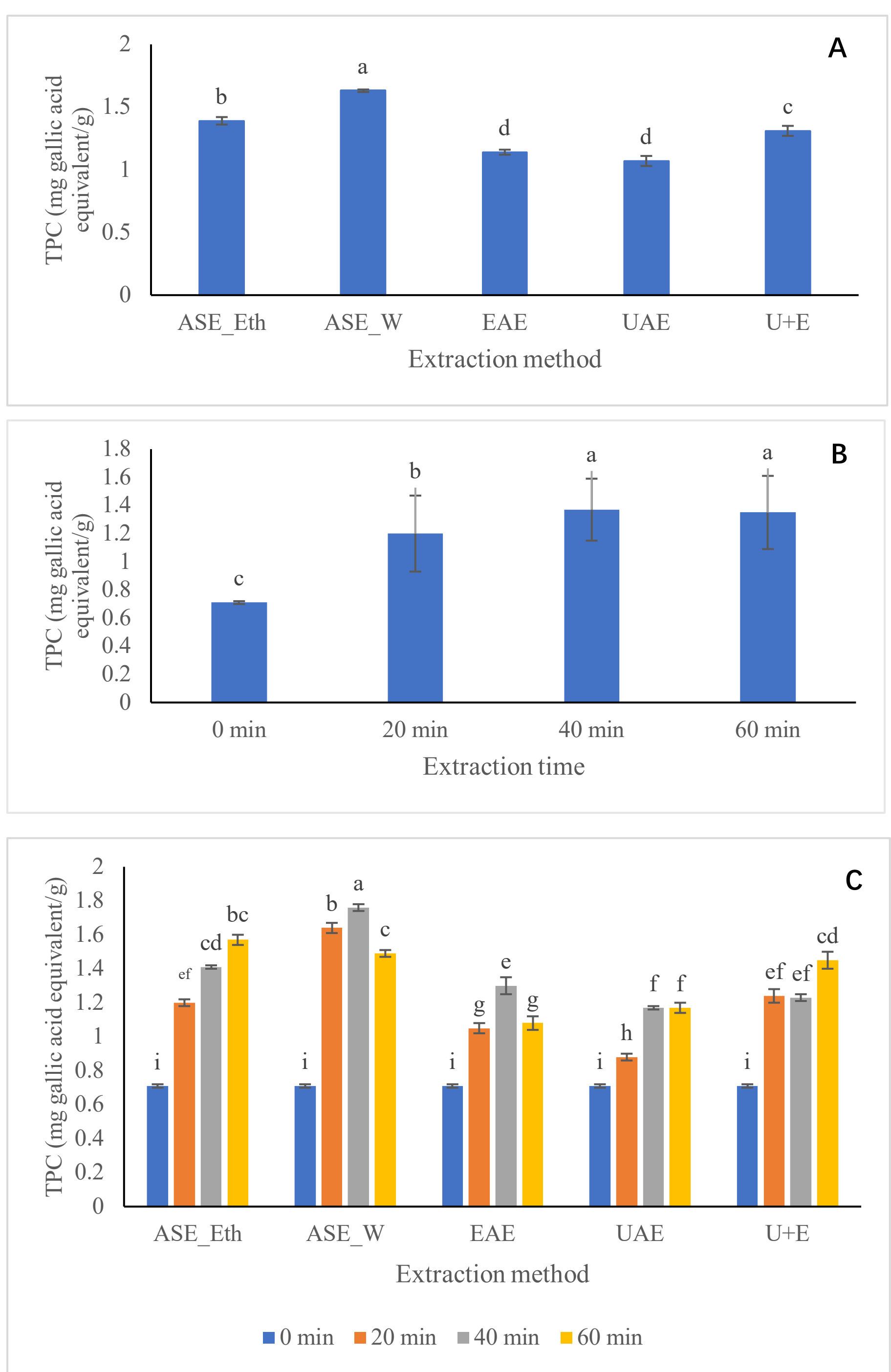


Figure 4. 1. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total phenolic content (TPC) from the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-i) are significantly different (p<0.05) using the Tukey method. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction.

Furthermore, there were statistically significant differences (*p*<0.05) between the means of TPC content of the plum samples treated for different extraction times. The TPC of the samples treated with different extraction methods for 20 min (1.20±0.27 mg GAE/g) was significantly lower than that extracted for 40 min (1.37±0.22) or 60 min (1.35±0.26). However, in the case of all five extraction methods, there were no significant differences (*p*>0.05) between the TPC values of the samples extracted for 40 and 60 min. It appears that the TPC extracted with UAE, E+UAE method, and ASE in ethanol had an increasing trend as a response to increasing the extraction time (Figure 4.1). Nevertheless, when the treatment time was extended from 40 min to 60 min, a further decrease in TPC was observed in the case of the samples extracted with EAE and ASE in water. The reason for such a decrease has previously been attributed to by Butnariu et al. (2013); enzyme hydrolysis of the lateral glycosidic chains of polyphenolic compounds could affect the stability of polyphenols in apple or strawberry juice samples. Similarly, in the case of the current research, the lower concentration of polyphenols could be extracted from the FDDPP samples due to the oxidation of low stability polyphenols since they were extracted using the EAE method. Additionally, the reason that the TPC values for the sample extracted by the E+UAE method did not show a decrease as extraction time extended, could be explained as follows. The acoustic cavitation produced by ultrasound could control enzymatic cell wall breakage and more soluble antioxidant polyphenols were released into the extracted solution because of this (Butnariu et al., 2013; Machado et al., 2019; Nguyen & Nguyen, 2018b).

Among UAE, EAE, and their combination method, EAE for 40 min (1.30±0.05 mg (GAE)/g of FDDPP) was considered the optimal single treatment. 60 min (1.45±0.05 mg GAE/g) was considered the optimal time for the E+UAE ultrasound and enzyme-assisted treatment. Olawuyi et al. (2021) reported that 30 min ultrasound and 60 min of enzymatic extraction achieved the highest TPC extraction in mashed plums. However, the trend for the TPC extracted from FDDPP using EAE in the current study was similar to the results reported by Olawuyi et al. (2021) where it was reported that the TPC started to decrease as the extraction time extended from 60 to 120 min. The UAE for 40 min (1.17±0.01 mg GAE/g) and 60 min (1.17±0.03 mg GAE/g) also resulted in a comparatively high TPC than EAE for a shorter or longer time.

The ASE in water performed the best results in terms of TPC in this trial, and 40 min (1.76±0.02 mg GAE/g) was the optimal time for this method of extraction. A significant decrease in TPC occurred when the extraction time was extended from 40 to 60 min for ASE in water, but it showed a noticeable increase for ASE in ethanol as extraction time was extended to 60 min (Figure 4.1).

No evidence was found in the literature that compared the extraction efficiency of phenolic compounds from damson plums using ASE between the extraction solvents of water and ethanol. However, some literature compared water to other extraction solvents using the ASE method in the case of other plant samples. For example, the TPC of 15.14±0.10 mg/GAE g DW in 50% acidified methanol with 0.5% acetic acid was extracted using the ASE method by Yang et al. (2017). These researchers reported

that this method was a relatively new extraction technique with a significant higher extraction efficiency compared to the UAE method (TPC of 13.40±0.83 mg GAE/g DW in 50% acidified methanol) for the extraction of the phenolic compounds and antioxidants from mulberry (*Morus atropurpurea Roxb.*). Additionally, they suggested that the extraction efficiencies of TPC with different extraction solvents using the ASE method were as: 100% methanol ≥ 50% acidified methanol ≥ 50% acidified acetone ≥ 100% acetone ≥ 99.5% acidified acetone ≥ 50%, acetone ≥ 99.5% acidified methanol > water ≥ 50% methanol. In their study, Yang et al. (2017) found that in the case of the ASE method, the extraction efficiency of phenolic compounds with 50% methanol was lower than that with water. However, 100% of methanol, 50% of acidified methanol, and 95.5% acidified methanol showed significantly higher extraction efficiency than both water and 50% methanol. Even a small concentration of acetic acid could considerably improve the extraction efficiency of methanol, but ethanol was not used in this study, making it difficult to compare with the findings of the current study.

The extraction efficiency of methanol and ethanol was also assessed in another study by Azlim Almey et al. (2010) who extracted phenolic compounds with ethanol and methanol from four aromatic plants (leaves) including knotweed (*Polygonum minus*), curry (*Murraya koenigii*), kaffir lime (*Citrus hysrix*), and fragrant screwpine (*Pandanus odurus*). The results varied among different plants' leaves so in the case of the former three plants, the methanol extraction presented a significantly higher efficiency compared to ethanol, but TPC values of the fragrant screwpine samples extracted by

ethanol were considerably higher than those extracted by methanol.

In another study, Johnson et al. (2020) studied the TPC and antioxidant content of six damson plum varieties extracted with ethanol and methanol. They concluded that the methanolic extraction obtained higher levels of TPC and antioxidant capacity for three of the plum varieties. However, in their study the concentration and content of methanol (100 mL, 90%) and ethanol (50 mL, 50%) were different, and the specific methods were also various. To sum up, according to the most related studies, 50% methanol performed slightly lower extraction efficiency compared to water. Ethanol could be better than methanol in terms of extraction efficiency for different plants and phenolics, depending on the conditions of the extraction process. Therefore, in the case of the current study, the TPC values for the samples extracted by water using the ASE method were reasonably higher than those extracted by 50% ethanol, as the extraction efficiency of ethanol or methanol varied from different plant materials (Figure 4.1).

4.1.2 Total flavonoid content (TFC)

Figure 4.2 shows the effect of the extraction method and time (and the combination of both) on extractable TFC from the FDDPP. Significant differences were seen between groups of means of TFC content for the samples extracted with five various extraction methods ($p<0.05$). However, the TFC of the FDDPP samples extracted using the ASE method did not perform as efficiently as EAE and the E+UAE method. Only the

samples extracted with ASE in water (26.22±0.22 mg RE/g FDDPP) showed slightly higher TFC than those extracted with the UAE method (25.44±0.22 mg RE/g). Additionally, the FDDPP with the E+UAE method (28.44±0.63 mg RE/g) showed a significantly higher ($p<0.05$) TFC value than that extracted with the UAE method ($p<0.05$), but only slightly higher than that extracted using the EAE method (28.16±0.25 mg RE/g). As seen in Figure 4.2, the damson plum sample extracted using the ASE method with ethanol presented the lowest TFC (21.16±1.00 mg RE/g).

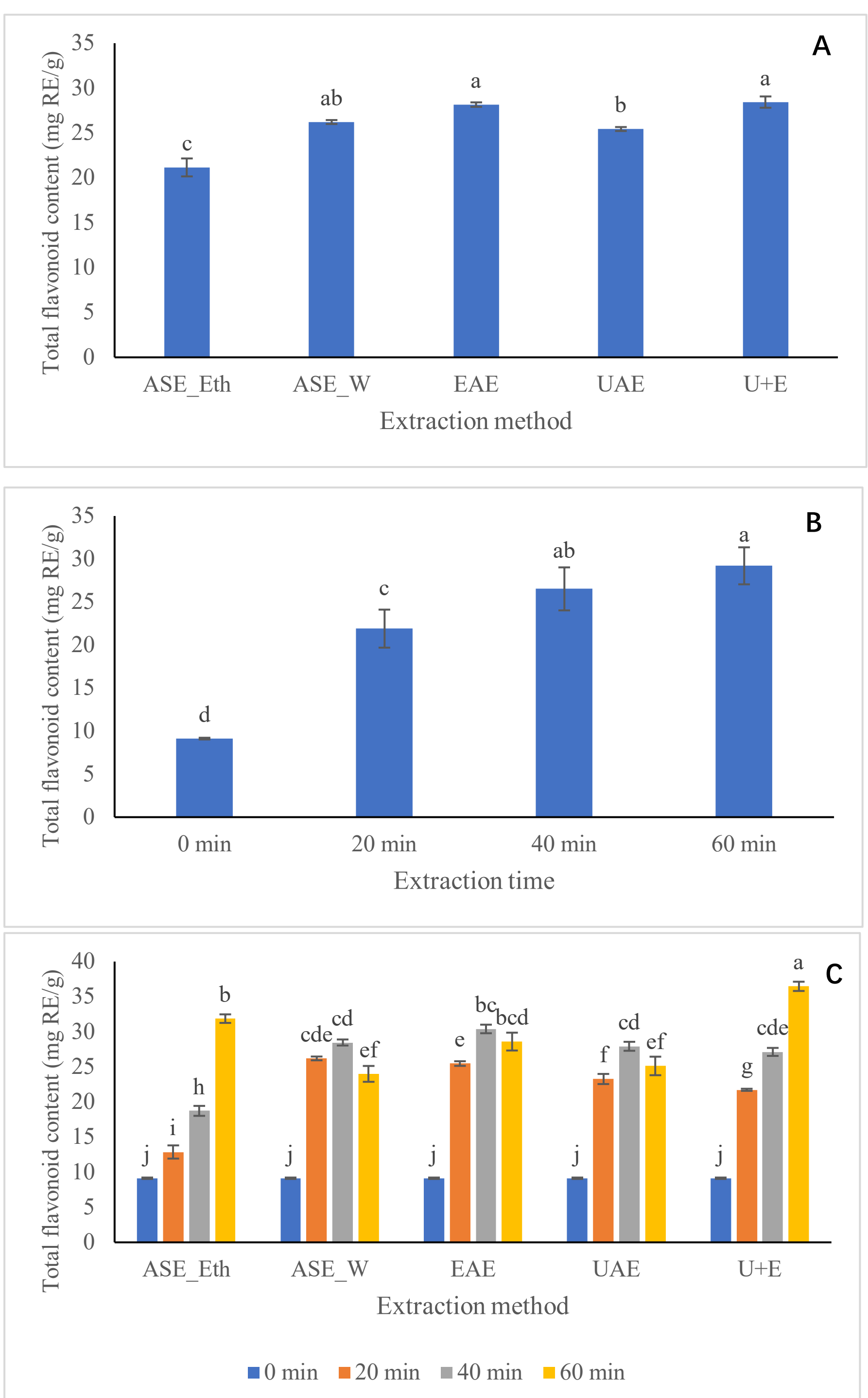


Figure 4. 2. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total flavonoid content (TFC) from the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-j) are significantly different (p<0.05) using the Tukey method. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction.

The extraction time also showed a significant (p<0.05) impact on the TFC extracted from FDDPP with five extraction methods. Overall, the TFC values increased as the extraction time extended to 60 min (Figure 4.2). Both extraction time and extraction method affected the TFC of the FDDPP samples.

The E+UAE method and ASE in ethanol showed an increasing trend as extraction time extended, and both methods showed a significant leap in extracted TFC when the extraction time extended from 40 to 60 min (E+UAE method: 21.13 to 36.47 and ASE-Eth: 18.73 to 31.87 mg RE/g). However, the EAE and UAE methods all had slight decreases in TFC when the extraction time was extended from 40 to 60 min. Particularly, the ASE method with water showed a significant drop in TFC in this case. The optimal extraction method and time for TFC was the E+UAE method for 60 min of extraction (36.47±0.66 mg RE/g).

In comparison, the TFC of the samples of plum (*Prunus salicina* L.) juice treated with the E+UAE method (224.07-246.10 mg RE/100 mL plum juice) also showed a significantly high value compared to either EAE (215.8±1.32 mg RE/100 mL) or UAE (202.89±1.32 mg RE/100 mL) method in the study conducted by Olawuyi et al. (2021). However, in the case of the data of the current study, the E+UAE method showed the highest extraction efficacy in 15 min, and a significant drop in TFC showed as the extraction time was extended to 30 or 60 min. The differences could result from the various plum species and various forms of plum materials.

Regarding the UAE method, it could improve the extraction yield due to the propagation of ultrasound waves and cavitation, as cavitation bubbles attack the plant cell wall, and thus, enhancing the solvent diffusion through the cell wall (Saleh et al., 2016). However, according to the results reported by Dent et al. (2015), a high frequency of UAE could reduce the TFC extraction efficiency from sage in 10 (2072.21 to 1884.66 mg RE/100 g dry matter) and 11 min (2122.96 to 1928.79 mg RE/100 g), as the frequency of ultrasonic device was changed from 24 kHz to 30 kHz. Prolonged extraction under ultrasound obtained a lower TFC content when the extraction process was extended to 12 min (1412.32±14.79 mg RE/100 g) under an ultrasonic frequency of 24 kHz. A similar trend was shown in the current study; i.e., when the extraction time was extended from 40 to 60 min, a significantly dropped TFC was quantified ($p<0.05$). A higher extraction yield was also obtained using the EAE method, because the enzyme could break the cell wall and the structural polysaccharides under moderate conditions. Consequently, this could increase the effectiveness of a vast group of secondary plant metabolites with antioxidant characteristics, including flavonoids (Selvamuthukumaran & Shi, 2017). However, a decrease in TFC occurred during the prolonged extraction for 60 min, and no evidence was found in the literature to provide similar results in other studies. Regarding the ASE method, Tomsone et al. (2015) demonstrated that the TFC values of most samples of horseradish and lovage extracted by ASE were lower than the Soxhlet method. However, no evidence was found in terms of the comparison of the TFC values for the samples extracted using the ASE method with those extracted using

other methods, or different extraction solvents.

