DEVELOPMENT OF LIQUID BIPHASIC SYSTEM FOR BETACYANINS EXTRACTION FROM PEEL AND FLESH OF *HYLOCEREUS POLYRHIZUS* AND ITS APPLICATIONS

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Abstract

In the current scenario, development of a green, simple, economically viable and effective bioseparation technology, such as liquid biphasic system, is a rapidly growing field in the biotechnology industries. Betacyanins are presently gaining higher interest in the food industries, as driven by their high tinctorial strength and healthy functional properties. In this research work, various liquid biphasic systems were utilised for the extraction of betacyanins from peel and flesh of red-purple pitaya. In particular, alcohol/salt-based liquid biphasic flotation (LBF) system and liquid biphasic partitioning system (LBPS) were first applied to the betacyanins extraction, and the satisfactory efficiencies were achieved by these approaches. With the utilisation of LBF system, the highest values of betacyanins concentration in alcohol-rich top phase (C_t) (95.989% and 95.488%), separation efficiency (E) (88.361% and 94.886%) and partition coefficient (K) (24.168 and 21.195) of betacyanins from the peel and flesh were obtained. On the other hand, the highest values of C_t (98.080% and 96.256%), phase volume ratio (V_r) (1.667 and 2.167) and K (51.097 and 25.764) of betacyanins from the peel and flesh were obtained by the application of LBPS. Subsequently, an integration of LBF system and LBPS with electricity treatment were employed for the betacyanins extraction. Electricity treatment not only ameliorates the extraction efficiency of biomolecules but also acts as a green extraction technique. Collectively, the betacyanins extraction from the peel and flesh using liquid biphasic electric flotation (LBEF) system showed the significant values of C_t (99.014% and 96.132%), E (98.383% and 96.576%) and K (100.814 and 24.883) of betacyanins compared to that of the LBF system. Similarly, the betacyanins extraction from the peel and flesh using liquid biphasic electric partitioning system (LBEPS) also demonstrated the high values of C_t (99.256% and 97.189%) and K (133.433 and

34.665) of betacyanins compared to that of the LBPS. Nevertheless, their V_r value remained the same. In addition, the peel and flesh extract obtained from these systems displayed different variations of red colour and their antioxidant properties were well retained. The 10-days stability study on the betacyanins extracted from the peel and flesh of red-purple pitaya was conducted. Our results revealed that the peel and flesh extract added with 0.5% (w/v) of ascorbic acid and without any pH adjustments showed a good stability of betacyanins under different storage conditions. Especially, the retention of betacyanins' activity in peel and flesh extract at 4 °C dark storage were the highest (114.976% and 105.903%). Lastly, two different applications were successfully accomplished in the present study. The salt-rich waste feedstock obtained from the liquid biphasic systems was used in the *Aurantiochytrium limacinum* SR21 wild type cultivation, and a higher lipid accumulation (8.29%) and docosahexaenoic acid (DHA) production (128.81 $mg.L^{-1}$) were noted. Whereas, the natural plant pigment products containing the stabilised betacyanins were applied as the natural red colourants for hard candy production. To conclude, this dissertation presents the reliable and effective separation approaches for the extraction of biomolecules like betacyanins valuable for food processing. In addition, this study shows a responsible practice on waste management.

List of Publications and Achievements

Published journals

1. **Hui Yi Leong**, Chien Wei Ooi, Chung Lim Law, Advina Lizah Julkifle, Tomohisa Katsuda, Pau Loke Show*. (2019) "Integration process for betacyanins extraction from peel and flesh of *Hylocereus polyrhizus* using liquid biphasic electric flotation system and antioxidant activity evaluation" *Separation and Purification Technology*, 209, 193–201. DOI: 10.1016/j.seppur.2018.07.040 [Accepted: 15 July 2018; 2017 IF: 3.927, Q1(22/137)]
2. **Hui Yi Leong**, Chien-An Su, Bo-Sheng Lee, John Chi-Wei Lan, Chung Lim Law, Jo-Shu Chang, Pau Loke Show*. (2019) "Development of *Aurantiochytrium limacinum* SR21 cultivation using salt-rich waste feedstock for docosahexaenoic acid production and application of natural colourant in food product" *Bioresource Technology*, 271, 30–36. DOI: 10.1016/j.biortech.2018.09.093 [Accepted: 17 September 2018; 2017 IF: 5.807, Q1(13/160)]
3. Yee Meng Wong, Pau Loke Show, Ta Yeong Wu, **Hui Yi Leong**, Shaliza Ibrahim, Joon Ching Juan*. (2019) "Production of bio-hydrogen from dairy wastewater using pretreated landfill leachate sludge as an inoculum" *Journal of Bioscience and Bioengineering*, 127, 150–159. DOI: 10.1016/j.jbiosc.2018.07.012 [Accepted: 15 July 2018; 2017 IF: 2.015, Q3(87/160)]
4. **Hui Yi Leong**, Pau Loke Show*, Miang Hoong Lim, Chien Wei Ooi, Tau Chuan Ling. (2018) "Natural red pigments from plants and their health benefits: A review" *Food Reviews International*, 34(5), 463–482. DOI: 10.1080/87559129.2017.1326935 [Accepted: 10 May 2017; 2017 IF: 3.100, Q1(28/133)]

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<u>Submitted paper</u>

1. **Hui Yi Leong**, Yu-Kaung Chang, Chien Wei Ooi, Chung Lim Law, Advina Lizah Julkifle, Pau Loke Show*. "Liquid biphasic electric partitioning system as a novel integration process for betacyanins extraction from red-purple pitaya and antioxidant properties assessment" *Frontiers in Chemistry*. (Status: Under review)

Conference presentations

1. **Hui Yi Leong**, Pau Loke Show**, Hon Loong Lam, Azizon Abdullah*. (2016) "Amino acids recovery from swiftlets (*Aerodramus fuciphagus*) guano" The 3rd Edible Birdnest Industry Conference (EBNIC 2016), Empowering the Essence of Edible Birdnest, 11–12 October 2016, Marriott Hotel, Putrajaya, Malaysia.
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Research showcase & seminars

- CFF-UNMC Doctoral Training Partnership (DTP) Week 2015 (21–25 September 2015), 2016 (13–16 December 2016) and 2017 (12–15 December 2017), CFF Gallery, Semenyih, Selangor, Malaysia.
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Table of Contents

Abstract ii

List of Publications and Achievements iv

Acknowledgements viii

Table of Contents ix

List of Figures xix

List of Tables xxviii

List of Abbreviations and Nomenclatures xxxi

List of Appendices xxxv

CHAPTER 1: INTRODUCTION 1

1.1 Research background 1

1.2 Problem statement 3

1.3 Justification and novelty of the research 3

1.4 Objectives 4

1.5 Summary of the research 4

1.6 Flow diagram of the research 8

CHAPTER 2: LITERATURE REVIEW 9

2.1 Natural red pigments 9

2.1.1 Carotenoids 9
2.1.2 Lycopene 12
2.1.2.1 Features of lycopene 12
2.1.2