4.1.3 Total anthocyanins (TAC)

Figure 4.3 presents the effect of extraction methods and time on the TAC values from the FDDPP. The extraction method is the significant factor in the extracted TAC (p<0.05). TAC of the sample extracted with the UAE method (8.83±0.18 mg cyn-3-glu/g) was significantly lower than that extracted with EAE (51.49±1.21 mg cyn-3-glu/g) and their combined method (40.88±0.83 mg cyn-3-glu/g). However, the TAC of the sample extracted with the E+UAE method was also notably lower than that extracted using EAE. Similar results were also reported by Olawuyi et al. (2021). In their results, the TAC of plum juice extracted with UAE at 38 kHz frequency was 0.13±0.01 (mg/100 mL), which was significantly lower than the sample extracted using EAE (0.91±0.02 mg/100 mL) and their combined method (15 min: 0.66±0.23, 30 min: 0.76±0.05, and 60 min: 0.89±0.05). Olawuyi et al. (2021) explained that low extracted TAC value from ultrasound-treated samples could be consequently caused by the degradation of anthocyanin glucosides induced by the action of polyphenol oxidase (PPO). Their results were also in line with those reported by Olawuyi et al. (2021) who showed a negative relationship (r=-0.912) between TAC and PPO activity. Additionally, many studies suggested that enzymatic treatment could significantly increase the TAC of various fruit juices (Nguyen & Nguyen, 2018a; Wang et al., 2009). This treatment could also positively relate to the retardation of hydrolysis of anthocyanins by the

inhibition of PPO (Khandare et al., 2011). Furthermore, in the case of the current study, water (64.74±0.68 mg cyn-3-glu/g) appeared to be a more effective solvent for the extraction of anthocyanins than ethanol (37.83±1.01 mg cyn-3-glu/g) for the ASE method.

Muangrat et al. (2018) showed that in the case of the extraction of anthocyanins from dried cob of purple waxy corn using the UAE method, the solvent ratio of 50% ethanol (139.550-238.405 μg cyn-3-glu/g DW) and 50% water performed much better than water (115.240-155.715 μg cyn-3-glu/g DW) or ethanol (37.095-112.900 μg cyn-3-glu/g DW) alone. In their study, pure water (115.240-155.715 μg cyn-3-glu/g DW) also achieved a higher TAC than pure ethanol (37.095-112.900 μg cyn-3-glu/g DW) at all amplitude levels of ultrasound (25%, 50%, and 100%). This could also provide evidence that pure water performed better than pure ethanol to extract anthocyanins from plant materials, as the similar results presented in the current study for the extraction of anthocyanins from the FDDPP.

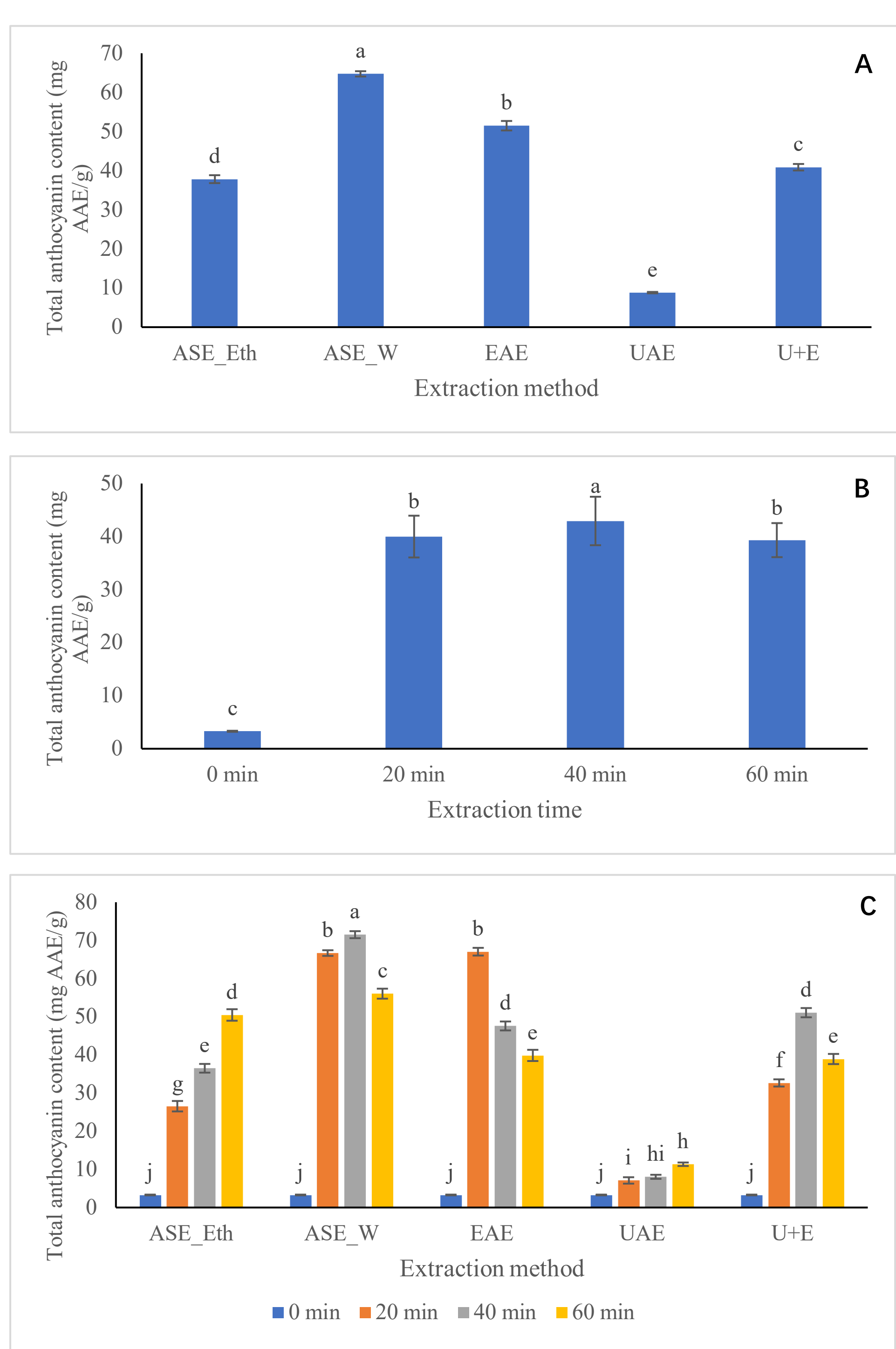


Figure 4. 3. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total anthocyanin content (TAC) from the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-j) are significantly different ($p<0.05$) using the Tukey method. AAE: ascorbic acid equivalent. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction.

Overall, in the current experiment, the FDDPP sample extracted using TAC for 40 min (42.94±4.57 mg cyn-3-glu/g) showed a significantly (p<0.05) higher TAC value than that extracted using the same method but for 20 min (40.00±3.94 mg cyn-3-glu/g) or 60 min (39.33±3.21 mg cyn-3-glu/g) (Figure 4.3). This means that although increasing extraction time could be beneficial for up to 40 minutes, increasing extraction time beyond 40 minutes may not be beneficial in this case. Additionally, the E+UAE method presented a notable increase trend in the extraction of TAC from the samples exposed to extraction 0 (3.29±0.10 mg cyn-3-glu/g) to 40 min (51.06±1.21 mg cyn-3-glu/g), but there was a significant drop when the extraction time was extended to 60 min (38.92±1.33 mg cyn-3-glu/g) (Figure 4.3). Muangrat et al. (2018) also added that TAC reduced as the amplitude level of ultrasound increased from 50% to 100 % for all the samples extracted with 100% ethanol, 50% ethanol, or 100% water. This could also explain the considerably low TAC content in the samples extracted by UAE in this experiment, which were lower than the values seen for the other methods.

4.1.4 Antioxidant capacity and radical-scavenging activity (DPPH and ABTS)

4.1.4.1 DPPH

Both extraction methods and the extraction time tested in the current study were significant (p<0.05) factors that could affect the concentration of antioxidants extracted from the FDDPP using the DPPH assay (Figure 4.4).

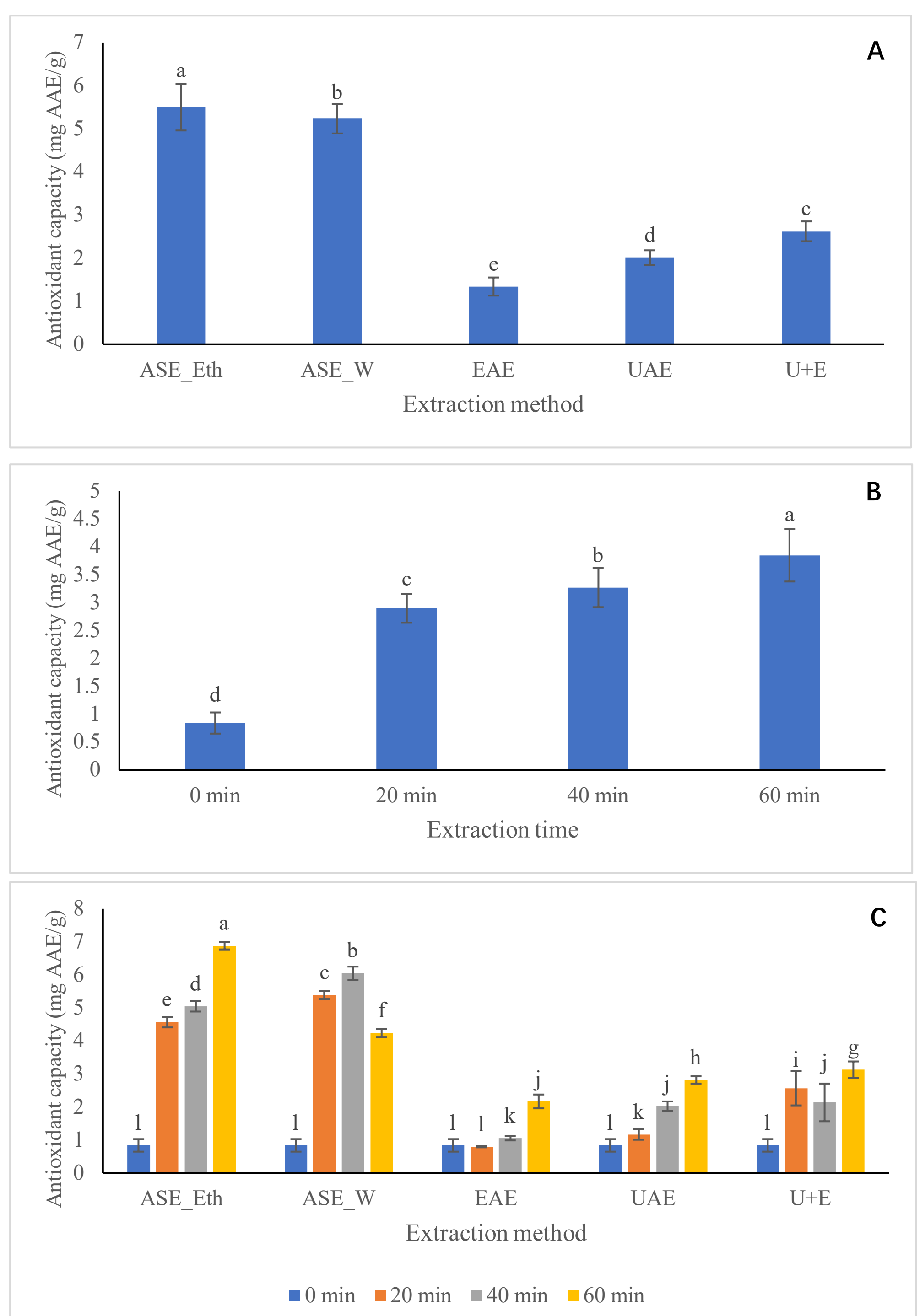


Figure 4. 4. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total antioxidant properties (measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay presented as ascorbic acid equivalence (AAE)) of the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-l) are significantly different ($p<0.05$).

Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction.

Among EAE, UAE, and their combined method (E+UAE), the antioxidant activity of the sample extracted with the E+UAE method presented notedly higher than that extracted with the UAE and EAE (2.62±0.23 vs 2.01±0.17 and 1.34±0.21 mg AAE/g, respectively; Figure 4.4). In terms of total antioxidants extracted from the FDDPP, the UAE showed a better performance than the EAE method. ASE method showed a significantly higher extraction efficiency than the other methods, and ethanol was a better solvent. Additionally, the total antioxidant activity exhibited an increasing trend with the increasing the extraction time, so that the four extraction timings showed significant $p<0.05$) differences in this regard (0.84±0.19 mg AAE/g t = 0, 2.90±0.26 mg AAE/g t = 20 min, 3.27±0.35 mg AAE/g t = 40 min, 3.85±0.47 mg AAE/g t = 60 min; Figure 4.4).

As seen in Figure 4.4, there were gradual increases in total antioxidants from 20 to 60 min in the case of EAE (0.80 to 2.17 mg AAE/g) and UAE (1.17 to 2.81 mg AAE/g). The antioxidant capacity was positively correlated with TAC and TFC, according to Olawuyi et al. (2021) who assessed the TPC, TFC, TAC, and antioxidant capacity of plum juice with different extraction methods. Similarly, in this study, the TAC extracted by the UAE method showed a similar trend as the antioxidant capacity assessed by using the DPPH assay. However, there was a significant ($p<0.05$) drop in the total antioxidants when time increased from 20 min to 40 min (2.57±0.52 to 2.14±0.57 mg AAE/g) for the E+UAE method, but it increased again for the 60 min extraction (3.13

mg AAE/g). Although generally speaking, the total extractable antioxidants significantly increased when the extraction time was extended from 40 min to 60 min, an opposite trend was seen when water was used as the solvent in the ASE method; i.e., the DPPH value dropped from 6.05±0.20 mg AAE/g to 4.24±0.12 mg AAE/g when the extraction time was extended from 40 min to 60 min. However, there is no available evidence about the effect of the prolonged ASE method on the extraction of total antioxidants from the damson plum or similar fruit or plant materials

4.1.4.2 ABTS

Figure 4.5 presents the effect of extraction method and time on total extractable antioxidants from the FDDPP determined using the ABTS assay. Both the extraction method and extraction time showed a significant effect ($p<0.05$) on the total antioxidants. However, the ABTS analysis showed slightly different results than those obtained from the DPPH assay; i.e., the value for the EAE method (2.16±0.22 mg AAE/g) was significantly ($p<0.05$) lower than that from the UAE method (2.54±0.19 mg AAE/g), and slightly higher than the extracted antioxidants using the E+UAE method (2.49±0.27 mg AAE/g) (Figure 4.5).

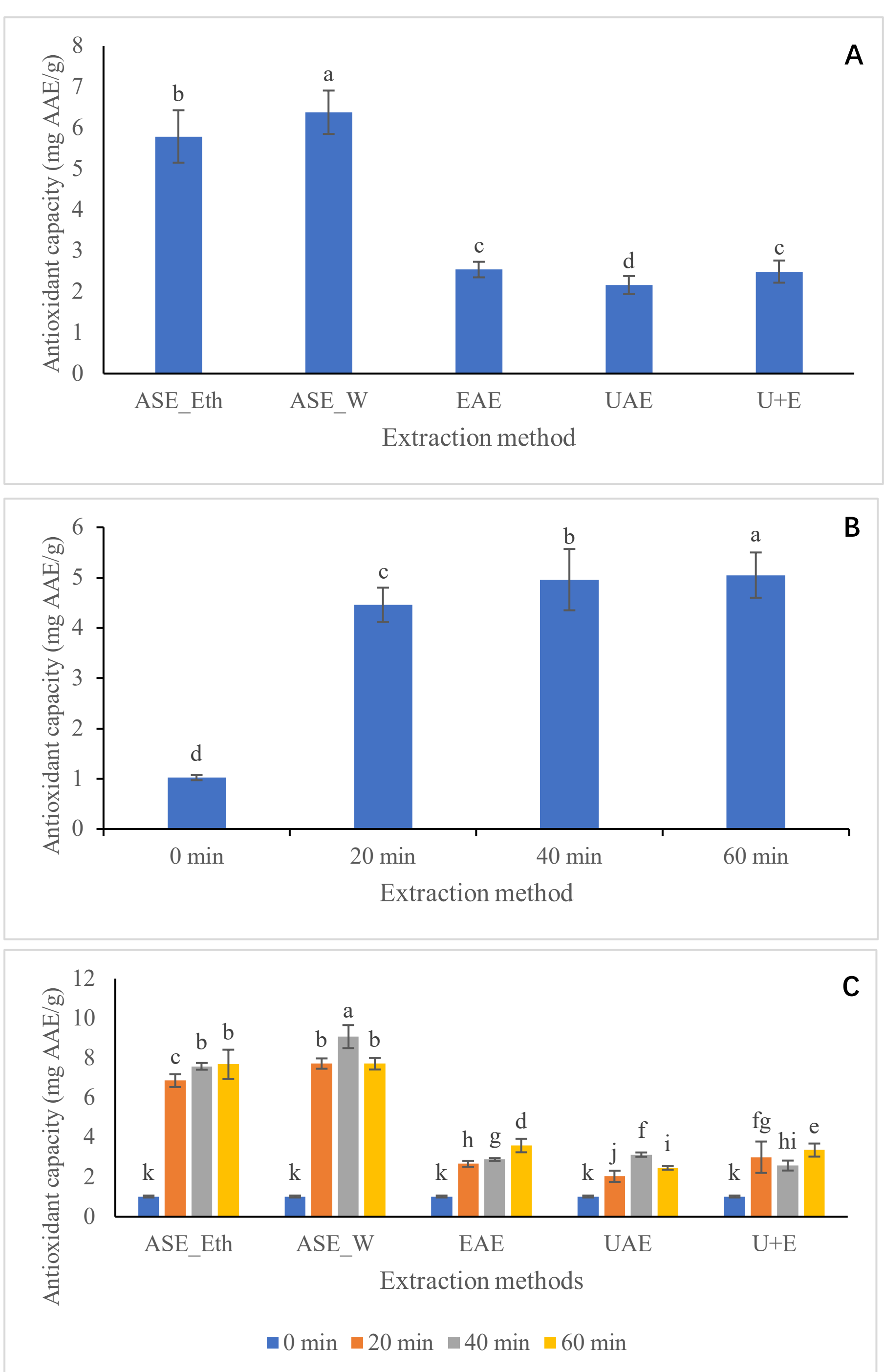


Figure 4. 5. The effect of extraction method (A), time (B), and a combination of both (C) on extractable total antioxidant properties (measured using 2,2'-Azinobis-3-ethylbenzothiazoline-6-Sulfonic acid (ABTS) assay and presented as ascorbic acid equivalence (AAE)) of the damson plum samples. Values are means±SD (n=9). Columns with different superscripts (a-k) are significantly different (p<0.05) using the Tukey method. Note: ASE_Eth: accelerated solvent extraction with ethanol; ASE_W: accelerated solvent extraction with water; EAE: Enzyme-assisted extraction; UAE: ultrasound-assisted extraction; U+E: ultrasound combined enzyme-assisted extraction.

The effect of extraction time on total antioxidants determined using the ABTS assay was not consistent. Although there remained a steady increasing trend when the extraction time was extended from 0 to 40 min, there was a significant (p<0.05) drop (from 5.05±0.45 to 4.96±0.61 mg AAE/g) when the extraction time was extended from 40 min to 60 min (Figure 4.5). In the case of all three extraction methods (ASE, EAE, and combination) and when water was used as the solvent, the effect in terms of antioxidant activity obtained from the ABTS assay was the same as those obtained from the DPPH assay. However, the effect was not the same when comparing the time increase from 40 min to 60 min. Additionally, in the case of ABTS values, there was a significant decrease when the time was increased from 40 min (3.13±0.11 mg AAE/g) to 60 min (2.46±0.09 mg AAE/g); whereas, such an effect was the other way around in the case of DPPH values (Figure 4.5).

Compared to the results obtained by Olawuyi et al. (2021), the E+UAE method also performed the best results compared to the single extraction of UAE or EAE. However, they obtained a higher antioxidant activity as the plum mash was extracted by EAE than UAE for both DPPH and ABTS. This is contrary to the results achieved in this study using DPPH analysis, but it is similar to that assessed by the ABTS method. It has previously been reported that the antioxidant activity assessed by DPPH in most cases is positively correlated with ABTS (0.85), but compared to the correlation between ABTS and FRAP (0.97), the trends by proportional of ABTS still have 15% differences

compared to DPPH (Thaipong et al., 2006).

In the study conducted by Olawuyi et al. (2021), all the E+UAE methods obtained higher values for antioxidant activity, as well as TPC and TFC. They also suggested that the TFC ($R \geq 0.860$) and TAC ($R \geq 0.787$) strongly correlated with the antioxidant capacity of plum juice. A similar strong correlation ($R \geq 0.790$ with 95% of the confidence interval for Pearson Correlation) was also presented in this study between antioxidant capacity assessed by ABTS and TFC excluding the samples extracted by ASE methods. However, it is reasonable that the antioxidant capacity of the FDDPP extract is related to the number of phenolic compounds and anthocyanins, as they play a significant role as antioxidants in various plum cultivars including damson plums (Kim et al., 2003).

4.1.5 Quantification of phenolic compounds in FDDPP using HPLC analysis

The FDDPP contains various phenolic compounds including phenolic acids and flavonoids (Moscatello et al., 2019). In this study, the concentration of seven phenolic compounds was individually assessed by the HPLC analysis. The phenolic compounds detected (under the 280 nm wavelength) in this study included neochlorogenic acid, gallic acid, rosmarinic acid, catechin, epicatechin, rutin, and naringenin. Different wavelengths were tested for the detection of all these seven phenolic compounds. Neochlorogenic acid and gallic acid were also separated at 320 nm. Rutin was also separated at both 320 nm and 360 nm. However, only catechin and epicatechin could

be detected at 520 nm. Accordingly, the data obtained using UV light at 280 nm wavelength was applied for the data analysis. While quercetin was tested as a standard for plotting the standard curve, it was not detected in any of the samples extracted with five methods under any of the wavelengths.

Phenolic compounds are relevant to the appearance (anthocyanin and pigment accumulation), taste (astringency), and antioxidant capacity of original damson plums (Tomás-Barberán et al., 2001). Overall, in the current experiment, the maximum extracted neochlorogenic acid (1393.2±29.7 μg/g) was found as the principal phenolic acid and antioxidant, followed by gallic acid (67.34±0.89 μg/g), while rosmarinic acid (12.85±0.75μg/g) relatively showed as a tiny concentration (Table 4.1-2). In addition, rutin (56.51±2.35 μg/g), catechin (67.51±1.52 μg/g), epicatechin (66.51±2.42 μg/g), and naringenin (52.91±1.42 μg/g) were predominantly found flavonoids in damson plums. These results agree with those found in a previous research by Will and Dietrich (2006) who found the first two (i.e., rutin and catechin) as the major flavonoids in plum samples.

The HPLC chromatograms of the standard compounds and the FDDPP extract samples against the control (20% methanol) are respectively shown in Figure 4.6. As indicated in this figure, apart from the quercetin that did not show up in any of the plum samples, the other seven phenolic compounds were successfully found and separated in the chromatograms of samples against the standards.

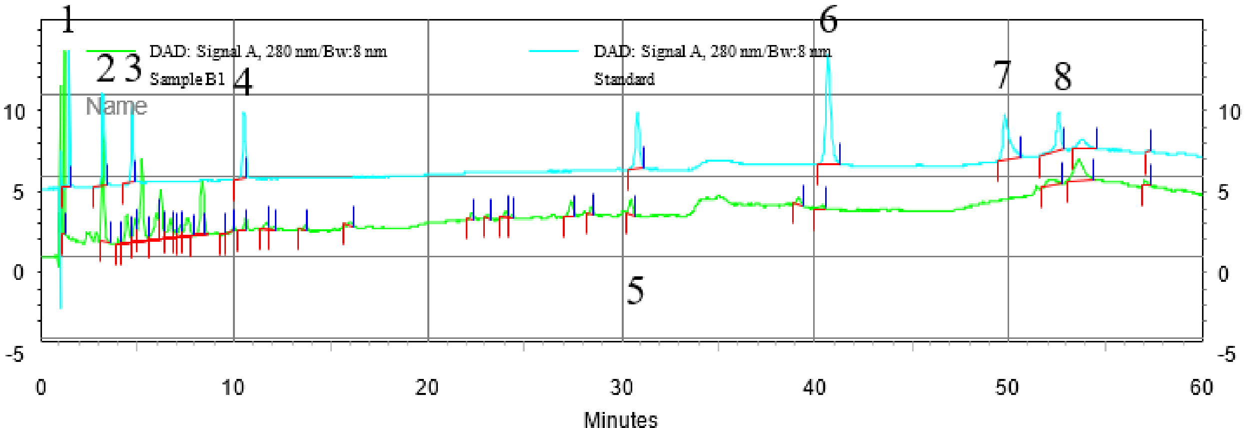


Figure 4. 6. The chromatogram of standards (blue line) and the damson plum sample treated by ultrasound-assisted extraction for 20 min (green line). The peaks on standards were detected by diode-array detection (DAD) with a wavelength of 280 nm and paired with the sample and numbered as 1: gallic acid monohydrate, 2: neochlorogenic acid, 3: catechin hydrate, 4: epicatechin, 5: rutin hydrate, 6: rosmarinic acid, 7: quercetin, and 8: naringenin.

Phenolic acids including gallic acid, neochlorogenic acid, and rosmarinic acid were significantly ($p<0.05$) affected by pre-treatments (Table 4.1). Enzyme-assisted extraction for 40 min showed a significant higher extraction efficiency than the other treatments for gallic acid (67.34±0.89 µg/g). Apart from the ASE treatment, the EAE also performed as the best extraction method in the case of neochlorogenic acid (1038.2±58.7 µg/g) and rosmarinic acid (11.57±0.71 µg/g). However, in the trials conducted by Olawuyi et al. (2021), the combination of EAE and UAE method showed a relatively higher extraction efficiency than other methods for the content of phenolic acids including chlorogenic acid, caffeic acid, and ferulic acids from *Prunus salicina*. Different plum cultivars could be a reason for this difference, because Azlim Almey et al. (2010) and Johnson et al. (2020) showed that the different extraction methods

showed various extraction effectiveness on distinct plant materials and damson plum cultivars, respectively.

In the case of the current research, the contents of neochlorogenic acid (1158.3-1393.2 μg/g) and rosmarinic acid (8.91-13.01 μg/g) presented higher in the case of the sample extracted using the ASE method when ethanol was used as a solvent than when water was used (neochlorogenic acid 1008.1-1105.0 μg/g and rosmarinic acid 8.44-12.85 μg/g). However, it showed contrary results for gallic acid, as water-extracted gallic acid (48.58-58.14 μg/g) was significantly higher than ethanol extracted one (36.29-39.67 μg/g). Additionally, for gallic acid, apart from the EAE, the other methods showed a steady increase as the result of the increase in the extraction time (from 20 to 60 min). In the case of the sample extracted using EAE, there was a significant drop in the concentration of gallic acid as the extraction time was extended from 40 to 60 min. Neochlorogenic acid was previously reported by Chun et al. (2003) to be one of the major phenolic compounds in damson plums. This phenolic acid could not be efficiently extracted with the UAE method. Even the E+UAE method showed a lower content of extracted gallic acid and neochlorogenic acid than that of the EAE method; i.e., the ultrasound frequency of 38 kHz could significantly suppress the extraction of these two phenolic acids. This could also be explained by the reasons mentioned in Section 4.1.1 that the prolonged ultrasound treatment could result in the degradation of phenolic compounds (Nguyen & Nguyen, 2018b). Such findings are also in line with the results obtained by Muangrat et al. (2018), who altered the amplitude level of the

ultrasound, and the phenolic contents extracted from dried cob of purple waxy corn decreased when the amplitude level of ultrasound increased up to 46.21% or higher.

Table 4. 1. Phenolic acids identified and quantified in the freeze-dried damson plum powder treated using different extraction methods/conditions.

Phenolic acid (μg/g)	Gallic acid	Neochlorogenic acid	Rosmarinic acid
Control	17.33±0.13^{j}	109.63±3.27^{k}	2.74±0.07^{g}
UAE20	60.26±0.59^{c}	185.35±11.78^{j}	8.45±0.80^{de}
UAE40	61.75±0.30^{c}	242.13±20.63^{i}	10.93±0.75^{b}
UAE60	64.46±1.32^{b}	204.35±10.2^{ij}	8.67±0.60^{de}
EAE20	63.75±1.59^{b}	896.0±55.8^{fg}	11.33±0.22^{b}
EAE40	67.34±0.89^{a}	1038.2±58.7^{de}	6.83±0.27^{f}
EAE60	52.08±2.38^{e}	769.7±42.7^{h}	11.57±0.71^{b}
U+E20	40.66±1.33^{g}	752.5±48.7^{h}	9.52±0.43^{cd}
U+E40	58.33±0.43^{d}	861.5±39.2^{g}	9.88±0.42^{cd}
U+E60	61.63±0.28^{c}	967.5±39.2^{ef}	9.71±0.66^{cd}
ASE_W20	48.58±0.09^{f}	1008.1±44.7^{e}	8.44±0.88^{de}
ASE_W40	52.39±0.93^{e}	1099.4±47.0^{cd}	9.91±0.22^{cd}
ASE_W60	58.14±1.76^{d}	1105.0±84.5^{cd}	12.85±0.75^{a}
ASE_Eth20	36.29±0.18^{i}	1295.9±25.9^{b}	10.32±0.59^{bc}
ASE_Eth40	37.62±0.19^{hi}	1393.2±29.7^{a}	13.01±0.44^{a}
ASE_Eth60	39.67±0.16^{g}	1158.3±40.2^{c}	8.91±0.60^{de}

Notes: Values are means±SD (n = 9). Values with different superscripts (a-j) within the same column are significantly different (p<0.05). Control: water and freeze-dried damson plum powder incubated at 45°C for 60 min; UAE20, UAE40, and UAE60: ultrasound extraction for 20, 40, and 60 min, respectively; EAE20, EAE40, and EAE60: enzyme assisted extraction for 20, 40, and 60 min, respectively; U+E20, U+E40, U+E60: enzyme combined ultrasound-assisted extraction for 20, 40, and 60 min, respectively; ASE_W20, ASE_W40, ASE_W60 ASE_Eth20, ASE_Eth40, and ASE_Eth60: accelerated solvent extraction with water for 20, 40, and 60 min, respectively, or 100% ethanol for 20, 40, and 60 min, respectively.

Table 4.2 presents the content of extracted flavonoids including catechin, epicatechin, rutin, and naringenin from the FDDPP extracted under different extraction methods and times. The concentration of these four flavonoids was significantly influenced by different treatments ($p<0.05$). ASE method with water in 40 min extraction time showed

the highest extraction efficiency for catechin (66.51±2.42 μg/g) and epicatechin (56.51±2.35 μg/g), and ethanol performed as a better extraction solvent for naringenin in the case of 20 min extraction and ethanol as the solvent. The ASE method showed the highest content of extracted naringenin (52.91±1.92 μg/g). Rutin was relatively higher in the case of EAE treatment (especially for 40 min) than in other samples.

Apart from the ASE method, extraction conducted with the EAE method for 40 min achieved the highest catechin and rutin. However, the E+UAE method for 15 min showed the highest extraction efficiency for these phenolics in the study conducted by Olawuyi et al. (2021). The different results could be due to the various frequencies of ultrasound during the extraction.

There are limited studies that compared the effectiveness of different frequencies of UAE on the extraction of phenolic compounds from damson plums or even other fruits. Dent et al. (2015) compared the effectiveness of extraction with the UAE method on phenolic compounds from sage (*Salvia officinalis* L.) with two different frequencies (24 and 30 kHz). The mass fraction of rosmarinic acid extracted with 30 kHz was significantly higher than that extracted with 24 kHz frequency ($p<0.05$), as the extraction time was extended to 11 and 12 min. However, the total phenols extracted under 24 kHz were significantly higher than that extracted under 30 kHz for the whole range of extraction time (8, 10, 11, and 12 min). Prolonged extraction time resulted in higher extraction capacity of phenolic compounds, while excessively prolonged extraction time in their study could also significantly reduce the content of extracted

phenolic compounds including romantic acid, hydroxy-cinnamic acid, hydroxy-benzoic acid, and flavonoids. For example, the content of romantic acid dropped from 3991.59±12.18 mg/100 g to 3549.36 ±13.75 mg/100 g of dry sage when the extraction time was extended from 11 to 12 min at 24 kHz (Dent et al., 2015). Additionally, the higher ultrasound frequency also improved the extraction capacity of phenolic compounds. For example, the concentration of extracted hydroxy cinnamic acids and benzoic acids was capped at 45.14±1.57 and 43.80±5.35 mg/100 g with the frequency of ultrasound of 24 kHz, and they were enhanced to 117.30±8.47 and 120.22±4.59 mg/100 g dry sage respectively with the frequency of 30 kHz. The timing of the extraction in their study was grouped in a shorter time interval, so the phenolic compounds could be suppressed or damaged by the longer time of extraction from 15 to 20 min (Dent et al., 2015; Nguyen & Nguyen, 2018b).

In the current study, the E+UAE method showed the highest efficiency among the UAE, EAE, and the E+UAE methods for extracting epicatechin from the FDDPP. It showed a stable increase trend for catechin (43.65-49.44 µg/g), epicatechin (36.64-52.33 µg/g), and rutin (51.35-61.43 µg/g) when the extraction time was extended from 0 to 60 min. However, the other methods mostly presented an increase when the extraction time was extended from 20 to 40 min, but a decreasing trend as the extraction time was further extended to 60 min (Table 4.2).

Table 4. 2. Flavonoids identified and quantified in the freeze-dried damson plum powder treated using different extraction methods/conditions.

Flavonoids (µg/g)	Catechin	Epicatechin	Rutin	Naringenin
Control	8.01±0.33^{j}	6.01±0.12^{h}	11.17±0.03^{h}	11.69±0.17^{g}
UAE20	46.51±1.85^{cd}	25.52±1.16^{f}	35.17±1.45^{f}	46.43±0.51^{cd}
UAE40	43.49±1.56^{de}	23.84±0.71^{f}	37.49±2.99^{f}	49.13±0.74^{ab}
UAE60	40.96±0.90^{ef}	23.17±1.36^{f}	44.81±2.38^{de}	49.12±1.95^{bc}
EAE20	46.99±1.27^{cd}	37.85±2.91^{d}	56.88±2.93^{bc}	44.52±1.32^{cd}
EAE40	56.33±2.36^{b}	43.88±2.27^{c}	67.51±1.52^{a}	45.11±1.39^{cd}
EAE60	44.65±1.66^{cd}	33.36±2.97^{d}	20.08±1.34^{g}	41.20±1.86^{e}
U+E20	43.65±2.30^{de}	36.64±1.99^{d}	51.35±2.27^{cd}	30.37±1.19^{f}
U+E40	43.76±2.59^{de}	44.56±0.66^{c}	55.57±2.77^{bc}	32.29±2.04^{f}
U+E60	49.44±2.56^{c}	52.33±1.23^{ab}	61.43±2.88^{b}	30.33±1.18^{f}
ASE_W20	20.64±0.34^{i}	37.55±1.48^{d}	43.97±3.41^{ef}	44.51±1.22^{cd}
ASE_W40	66.51±2.42^{a}	56.51±2.35^{a}	52.08±3.10^{cd}	46.67±2.53^{cd}
ASE_W60	29.44±1.61^{h}	50.24±1.16^{b}	43.15±1.36^{ef}	42.72±1.53^{de}
ASE_Eth20	45.25±1.37^{cd}	24.48±0.40^{f}	20.59±1.76^{g}	52.91±1.42^{a}
ASE_Eth40	42.00±0.60^{ef}	28.13±1.11^{e}	45.73±1.53^{de}	51.23±1.39^{a}
ASE_Eth60	36.69±1.53^{g}	19.52±0.55^{g}	21.65±0.59^{g}	47.52±0.32^{bc}

Notes: Values are means±SD (n = 9). Values with different superscripts (a-j) within the same column are significantly different ($p<0.05$). Control: water and freeze-dried damson plum powder incubated at 45°C for 60 min; UAE20, UAE40, and UAE60: ultrasound extraction for 20, 40, and 60 min, respectively; EAE20, EAE40, and EAE60: enzyme assisted extraction for 20, 40, and 60 min, respectively; U+E20, U+E40, U+E60: enzyme combined ultrasound-assisted extraction for 20, 40, and 60 min, respectively; ASE_W20, ASE_W40, ASE_W60 ASE_Eth20, ASE_Eth40, and ASE_Eth60: accelerated solvent extraction with water for 20, 40, and 60 min, respectively, or 100% ethanol for 20, 40, and 60 min, respectively.

4.2 Liposomal Encapsulation of FDDPP

The encapsulation process is essential to maintain the properties of phenolic compounds extracted from the FDDPP. Encapsulation of these compounds is a significant technique to preserve their properties for a longer period because the coating materials could function as a barrier to oxygen and water and protect phenolic compounds from being oxidised and improve their stability (Lavelli et al., 2016). In this research, liposomes were the ideal encapsulation method for the bioactive

compounds and antioxidants from the FDDPP, because of their high performance of encapsulation efficiency, non-toxicity, small particle size, biodegradability, high physical stability, and biocompatibility (Rashidinejad et al., 2014; Rashidinejad, Birch, Sun-Waterhouse, et al., 2016). This is also a simple and fast encapsulation method that can be considered to industrialise the production of encapsulated bioactive compounds of the FDDPP and its application in functional foods. Such vesicles (i.e, liposomes) can be produced using simple processes such as high-shear mixing and microfluidising with a coating material like soy lecithin in a short time (e.g., 3-5 min). The properties and the effect of the liposome-coated FDDPP extract before its incorporation into the standard milk are shown in the following sections.

4.2.1 Encapsulation efficiency (EE) and encapsulation yield (EY) determined with HPLC analysis

Sephadex gel filtration was used to separate the encapsulated and free phenolic compounds from liposome solutions. Some phenolic compounds such as gallic acid, catechin, epicatechin, and rutin could be filtered using this method, but other phenolic compounds and their encapsulated form were not showing a close value to the general EE of the liposomes or were even not presented after the Sephadex extraction.

Tables 4.3 and 4.4 present the EE and EY of the liposomes made by different methods for seven phenolic compounds, respectively. The calculated EE relied on the effect of

Sephadex extraction that removed the free phenolics and small particles, and the determination of the content of these seven phenolic compounds in the encapsulated samples (i.e., high-shear homogenisation, high-shear homogenisation, and microfluidisation, freeze-dried high-shear homogenised, and freeze-dried high-shear homogenised and microfluidised samples) before and after the Sephadex extraction. The purpose of using freeze drying was to explore and optimise the properties of liposomes in liquid and freeze-dried powder form. The data for rosmarinic acid and naringenin was missing because the content of these two phenolic compounds in the samples after Sephadex extraction showed zero with HPLC analysis.

Table 4. 3. The encapsulation efficiency of the liposomes manufactured with different methods for the encapsulation of different phenolic compounds from the freeze-dried damson plum power.

Phenolics	HSH	HSH+MF	FD-HSH	FD-HSH+MF
Gallic acid	64.8%	66.2%	57.5%	60.1%
Neochlorogenic acid	33.4%	24.4%	35.2%	26.4%
Catechin	88.9%	95.3%	86.5%	91.1%
Epicatechin	90.5%	81.9%	75.9%	95.7%
Rutin	84.3%	96.2%	95.0%	90.5%

Note: HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum powder extract loaded liposome; FD: Freeze-dried.

Table 4. 4. The encapsulation yield of the liposomes manufactured with different methods for encapsulation of the extract from damson plums.

Method	HSH	HSH+MF	FD-HSH	FD-HSH+MF
Encapsulation yield	79.1%	83.3%	76.2%	81.7%

Notes: HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum powder extract loaded liposome; FD: Freeze-dried.

The additional microfluidisation improved the EY of the damson plum extract for both freeze-dried and non-freeze-dried liposomal encapsulants, and the non-freeze-dried ones showed 1.6% higher EY compared to the freeze-dried ones (Table 4.4).

The EE results of catechin, epicatechin, and rutin looked reliable compared to the EE of gallic acid and neochlorogenic acid. The calculated EE of the latter two phenolic compounds could be affected by the incompatible filtration method. The EE of catechin using a high-shear homogeniser (88.9%) was slightly higher than the EE of catechin (around 80%), which was reported by Rashidinejad et al. (2014). The only difference was the form of the soy lecithin applied for the encapsulation in this study; i.e., soy lecithin granule instead of liquid soy lecithin. The additional microfluidisation did improve the EE of catechin and rutin by 7-8% and improved the EE of both catechin and epicatechin after freeze drying by 4.6% and 19.8% (Table 4.3). Freeze drying had a slightly negative effect on the EE of liposomes made by high-shear homogenisation for catechin and epicatechin and the EE of liposomes made by combined high-shear homogenisation and microfluidisation for catechin and rutin. However, it seemed to improve the EE of liposomes made by high-shear homogenisation by 5.7% for rutin and the combined method by 13.8% for epicatechin (Table 4.3).

Regarding the differences in the EE of various phenolic compounds, three factors could be considered. First, the encapsulation techniques and conditions can have a substantial effect on encapsulation performance, as the encapsulation systems are theoretically based on the interference of a dispersed phase constituted of bioactive compounds and

coating materials in form of small particles (or nanoparticles) through procedures including drying, rearranging surface layers or phase transition (Mishra, 2019). For example, microfluidisation, which is one of the methods applied in this study to make liposomes, was previously used by Ré et al. (2019), by which they could achieve a significantly high encapsulation efficiency. Zou, Peng, et al. (2014) specified that a high-pressure microfluidisation could achieve an EE of 94% and a particle size of 70 nm. The principle of this technique is to establish a direct flow through microchannels and an interaction chamber under high pressure. Shear forces and excessive heat could be generated because of the implementation of high pressure, the former leading to the decrease in droplet size (Mahdi Jafari et al., 2006), and the latter could potentially destroy the encapsulated materials if it is not controlled. That was also the reason for applying ice blocks during microfluidisation in the current study. Overall, in line with the previous evidence (Mahdi Jafari et al., 2006; Ré et al., 2019; Zou, Liu, et al., 2014) that showed microfluidisation could achieve a high EE, it was also proven in this study that the EE of liposomes was improved by the microfluidisation.

Different coating materials and their conditions could also alter the EE. Coating materials, as the name implies, are responsible for protecting phenolic compounds from external factors such as light and oxygen. Especially, light can induce photooxidation that damage the phytochemicals including phenolic compounds (Lu & Zhao, 2017). Therefore, an appropriate selection of coating materials for the encapsulation of phenolic compounds is also essential to developing an effective EE (Gadkari &

Balaraman, 2015) and its further application in functional foods and their stability. A large variety of coating materials can be used for the encapsulation of bioactive ingredients; e.g., polysaccharides, lipids, and proteins (Ray et al., 2016). Soy lecithin granule was used as the coating material in this study. Fan et al. (2007) compared the effects of five liposome methods on the encapsulation of salidroside (a phenolic glycoside of genus Rhodiola). They found the effectiveness of the method to prepare liposomes for encapsulation is freezing-thawing > thin-film evaporation > reverse-phase evaporation > melting > sonication. Furthermore, Fang et al. (2006) reported that the EE of the liposomes could be increased by the addition of 15% ethanol to the preparation of hydration solution, as the core material of (−)-epigallocatechin-3-gallate (EGCG) in the liposomes showed a EE close to 100% compared to the EE of 84.6% achieved by the conventional liposome. Additionally, the EE could also be affected by the ionic strength of the liposome solution, as lower ionic strength (liposomes in PB) showed a higher entrapment efficiency of 68.6±7.2% compared to the liposomes in PBS (7.3±4.85%) when 0.25 mg of core material (ovalbumin) was used (Brgles et al., 2008). Third, different core materials could also affect the properties of the liposomes. For example, Fan et al. (2007) suggested that salidroside is very important to prevent the aggregation and fusion of liposomes, as the liposomes with salidroside showed a slower increase in particle size than that without salidroside. The results from this study also showed the differences in EE for various phenolic compounds based on the encapsulation conditions and methods using the same technique.

4.2.2 Loading capacity

The loading capacity represents the maximum phenolic compounds encapsulated in a specific mass of liposomes - in other words, the maximum weight of phenolic compounds that could be encapsulated with one unit weight of coating material(s). Table 4.5 summarises the loading capacities of the liposomes obtained with different methods for various phenolic compounds from the FDDPP. The contents of rosmarinic acid and naringenin were not available with HPLC analysis for the samples before and/or after the Sephadex extraction that separated the loaded liposomal encapsulants from the free phenolic compounds in the samples. The loading capacity of gallic acid and neochlorogenic acid might not be reliable, as the Sephadex extraction was not suitable for the separation process of these two free compounds referred to in section 4.2.1.

Table 4. 5. The loading capacity of the liposomes manufactured with different methods for the encapsulation of different phenolic compounds from the freeze-dried damson plum power.

Phenolics	HSH	HSH+MF	FD-HSH	FD-HSH+MF
Gallic acid	13.7%	16.4%	14.7%	15.1%
Neochlorogenic acid	15.5%	24.4%	17.8%	23.2%
Catechin	37.0%	38.13%	27.6%	30.1%
Epicatechin	34.6%	36.4%	21.7%	25.7%
Rutin	47.3%	59.5%	36.7%	41.6%

Notes: HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum powder extract loaded liposome; FD: Freeze-dried.

The loading capacities of catechin, epicatechin, and rutin were higher in the non-freeze-

dried liposomes compared to the freeze-dried liposomes. The additional microfluidisation enhanced the loading capacity of these three phenolic compounds in liposomes. However, a limited related study reported the loading capacity of liposomes for phenolic compounds analysed in this study. Cheng et al. (2019) introduced a biosurfactant named rhamnolipids to enhance the encapsulation properties (loading capacity and efficiency) of the liposomes used to encapsulate curcumin, and they have achieved a loading capacity larger than 90% for this polyphenol. However, the previous studies only achieved a considerably low loading capacity of 1.4% of curcumin in whey protein-coated liposomes, 3.5% in guar gum-coated liposomes, and 4.4 in Pluronic-modified liposomes (Gómez-Mascaraque et al., 2017; Li et al., 2018; Pu et al., 2019). The loading capacities of liposomes are highly related to the mechanisms or techniques and solvent conditions during obtaining liposomal encapsulants, according to the evidence above.

4.2.3 <u>The particle size of liposomes</u>

Table 4.6 presents the particle sizes of the liposomes under different methods including freeze drying and microfluidisation and/or high-shear homogenisation and their changes during 28 days of storage at 4°C. The average particle size is one of the most important properties of liposomes for the investigation of colloidal dispersions. This metric could help with understanding the physical, chemical, and biological properties of liposomes such as solubility, stability, and release rate (Tamjidi et al., 2013).

Table 4. 6. Particle sizes of empty liposomes and liposomes containing freeze-dried damson plum powder extract during 28 days of storage at 4°C and in the dark.

Particle size (nm)	Day 0	Day 7	Day 14	Day 21	Day 28
HSH-LL	260.6±2.5A[d]	271.6±8.8A[e]	272.4±7.1A[d]	274.7±2.5A[d]	274.9±5.0A[c]
HSH+MF-LL	73.2±1.5B[e]	82.2±1.2A[f]	83.6±0.9A[e]	84.2±1.4A[e]	84.7±1.3A[d]
HSH-EL	281.5±2.0A[d]	290.2±3.2A[e]	297.4±2.8A[d]	299.0±6.0A[c]	301.0±5.8A[c]
HSH+MF-EL	78.3±0.2B[e]	83.5±0.9A[f]	86.5±0.8A[e]	86.9±1.1A[e]	87.1±1.2A[d]
FD-HSH-LL	481.8±1.9A[c]	504.8±1.8A[b]	493.9±15.4A[c]	496.6±18.3A[b]	504.8±16.7A[b]
FD-HSH+MF-LL	531.3±35.3A[b]	519.7±17.9A[b]	521.4±17.9A[b]	520.6±13.1A[b]	533.7±19.8A[b]
FD-HSH-EL	506.5±16.2A[b]	507.4±7.1A[b]	509.7±24.7bA[c]	508.9±19.0A[b]	510.9±14.5A[b]
FD-HSH+MF-EL	765.3±45.6A[a]	796.7±76.1A[a]	799.5±47.4A[a]	770.5±75.4A[a]	781.6±54.7A[a]

Notes: Values are means±SD (n = 3). Values with different scripts in capital letter within the same row are significantly different (p<0.05). Values with different superscripts (a-f) within the same column are significantly different (p<0.05). HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum powder extract loaded liposome; FD: Freeze-dried.

As seen in Table 4.6, overall, except for empty and non-freeze-dried liposomes containing damson plum extract that was obtained by additional microfluidisation, the particle size of the liposomes obtained using other methods did not change during storage ($p<0.05$). It only showed a significant ($p<0.05$) increase in particle size in the first week of storage (4°C) for the liposomes made by the former two methods (Table 4.6). In the case of the non-freeze-dried samples, the particle sizes of the liposomes made using microfluidisation showed a significantly ($p<0.05$) lower value than those that were only obtained by high-shear homogenisation (73.2-87.1 vs 260.6-301.0 nm, respectively). Though there were no significant differences between the particle sizes of the empty liposomes and the liposomes containing damson plum powder extract

under the same method for non-freeze-dried samples, the particle size of the former appeared slightly bigger than the latter. Li et al. (2019) also reported that they could significantly reduce the particle size of F127 modified liposomes and enhanced the storage stability and membrane stability of the liposomes by influencing the interaction of the liposomes and phospholipids.

Additionally, the freeze-dried samples obtained significantly larger particle sizes than that of non-freeze-dried samples ($p<0.05$). It showed contrary results to the non-freeze-dried samples, as for both empty liposomes and the FDDPP-loaded liposomes, the samples with the additional microfluidisation achieved larger particle sizes than the samples without it. The FDDPP extract contained polyphenols such as catechin and epicatechin. These two polyphenolic phytochemicals could interact with the lipid bilayer's surface and increase the size of loaded liposomes because of their affinity (Rashidinejad et al., 2016), and this could result in larger particles for the loaded liposomes compared to the empty liposomes.

There were no significant changes in particle sizes of liposomes from 0 to 28 days of storing at 4°C ($p<0.05$), excluding the first week for the non-freeze-dried liposome samples. There was still a slight increase in the size of non-freeze-dried liposomes as the storage time extended, but freeze-dried samples did not show an increase in their size over time. Overall, the stability of liposomes was performed appropriately in this study for both freeze-dried and non-freeze-dried liposomal samples. Gibis et al. (2013) showed that particle size distribution of the empty liposomes was broadened over time

during storage, which could be induced by the degradation of liposomes resulting from the unsaturated fatty acids degradation. Nevertheless, in the case of the current study, the changes in particle size of liposomes were not significant for both empty and loaded ones over the 28 days of storage at 4°C for 4 weeks in dark.

4.2.4 Zeta potential of liposomes

Table 4.7 presents the zeta potential of the liposomes manufactured using different methods and their changes during the 28 days of storage at 4°C. The negative values of zeta potential of both freeze-dried and non-freeze-dried liposomes slightly decreased over time during storage. This could be related to the structural integration in liposomes during storage, as Dag and Oztop (2017) also reported that the green tea extract-loaded liposomes prepared in distilled water showed a significant drop in the zeta potential values over 14 days.

Zeta potential is a common characterisation method to assess the surface charge of nanoparticles (Smith et al., 2017). In their study, it was suggested that several factors could affect the output value of zeta potential, but ionic strength and pH were considered the two major parameters that had a significant impact on the results. Smith et al. (2017) reported that the zeta potential of liposomes containing 1,2-distearoyl-sn-glycero-3-phosphocholine, cholesterol, and 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (61.8:31.3:6.9) shifted from -45 to -21 mV when the pH was adjusted from 7.7

to 3.8. In the current study, all the non-freeze-dried liposomes were manufactured in pH 3.8 acetate buffer (sodium acetate and acetic acid), and the freeze-dried liposomes were redispersed in the same buffer as well.

The other crucial factor for liposome stability is ionic strength. It was reported by Smith et al. (2017) that ionic strength could affect the zeta potential of liposomes, which may have been the case in the current study too. In their study, the zeta potential of liposomes shifted from -63 to -21 mV when the concentration of NaCl was increased from 0 to 0.05 mol/L (pH: 7.5-7.7). The nanoparticles with a large positive or negative zeta potential have good physical stability due to the electrostatic attraction between individual particles. Similar results were also reported by Brgles et al. (2008) that the zeta potential of the anionic liposomes was more negative in phosphate buffer (PB) than in phosphate buffer with 0.15 M NaCl (PBS), and the zeta potential of cationic liposomes was more positive in PB than in PBS.

Table 4. 7. Zeta potential of empty liposomes and liposomes containing freeze-dried damson plum extract during the storage for 28 days at 4°C and in the dark.

Zeta potential (mV)	Day 0	Day 7	Day 14	Day 21	Day 28
HSH-LL	-30.94±0.58A^{b}	-28.80±0.51B^{b}	-28.58±0.36B^{a}	-29.09±0.88B^{a}	-28.84±0.49B^{a}
HSH+MF-LL	-31.21±1.14A^{b}	-30.91±0.67A^{a}	-29.76±0.34A^{a}	-27.01±0.67B^{a}	-25.17±0.74C^{b}
HSH-EL	-31.04±0.38A^{b}	-30.81±0.40A^{a}	-29.05±0.13B^{a}	-28.46±1.00B^{a}	-27.69±0.57B^{ab}
HSH+MF-EL	-35.39±0.97A^{a}	-30.44±0.37B^{a}	-29.45±0.89B^{a}	-29.82±0.43B^{a}	-29.74±0.71B^{a}
FD-HSH-LL	-23.67±1.96A^{c}	-25.14±1.30A^{c}	-25.37±0.21A^{b}	-22.55±1.94A^{b}	-22.24±1.73A^{c}
FD-HSH+MF-LL	-29.92±0.60A^{b}	-26.6±1.05B^{c}	-25.25±0.89B^{b}	-24.25±1.58BC^{b}	-22.14±1.53C^{c}
FD-HSH-EL	-28.43±1.21A^{b}	-25.69±2.30A^{c}	-27.73±0.40A^{ab}	-24.85±3.11B^{b}	-23.42±2.00B^{bc}
FD-HSH+MF-EL	-31.64±2.00A^{b}	-30.93±1.23A^{a}	-29.22±0.75A^{a}	-26.77±1.71B^{ab}	-25.14±1.44B^{b}

Notes: Values are means±SD (n = 3). Values with different scripts (A-C) within the same row are significantly different ($p<0.05$). Values with different superscripts (a-f) within the same column are significantly different ($p<0.05$).

HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum extract loaded liposome; FD: Freeze-dried.

In this experiment, the initial value of zeta potential was in the range of -30.94 to -35.39 mV for non-freeze-dried liposomes and -23.67 to -31.64 mV for freeze-dried liposomes (Table 4.7). It was suggested by Tamjidi et al. (2013) that the value of zeta potential approximate to or higher than ±30 mV could stabilise steric and electrostatic repulsions and prevent the liposomes from aggregation. Most encapsulation methods in this study could achieve a more negative zeta potential than -30 mV except for freeze-dried high-shear homogenised FDDPPE-loaded liposomes (-23.67±1.96 mV). Overall, the zeta potential of liposomes obtained in this study (30±2 mV or higher) means high stability of liposomes, which are much higher than the values reported by some previous researchers; e.g., Kanlayavattanakul et al. (2013) and Figueroa-Robles et al. (2021).

In the current research, the empty liposomes showed higher physical stability than the FDDPPE-loaded liposomes for both microfluidised and/or high-shear homogenised samples. The microfluidisation did significantly improve the stability of the empty non-freeze-dried liposomes and the FDDPPE-loaded freeze-dried liposomes; i.e., the zeta potential values of the samples achieved by additional microfluidisation were more negative than those obtained by high-shear homogenisation only (Table 4.7). Freeze drying, on the other hand, showed a significant negative effect on the value of the zeta potential of liposomes ($p<0.05$). However, microfluidisation could make up for the negative effect brought by freeze drying, so that the zeta potential value was still close to -30 mV for loaded liposomes, which showed good physical stability.

Further study on improving the stability of solid freeze-dried liposomes is required, as the zeta potential value for this type of vesicle dropped significantly after 21 days ($p<0.05$). Nonetheless, the solid-formed FDDPPE-loaded liposomes are still applicable in the food and pharma industry for solid functional products, even though the liposomes dispersed in the buffer appeared to perform better physical properties and stability compared to the freeze-dried liposomes.

Table 4.8. shows the appearance of the aqueous medium containing both empty liposomes and the liposomes loaded with the plum extract over the storage of 28 days at 4°C. Some obvious changes could be seen in colour during the 28 days of storage for both empty and FDDPPE-loaded liposomes for non-freeze-dried liposomal encapsulants. The reason for the colour change of FDDPPE-loaded liposomes could be

the release or leakages (to a reasonable degree) of core materials over time during storage. Guldiken et al. (2018) assessed the physical and chemical stability of anthocyanin-rich black carrot extract-loaded liposomes during storage for 21 days and found that some discolouration of liposomes occurred during this time, possibly related to the leakage of encapsulated materials.

Table 4. 8. The appearance of empty liposomes and liposomes containing freeze-dried damson plum extract during 28 days of storage at 4°C and in the dark.

Methods	**Empty liposomes**			**Loaded liposomes**		
	Day 0	Day 14	Day 28	Day 0	Day 14	Day 28
High-shear homogenisation						
High-shear homogenisation and microfluidisation						

4.2.5 Morphology of liposomes

Transmission electron microscopy (TEM) was used to observe the size appearance and distribution of liposomes manufactured in this research. High magnification and high-resolution images could help with showing the laminar structure of liposomes containing coating and core materials (Rashidinejad et al., 2014).

The TEM micrographs of empty and FDDPPE-loaded liposomes obtained with microfluidisation and/or high-shear homogenisation are shown in Figure 4.7. The TEM micrographs of empty liposomes obtained with high-shear homogenisation under various magnifications are also shown in Figure 4.8. The particle size of the liposomes viewed using TEM confirmed the values reported for particle size and zeta potential (Tables 4.6-7). Compared to the study conducted by Rashidinejad et al. (2014), who delivered the encapsulated green tea catechin into cheese, the double layer microstructure of liposomes was clearly shown their studies, which agrees with the findings of the current research. They obtained an average diameter of 133 nm for empty liposomes, but 139-169.7 nm for various concentrations of catechin-loaded liposomes. However, more than doubled average particle sizes were presented in this study (see Section 4.2.3). This could result from differences in soy lecithin used for obtaining liposomes in the two different studies; i.e., solid soy lecithin granule in this research and liquid soy lecithin in the research carried out by Rashidinejad et al. (2014).

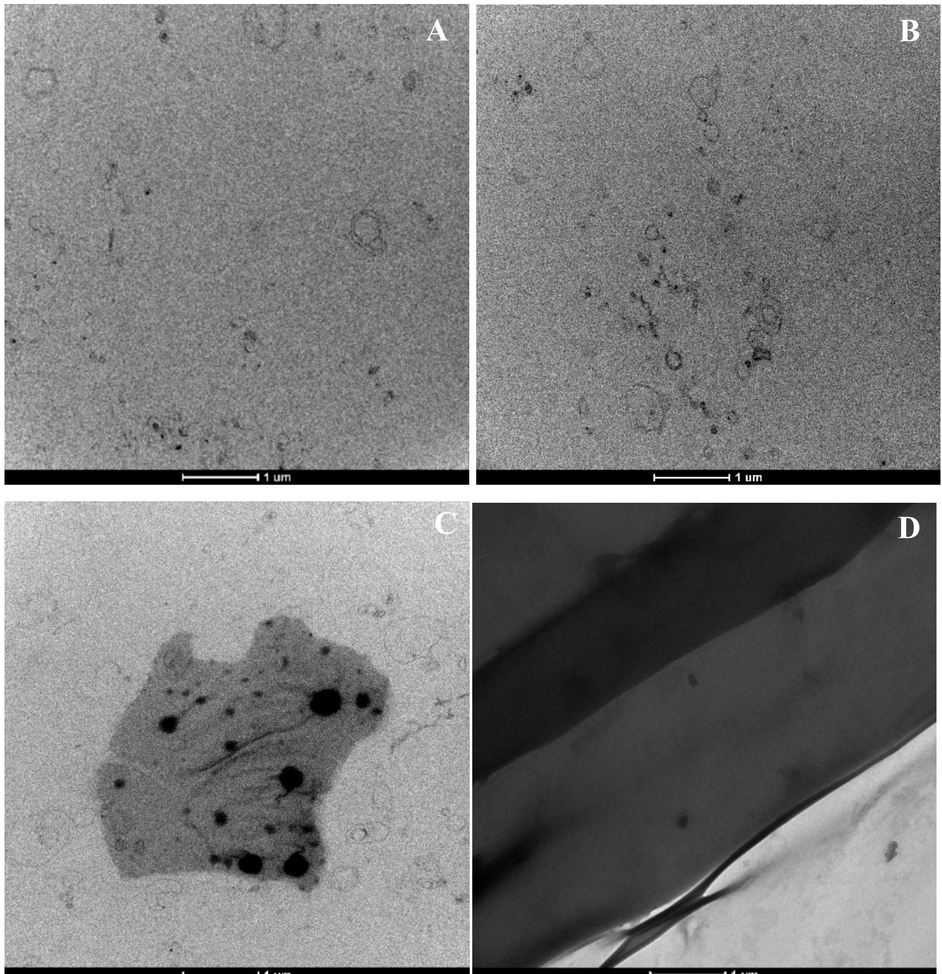


Figure 4. 7. Transmission electron micrographs of liposomes containing freeze-dried damson plum extract. A: Liposomes containing plum extract prepared using high-shear mixer, B: Liposomes containing plum extract prepared using high-shear homogeniser and microfluidiser, C: Control/empty liposomes prepared using high-shear mixer, and D: Control/empty liposomes prepared using high-shear mixer and microfluidiser under same magnification of 16500×.

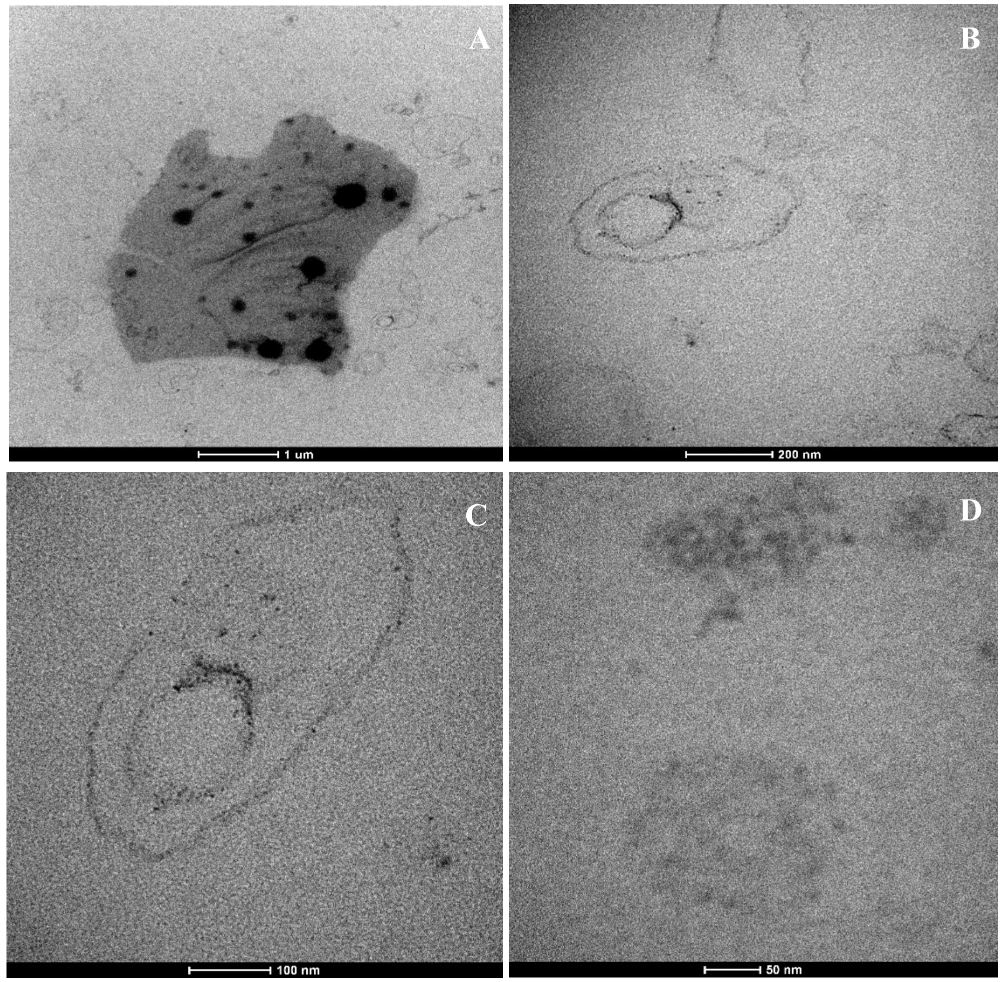


Figure 4. 8. Transmission electron micrographs of empty nanoliposomes prepared with high-shear homogeniser viewed under different magnifications. A: 16,500×, B: 82,500×, C: 165,000×, and D: 285,000×.

4.3 Physical properties of liposomes before and after incorporation into milk

Encapsulated FDDPPE with four encapsulation methods and control were applied to the standard milk to enrich its nutritional value. The physical properties, nutritional value, and phenolic compounds of the milk mixes were assessed. Particle size, zeta potential, and pH value could indicate the stability of the functional milk, and proximate analysis and HPLC analysis of seven phenolic compounds indicate the function of

alteration of nutrition value by the application of the encapsulated FDDPPE and their chemical stability in liposomes after their dispersion in milk. The following sections present the results of the effect of added encapsulated FDDPPE on the physical properties, nutritional value, and phenolic properties of functional milk.

4.3.1.1 Particle size, zeta potential, and their correlation with pH value

Table 4.9 presents the pH value, average particle size, and zeta potential of the control milk and the milk containing liposomes loaded with the damson plum extract.

Table 4. 9. The pH value, average particle size, and zeta potential of the control milk and the milk containing liposomes loaded with the damson plum extract.

Samples	pH value	Average particle size (nm)	Zeta potential (mV)
Milk control	6.61±0.01^{a}	375±13.7^{c}	-32.17±0.84^{b}
Milk with unencapsulated damson plum extract	6.68±0.01^{a}	387±12.1^{c}	-31.71±0.57^{b}
Milk with HSH-EL	5.27±0.01^{b}	423±15.7^{b}	-35.49±0.62^{ab}
Milk with HSH+MF-EL	5.30±0.01^{b}	405±14.8^{b}	-37.61±0.95^{a}
Milk with HSH-LL	5.28±0.01^{b}	416.6±13.8^{b}	-34.75±0.57^{ab}
Milk with HSH+MF-LL	5.29±0.01^{b}	412.9±9.9^{b}	-35.17±1.00^{ab}
Milk with FD-HSH-LL	5.17±0.01^{c}	800.5±72.1^{a}	-27.42±1.79^{c}
Milk with FD-HSH+MF-LL	5.19±0.01^{c}	751.0±41.2^{a}	-30.43±1.58^{bc}

Notes: Values are means±SD (n = 3). Values with different superscripts (a-c) within the same column are significantly different ($p<0.05$). HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum powder extract loaded liposome; FD: Freeze-dried.

The particle size of the milk fortified with FDDPPE-loaded liposomes was determined to assess the stability of liposomes dispersed in the milk. According to the determined average particle size of control and fortified samples, only the particle sizes of milk

samples with freeze-dried liposomes were significantly ($p<0.05$) larger than the others (Table 4.9). The addition of non-freeze-dried empty and FDDPPE-loaded liposomes obtained by microfluidisation and/or high-shear homogenisation significantly ($p<0.05$) increased the average particle size of the milk mix. However, the liposomes obtained with the addition of microfluidisation step showed no significant ($p>0.05$) impact on the milk compared to the ones obtained with high-shear homogenisation. There were no significant differences between the particle size of milk mix with empty liposomes and FDDPPE-loaded liposomes. Therefore, the average particle size and core materials of the liposomes did not significantly ($p>0.05$) affect the average particle size of the milk mix.

Cations in milk are capable to regulate electrostatic interactions and could interfere with the stability of liposomes (Wu et al., 2020). In this trial, the cations in milk slightly improved the ionic strength, as the pH condition of milk (6.61 at 22.5°C) are much higher than the acetate buffer (3.8 at 22.5°C). These two factors were discussed in Section 4.2.4, which could significantly affect the stability of liposomes with slight changes, as the high pH value could significantly improve the stability of liposome. However, high ionic strength would notedly reduce the stability of liposomes.

The addition of encapsulated plum extract significantly reduced the pH value due to the acetate buffer that was in the dispersion containing liposomes (see Section 3.2.5). The pH value could also significantly affect the coagulation rate of the milk. Castillo et al. (2000) showed that the clotting time and cutting time of goat's milk reduced

significantly from 24.5 to 11.8 min and from 27.2 to 15.1 min, respectively, when the pH value was reduced from 6.5 to 5.5. Therefore, a lower pH of 5.28±0.02 could accelerate the coagulation of milk compared to the pH value of 6.65±0.04. The increase in particle size of the milk mix with non-freeze-dried samples could be the coagulation of milk due to the significant drop of pH value with the introduction of accompanied acetate buffer.

The pH values of milk with freeze-dried liposomes were only 0.1 lower than the milk samples with non-freeze-dried liposomes, but the average particle size doubled for milk samples with freeze-dried liposomes. Xu et al. (2022) showed scaled-up evidence that a significant change in particle size of the skim milk samples was observed, as the large size population of 0.1 μm and minor populations at 10 μm when the pH values were 6.6, 5.6, and 5.3. However, the major size population shifted from 0.1 μm to 10 μm when the pH value was altered from 5.3 to 5.2. This was explained by the beginning of gelation and solubilisation of the 'cementing agent' colloidal calcium phosphate (CCP) from casein micelles in milk because casein micelles become unstable at pH 5.2 at 20 °C due to the full solubilisation of CCP (Guinee & O'Brien, 2010).

The zeta potential of the FDDPPE-loaded liposomes under different liposomal forming methods in milk is presented in Table 4.9. Interestingly, the zeta potential of the milk control was lower than that of the milk fortified with non-freeze-dried liposomal encapsulants; nonetheless, it was higher than that of the milk fortified with freeze-dried liposomal encapsulants. This indicated that the addition of the redispersed freeze-dried

liposomes decreased the stability of the colloidal dispersion. One of the reasons could be the low pH value below 5.2, which was explained in the correlation between particle size and pH value. The other reason could be the unstable lipid structure in the redispersed freeze-dried liposomes (due to the freeze drying without cryoprotectant), which caused the leakage of core material that could in turn react with the casein micelles in the milk, thus resulting in the aggregate of caseins. This was hypothesised referring to Han et al. (2019) who studied the interactions between phenolic compounds, such as catechin, tannic acid, homovanillic acid, and hesperetin, and milk proteins. They found that not only pure phenolic compounds but also natural crude polyphenols, such as green tea, grape, and cranberry extracts, had strong hydrophobic bindings with calcium caseinate in milk.

4.3.1.2 <u>Proximate analysis</u>

As mentioned in the methods section, this analysis was done by a certified laboratory and no statistical analysis was carried out on the corresponding data. Nonetheless, based on the results presented in Table 4.10, the control milk showed a higher solid content and lower moisture content than the other samples. This is the consequence of the addition of buffer in other samples that diluted the solid proportion, meaning that these parameters were not affected by the addition of the free FDDPPE or its encapsulated form. The relative comparison between the milk with buffer and the rest of the samples

showed that the addition of encapsulated or non-encapsulated FDDPPE improved the total DM of the milk mix by 0.64% for both freeze-dried and non-freeze-dried liposomes and 0.54% for the non-encapsulated FDDPPE. The introduced soy lecithin only altered the total solid by 0.1% (changing the crude fat by only 0.1%, as the result), which can be negligible. There was no considerable effect of FDDPP on other parameters such as minerals, fibre, crude protein, and carbohydrates.

Table 4. 10. Proximate analysis of control milk and the milk containing liposomes loaded with the damson plum extract.

Proximate analysis	Moisture%	Total solid%	Ash%	Crude Protein%	Crude Fat%	Carb%
Milk control	89.8	10.2	0.6	2.8	3	3.8
Milk+buffer	91.84	8.16	0.48	2.24	2.4	3.04
Milk+extract	91.3	8.7	0.5	2.4	2.5	3.3
Milk+Encap	91.2	8.8	0.5	2.4	2.6	3.3
Milk+FD Encap	91.2	8.8	0.5	2.4	2.6	3.3

Notes: Milk control: 100% milk; Milk+buffer: 80% milk mixed with 20% acetate buffer (pH 3.8); Milk+extract: 80% of milk mixed with 20% of buffer containing unencapsulated/free freeze-dried damson plum extract (same amount of damson plum extract to the encapsulated ones); Milk+Encap: 80% of milk mixed with 20% buffer containing liposomal damson plum extract; Milk+FD Encap: 80% of milk mixed with 20% freeze-dried liposomes containing the damson plum extract.

4.3.1.3 Viscosity

The viscosities of the blank milk, milk fortified with unencapsulated and non-freeze-dried, and freeze-dried liposomes containing damson plum extract are presented in Figure 4.9. The viscosity of milk samples was assessed using a rheometer (AR-G2, TA Instruments, UK) equipped with a Din Rotor concentric cylinder geometry. A water bath was used to maintain the temperature during the data acquirement of the viscosity

of milk samples. This purpose was because the dynamic viscosity of milk is responsible for the temperature. Milk behaves as a Newtonian fluid under most conditions, so the shear stress is proportional to the shear rate (Park, 2007), and the viscosity of milk is not responsible for the shear stress applied to milk. Park (2007) suggested that the contribution of casein micelles in milk appears to closely depend on temperature. It was previously explained by Walstra (1999) that a considerable increase in the viscosity of milk at low temperatures resulted from the marked increase of voluminosity of micelles and the dissociation of a part of caseins from the micelles. Additionally, Jenness and Patton (1959) showed the viscosity of milk increase when the heat reaches the point of coagulation of the proteins during the production of superheated condensed milk.

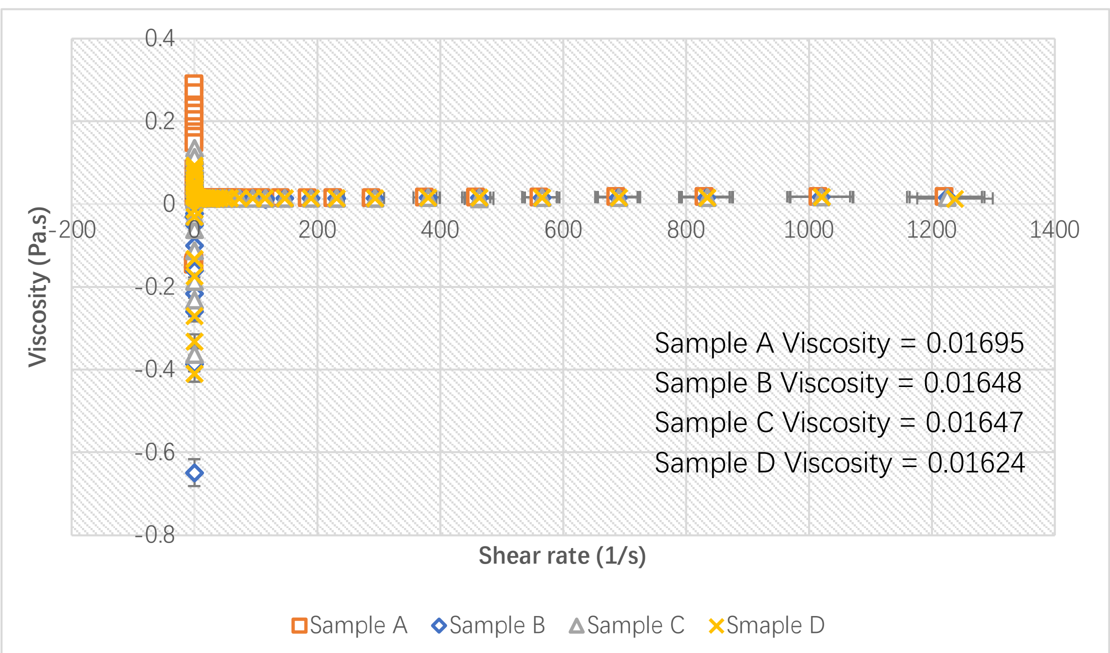


Figure 4. 9. The viscosity of the control milk (Sample A) and the milks containing free damson plum extract (Sample B), non-freeze-dried (Sample C), and freeze-dried (Sample D) liposomes loaded with the damson plum extract.

In the case of this experiment, there were no significant differences seen in the viscosity of the four samples. According to Figure 4.7, the viscosity of milk samples was stable at 0.01648±0.0005 Pa-s, as the shear rate was increased from 0.1 to 1200 s^{-1}. This indicated that the addition of both unencapsulated FDDPPE and the liposomes containing FDDPPE did not alter the viscosity and the fluid property of the milk.

4.3.1.4 The recovery of encapsulated FDDPPE in milk determined using HPLC

Tables 4.11 and 4.12 show the content and recovery rate of various phenolic compounds in the blank milk and the milk samples fortified with liposomes, respectively. Two phenolic compounds, including gallic acid and naringenin, were not found in any of the milk samples fortified with FDDPPE-loaded liposomes; i.e., the recovery rates of these two phenolic compounds were zero in the milk fortified with four different liposomes and stored for 28 days at 4°C. However, the other five compounds were all found in the milk fortified with freeze-dried liposomal encapsulants, although rutin was not present in the milk fortified with non-freeze-dried liposomal encapsulants, and epicatechin was not found in the milk sample fortified with the non-freeze-dried liposomal encapsulant obtained by high-shear homogenisation (Tables 4.11 and 4.12).

Table 4. 11. The contents of seven phenolic compounds in control milk and the milk fortified with different liposomes containing plum extract after the storage at 4°C for 28 days.

Phenolic compounds (µg/mL)	Milk blank	Original extracted in same ratio dilution	HSH-LL in milk	HSH+MF-LL in milk	FD-HSH-LL in milk	FD-HSH+MF-LL in milk
Gallic acid	0	0	0	0	0	0
Neochlorogenic acid	0	5.27	3.98	4.29	4.97	5.21
Rosmarinic acid	0	0.25	0.17	0.21	0.22	0.24
Catechin	0	1.04	0.51	0.54	0.63	0.81
Epicatechin	0	0.55	0	0.12	0.38	0.47
Rutin	0	1.13	0	0	0.98	1.07
Naringenin	0	1.14	0	0	0	0

Note: HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum powder extract loaded liposome; FD: Freeze-dried.

Table 4. 12. The recovery rates of five phenolic compounds in control milk and the milk fortified with different liposomes containing plum extracts after they were stored at 4°C for 28 days.

Recovery rate (%)	HSH-LL In milk	HSH+MF-LL in milk	FD-HSH-LL in milk	FD-HSH+MF-LL in milk
Neochlorogenic acid	75.52	81.40	94.31	98.86
Rosmarinic acid	68.00	84.00	88.00	96.00
Catechin	49.04	51.92	60.58	77.88
Epicatechin	0	21.82	69.09	85.45
Rutin	0	0	86.73	94.69

Note: HSH: high-shear homogenisation; MF: microfluidisation; EL: empty liposome; LL: freeze-dried damson plum powder extract loaded liposome; FD: Freeze-dried.

The freeze-dried liposomal encapsulants showed a considerably higher recovery rate than the non-freeze-dried liposomal encapsulants for these five phenolic compounds. Stark et al. (2010) stated that the application of cryoprotectants such as a mixture of glycerol and carbohydrate at the concentration of about 1% during freezing could obtain

a very stable structure of liposomes, because the freeze drying could significantly change their bilayer organisation and the transition behaviour of lipids.

The encapsulated phenolic compounds were more stable in freeze-dried power form than in the liquid form after 28 days of storage, though the physical stabilities of the freeze-dried liposomes were not good as the liquid form since they were redispersed in the acetate buffer (pH 3.8). In this case, the freeze drying method could be applicable to extend the shelf life of the encapsulated phenolic compounds from the FDDPP, as it could achieve up to 98.86% recovery rate for neochlorogenic acid, 96% for rosmarinic acid, and 94.69% for rutin, if they were encapsulated with the additional microfluidisation step (Table 4.12).

The recovery rates of the catechin and epicatechin were not as high as the other three phenolic compounds, but the freeze-dried liposomal encapsulant still performed the best recovery rate of phenolic compounds after they were added to milk. The recovery rate of epicatechin was higher than that of catechin for the milk fortified with freeze-dried liposomes. Xu et al. (2004) explained that the non-epimerase catechins such as catechins, catechin gallate, and gallocatechin rapidly degrade because of their poor stability compared to epicatechin. Additionally, the microfluidisation also improved the chemical stability of phenolic compounds extracted from FDDPP in the freeze-dried and non-freeze-dried liposomal encapsulants. It enhanced the recovery rate of catechin and epicatechin by 17.3% and 16.36% for the milk sample fortified with the freeze-dried liposomal encapsulants. This technique was also suggested by Zou, Liu, et al.

(2014) to decrease the degradation rate of tea polyphenol solution because of the slow release ratio of tea polyphenols controlled by the nanoliposomes compared to the solution of free tea polyphenol. Some other research has reported similar results that microfluidisation could obtain the liposomes with better stability and smaller particle size than only high-shear mixing (Li et al., 2019; Wagner & Vorauer-Uhl, 2011). However, no study compared the effect of microfluidisation and high-shear homogenisation on the chemical stability of core materials of liposomes.

The other important point to make in the case of the current study is the extraction method used for the preparation of milk samples before HPLC analysis. The ethanolic extraction before the HPLC analysis may not be suitable for all the phenolic compounds that were encapsulated in liposomes. It was previously reported that the ethanol extracted three times rosmarinic acid from *Orignum hirtum* (oregano) compared to ethyl acetate, but this solvent (i.e., ethanol) did not extract any naringenin while ethyl acetate was effective for this purpose. Therefore, various phenolic compounds in liposomal encapsulants were differently extracted by the ethanolic extraction system used before HPLC analysis. This could also affect the results of recovery rate differences of various phenolic compounds. Additionally, the potential interaction between polyphenols extracted from FDDPP and protein from milk could affect not only the physiochemical properties of milk but also the extracted phenolic compounds during the ethanolic extraction process of milk mix before the HPLC analysis. For example, an *in vitro* study conducted by Yuksel et al. (2010) demonstrated that casein

from milk could interact with polyphenols extracted from green tea by hydrophobic binding. Binding between polyphenol and proteins may result in a diminished digestibility of the proteins (Petzke et al., 2005).

5 Conclusions, recommendations, and future directions

5.1 Conclusions

In this study, the extraction of various phenolic compounds, including neochlorogenic acid, gallic acid, rosmarinic acid, catechin, epicatechin, naringenin, and rutin from freeze-dried damson plum powder (FDDPP) was investigated using different extraction techniques, solvents, and timings. Based on the results from the corresponding analyses (e.g., TPC, TFC, TAC, DPPH, ABTS, and HPLC), the New Zealand damson plum used in this experiment is a rich source of bioactive compounds with strong antioxidative properties. The accelerated solvent extraction (ASE) was the most effective method for the extraction of phenolics and anthocyanins from the plum powder, especially, when water was used as the solvent and the extraction lasted for 40 min. The enzyme-assisted extraction (EAE) improved the extraction of anthocyanins, possibly due to its function of retardation of hydrolysis of anthocyanins by the inhibition of polyphenol oxidase. However, high frequency or extended ultrasonic extraction could suppress the extraction of total anthocyanin content, due to the degradation of anthocyanin glucosides caused by the action of polyphenol oxidase (induced by the ultrasonication). Additionally, the antioxidant activity values for the samples extracted using the ASE

method were higher than those extracted using the other methods. Nevertheless, the extraction method that combined both ASE and ultrasound enzyme assisted (UEA) extraction (60 min) showed the highest extraction efficacy for the total flavonoid content.

Neochlorogenic acid was the predominant phenolic compound found in the FDDPP and played a significant role as an antioxidant, followed by gallic acid, catechin, epicatechin, naringenin, rutin, and rosmarinic acid. The different extraction methods showed various extraction efficacies for these seven phenolic compounds found in the FDDPP. For instance, neochlorogenic acid was found to be substantially ($p<0.05$) greater in the samples extracted using the ASE method with ethanol as the solvent for 40 min than in the other samples. The concentration of rutin, one of the main flavonoids in damson plums, was higher when samples were exposed to the EAE extraction for 40 min than when they were exposed to the other extraction methods.

The liposomal encapsulation technique was applied to entrap and protect the extracted phenolic compounds from the FDDPP, where the two different techniques (high-shear homogenisation and microfluidisation) applied for liposome manufacture presented various values for encapsulation efficiency (EE), encapsulation yield (EY), and loading capacity (LC) for catechin, epicatechin, and rutin (analysed by HPLC). The additional microfluidisation step in the encapsulation process enhanced the EE, EY, and LC of all phenolic compounds in the manufactured liposomes. The freeze drying technique improved the shelf life of the liposomes during storage in solid form compared to the

liquid form, but the physical stabilities (measured by zeta potential and particle size analyses) of the redispersed liposomes were not as satisfactory as the non-freeze-dried liposomal encapsulants. The microstructure of both empty and loaded liposomes was seen under the TEM micrographs, indicating the successfulness of the encapsulation process in this study.

Milk was chosen as a suitable delivery functional food for the incorporation of the liposomes containing plum extract, due to its availability, convenience, and potential nutritional benefits. The recovery rates of neochlorogenic acid, rosmarinic acid, catechin, and rutin in liposomes obtained with different methods were determined using HPLC. The freeze drying method as well as the additional microfluidisation step appeared to improve the recovery rate of these five phenolic compounds.

Taken together, this research confirmed the efficient extraction of potent high-value bioactive compounds (neochlorogenic acid, gallic acid, catechin, epicatechin, rosmarinic acid, and naringenin) from the New Zealand damson plums using environmentally friendly (water), simple, and efficient extraction methods. It also proved the successful liposomal encapsulation of this extract for its incorporation into functional food/beverage products as the most convenient way of delivering the functionality of bioactive compounds from New Zealand damson plum.

5.2 Recommendations and future directions

This research applied ASE as a novel method of the extraction of bioactive compounds

from New Zealand damson plums, and comparisons between its extraction efficiency with other extraction methods (e.g., UAE and EAE) were made. Nevertheless, some results such as the extraction efficacy of the ASE method in this study are yet to be explained by further investigation of this method. Further evidence is also needed to compare the encapsulation efficiencies of various encapsulation methods for various phenolic compounds from damson plums. For example, in the current research, no cryoprotectant was applied to obtain the freeze-dried liposomal powder. Thus, further study may apply this agent and research the physical and chemical stability of core materials during storage for a longer period. Additionally, the *in vitro* release of the seven phenolic compounds extracted from the damson plum extract in this study, along with the assessment of their bioavailability, bioaccessibility, and toxicity, are yet to be studied.

Ultimately, future research through the implementation of some corresponding clinical trials can address the evolution of the bioefficacy (e.g., prevention of cardiovascular diseases, cancer, obesity, diabetes, and muscle wastes) of the phenolic compounds from damson plums (in both free and encapsulated form and incorporated into functional food products). The effect of the phenolic compounds on the sensorial properties of the functional food products containing encapsulated damson plum extract is also a critical factor to be assessed before any further development of such products. Last but not least, the regulations and food safety for functional food products and their corresponding health claims are clear neither in New Zealand nor globally. Therefore, this needs to be

considered for the further development of functional products containing liposomal encapsulated phenolic extract from New Zealand damson plums.

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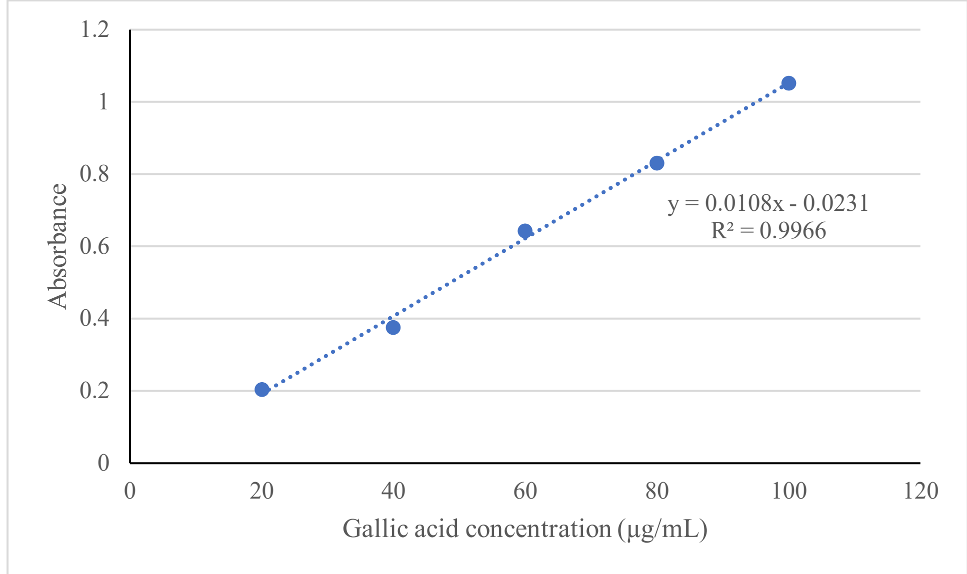
Appendices

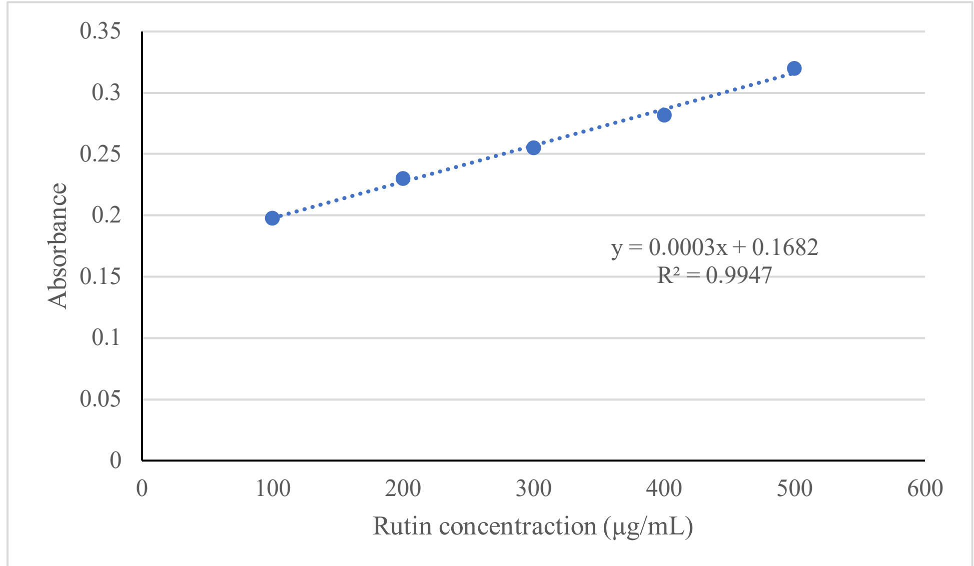
Appendix 1.

A calibration curve for different concentrations of gallic acid using the Folin-Ciocâlteu method for total phenolic content of damson plums.



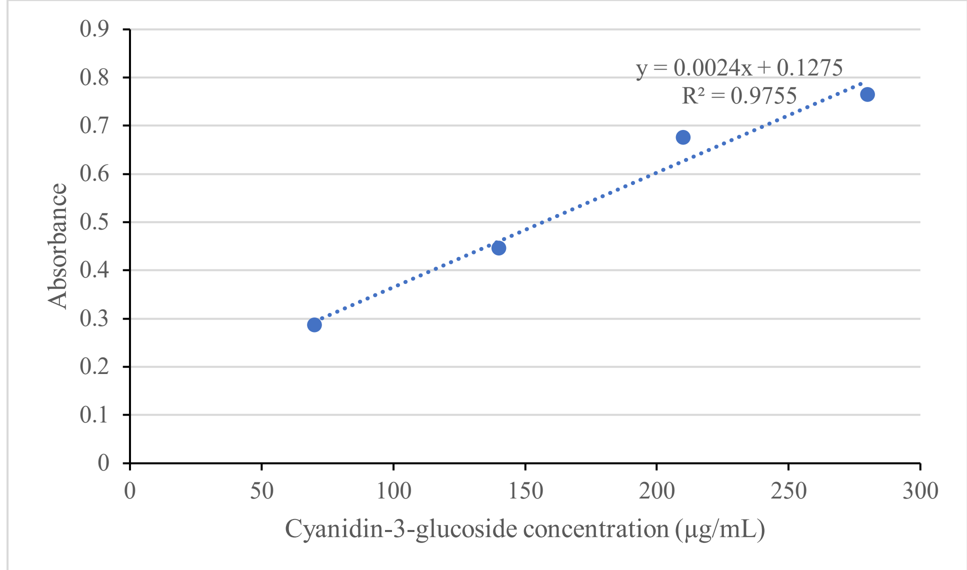
Appendix 2.

A calibration curve for different concentrations of rutin using the aluminium colourimetric assay for total flavonoid content of damson plums.



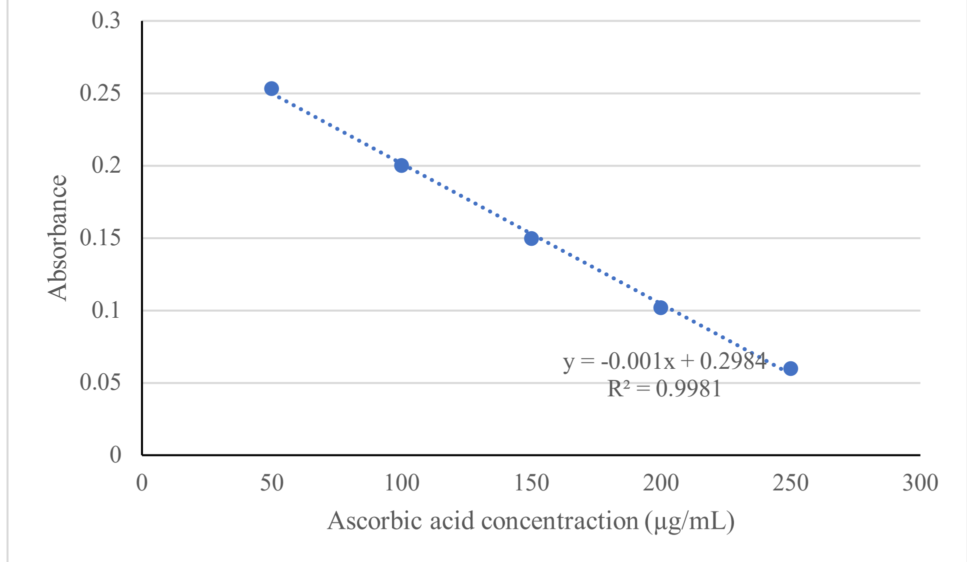
Appendix 3.

A calibration curve for different concentrations of cyanidin-3-glucoside using the AOAC pH-differential method for total anthocyanin content of damson plums.



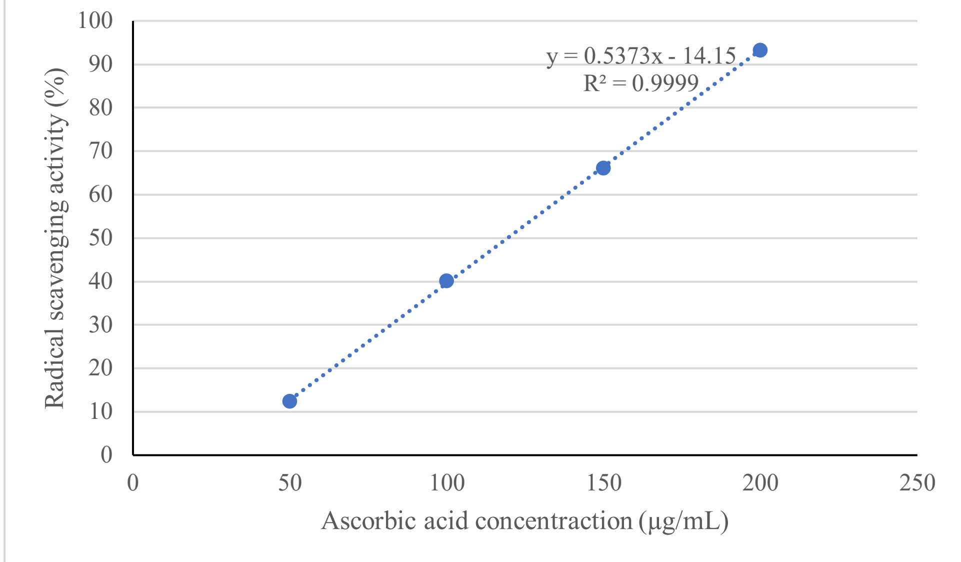
Appendix 4.

A calibration curve for different concentrations of ascorbic acid for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay.



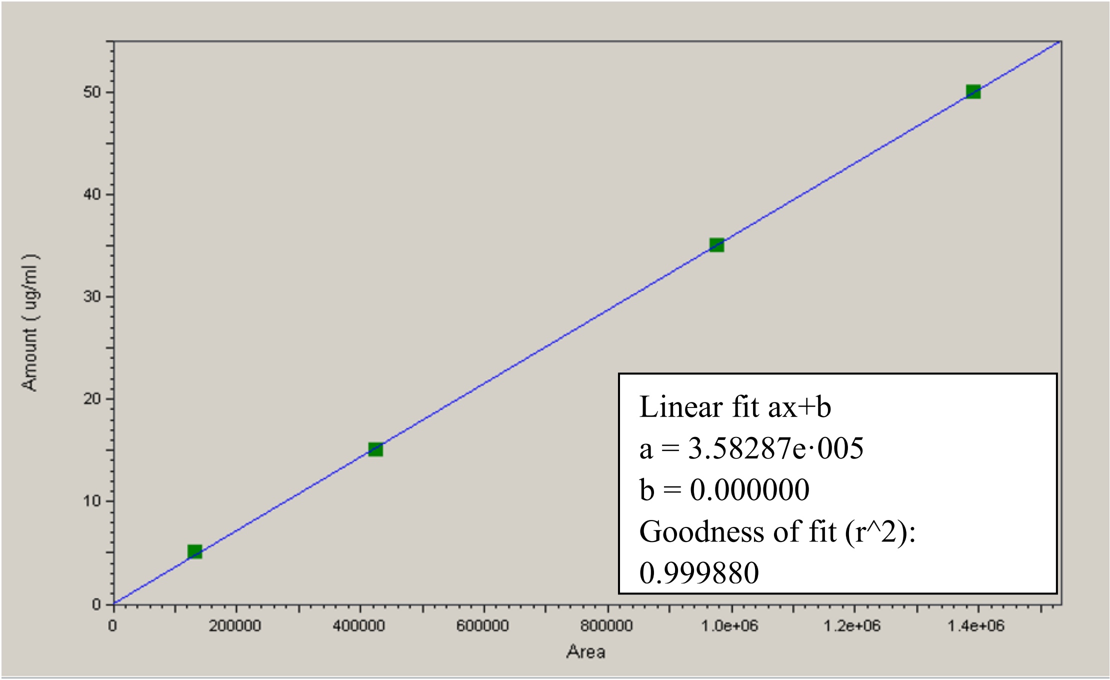
Appendix 5.

A calibration curve for different concentrations of ascorbic acid for 2,2'-Azinobis-3-ethylbenzothiazoline-6-Sulfonic acid (ABTS) radical assay.



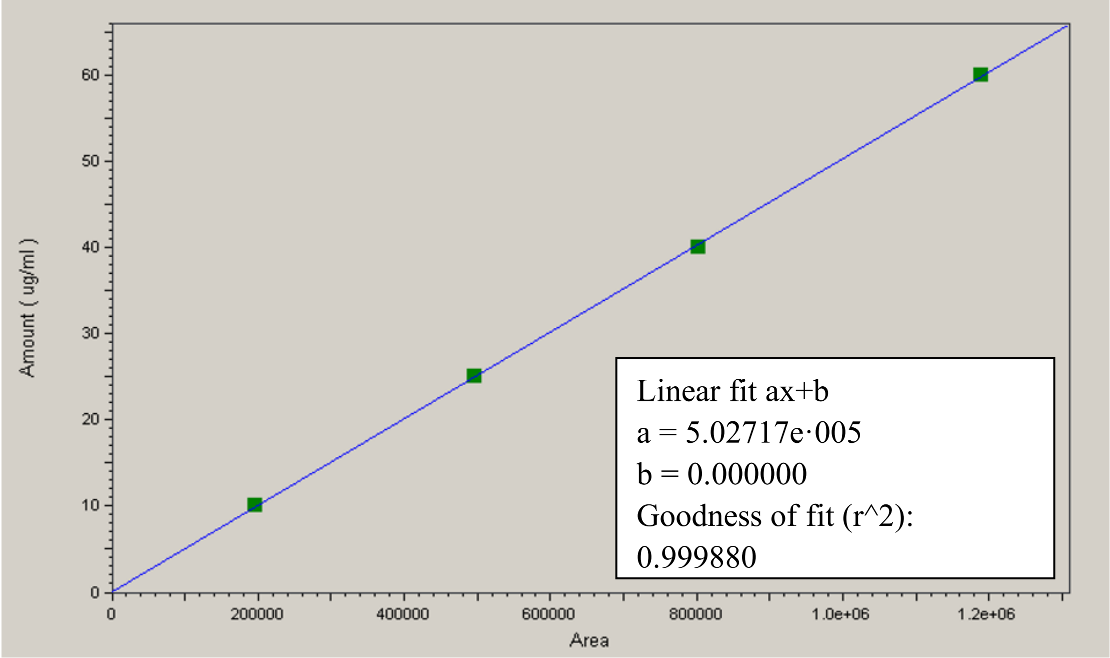
Appendix 6.

A calibration curve for different concentrations of gallic acid monohydrate for high-performance liquid chromatography (HPLC) assay.



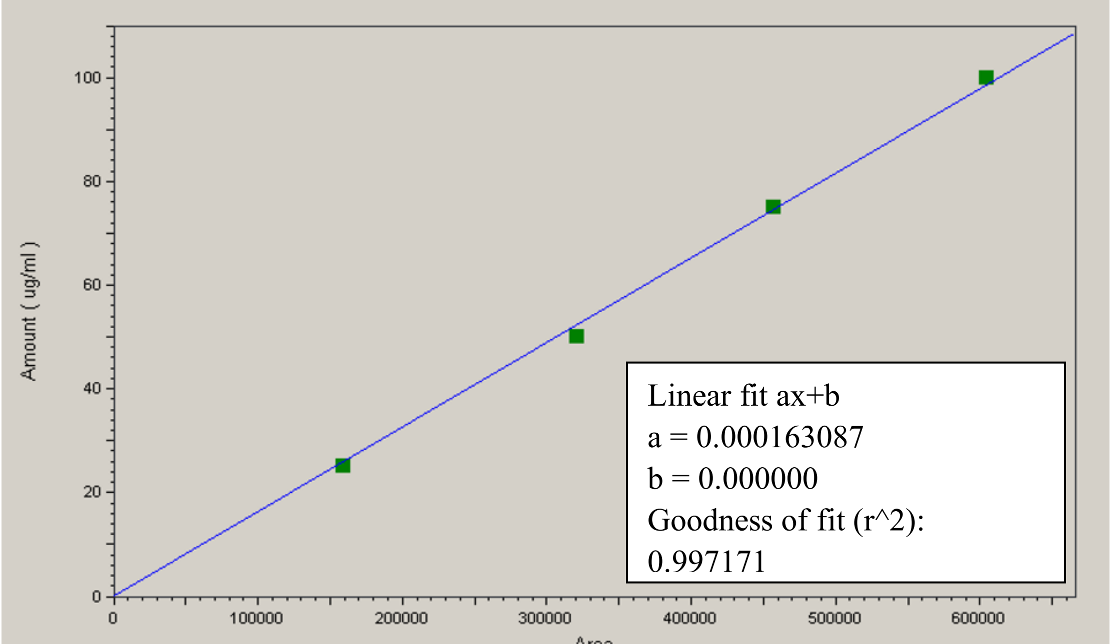
Appendix 7.

A calibration curve for different concentrations of neochlorogenic acid for high-performance liquid chromatography (HPLC) assay.



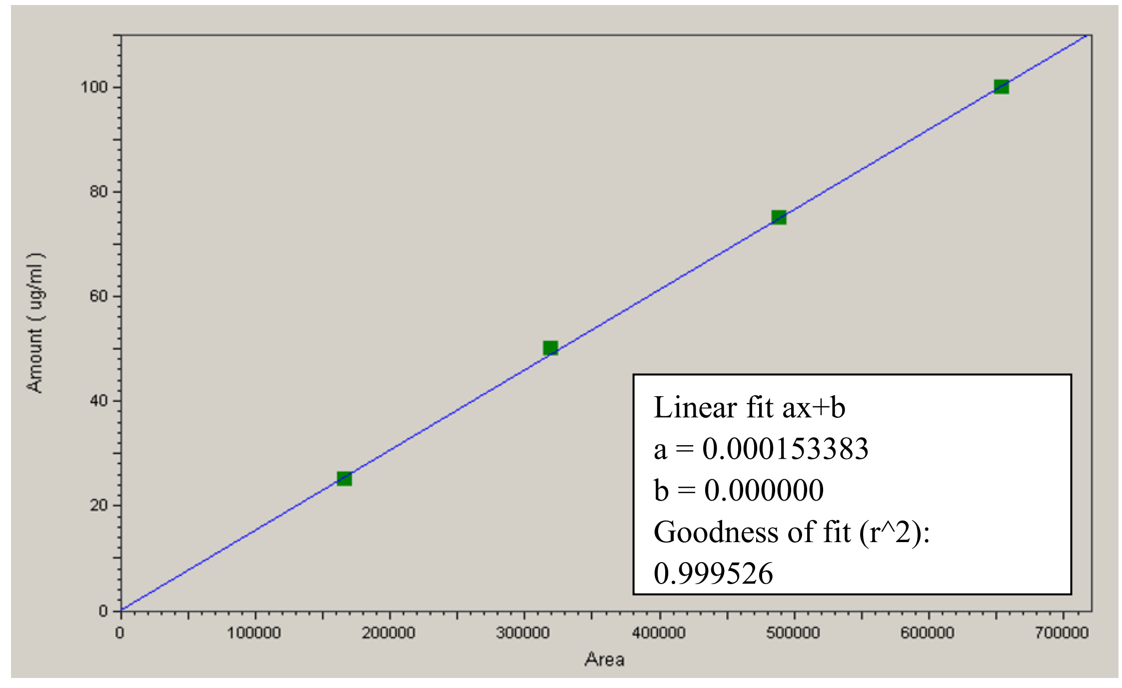
Appendix 8.

A calibration curve for different concentrations of (+)-catechin for high-performance liquid chromatography (HPLC) assay.



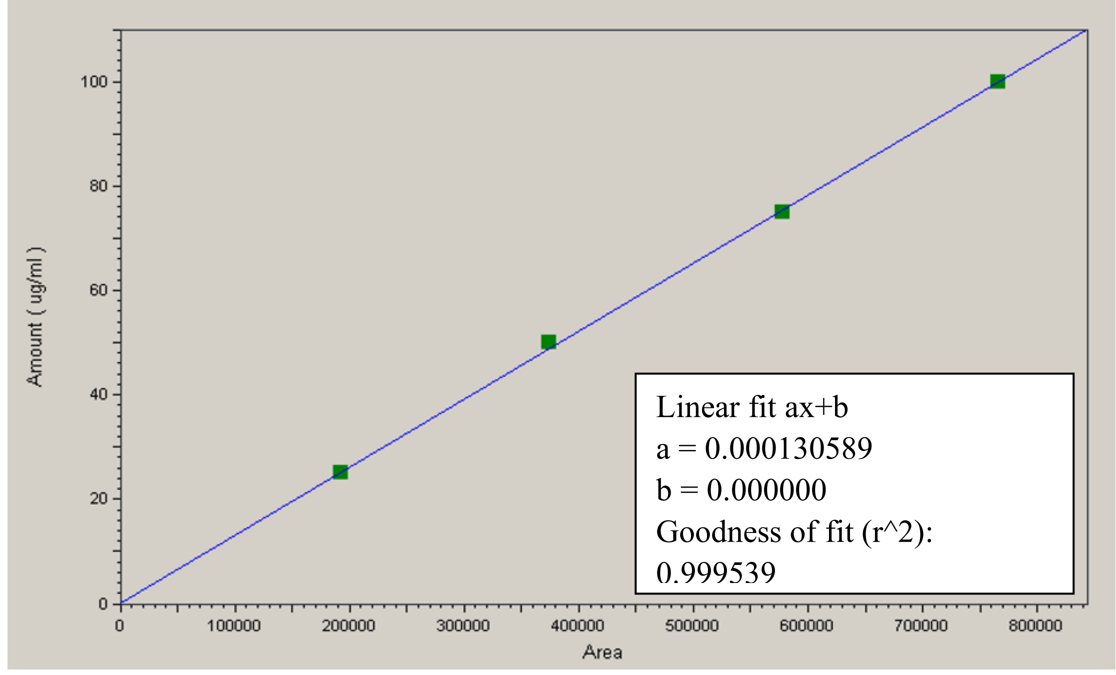
Appendix 9.

A calibration curve for different concentrations of (-)-epicatechin for high-performance liquid chromatography (HPLC) assay.



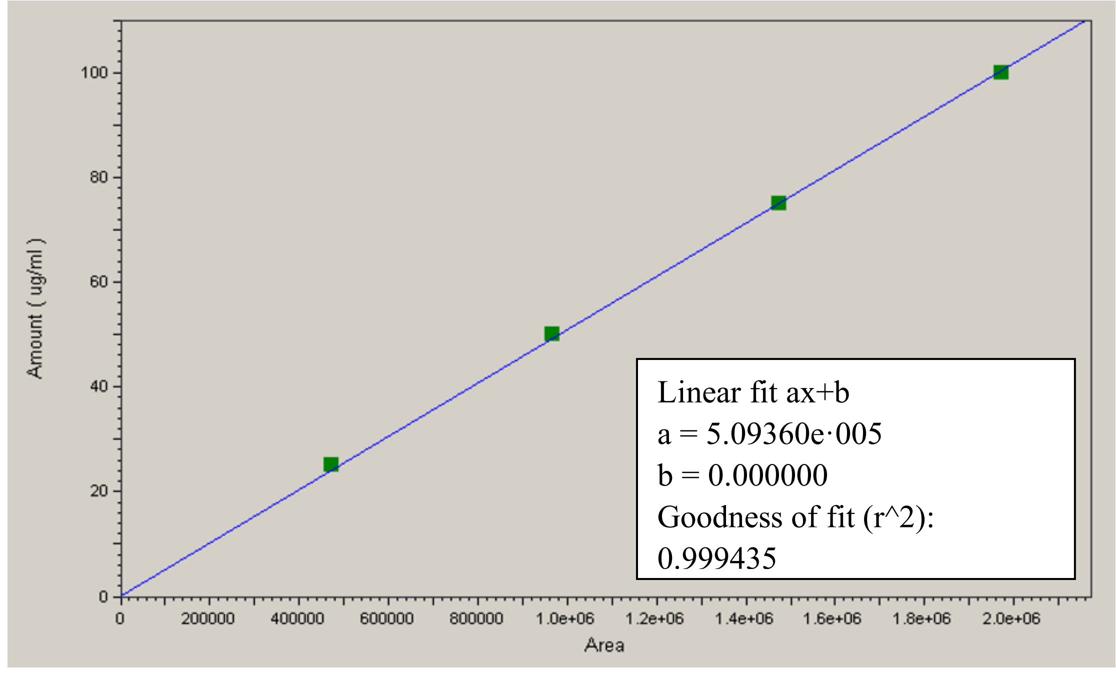
Appendix 10.

A calibration curve for different concentrations of rutin for high-performance liquid chromatography (HPLC) assay.



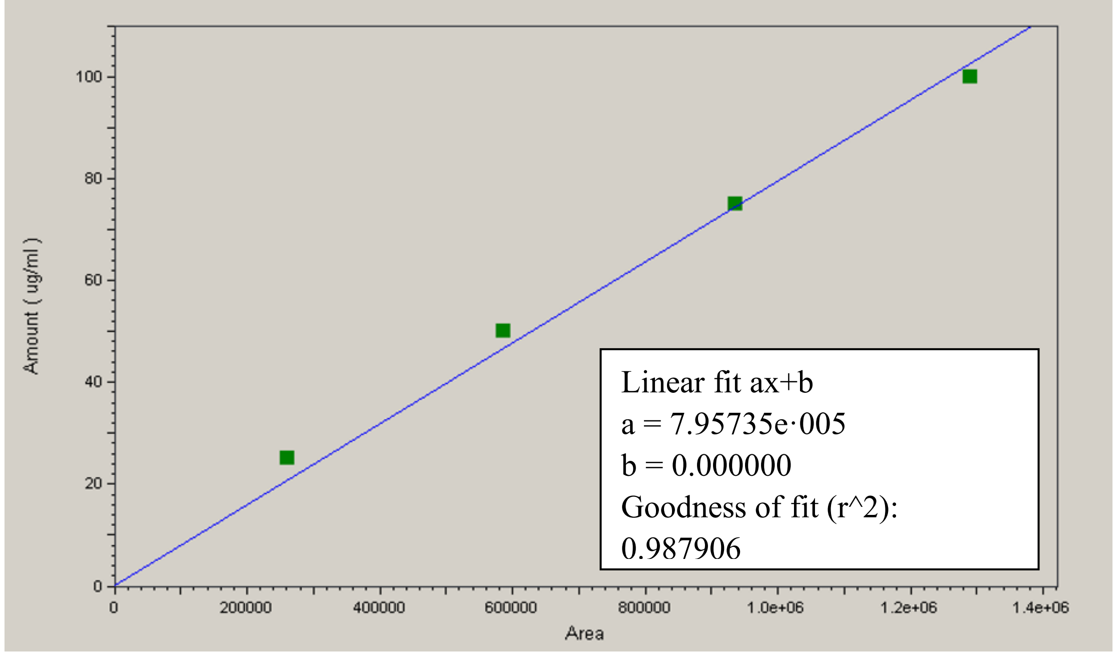
Appendix 11.

A calibration curve for different concentrations of rosmarinic acid for high-performance liquid chromatography (HPLC) assay.

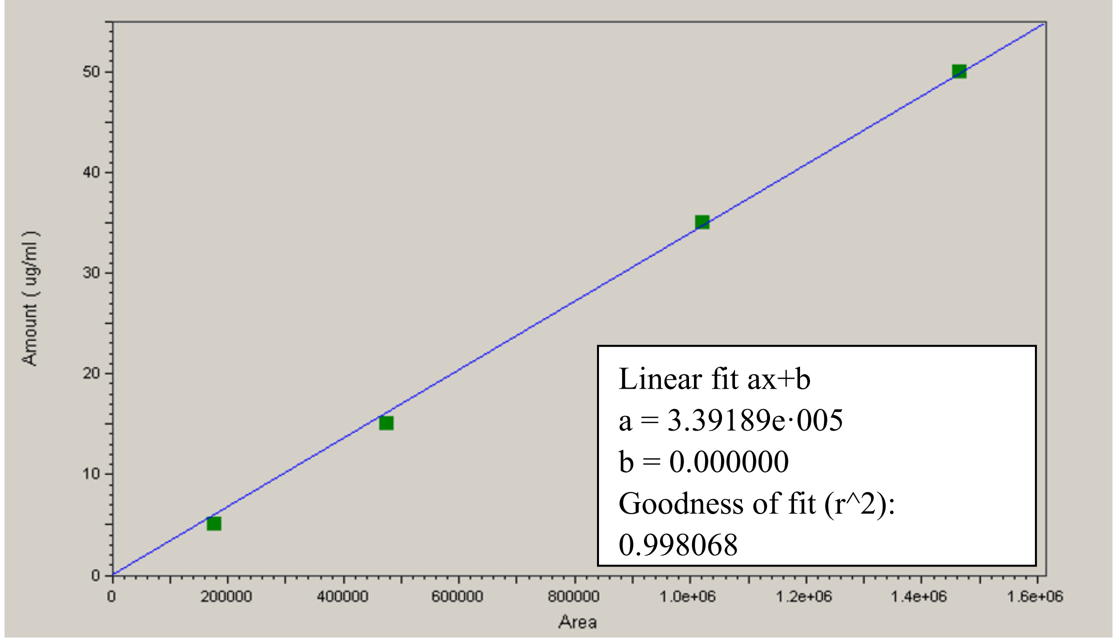


Appendix 12.

A calibration curve for different concentrations of quercetin for high-performance liquid chromatography (HPLC) assay.

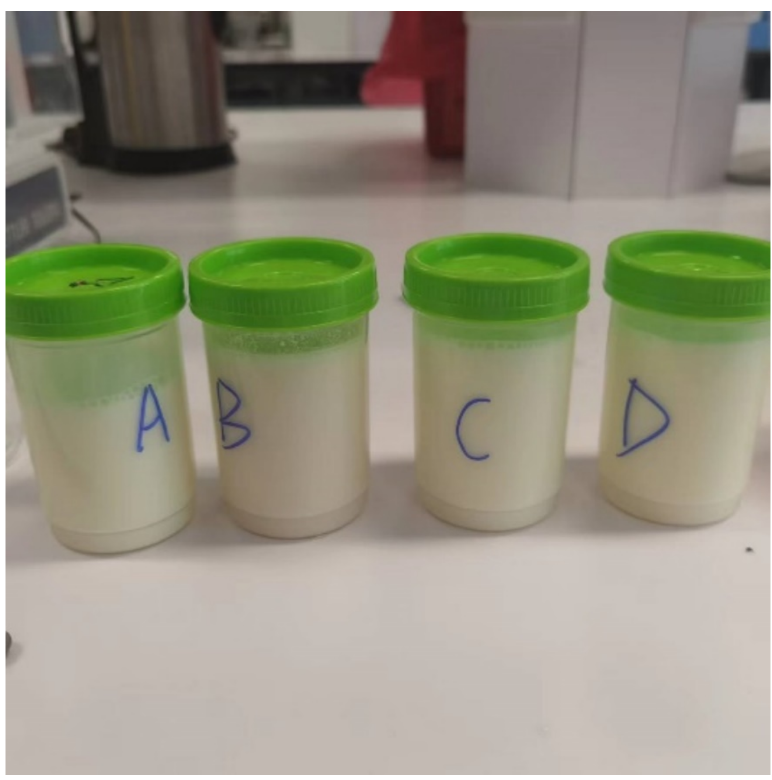


Appendix 13.
A calibration curve for different concentrations of naringenin for high-performance liquid chromatography (HPLC) assay.



Appendix 14.

The appearance of the control milk (A) and the milk after the addition of unencapsulated damson plum extract (B), freeze-dried (D), and non-freeze-dried (C) damson plum extract-loaded liposomes.



Appendix 15.
Permissions for Figure 2.5, used in literature review of this thesis without significant adaptation.

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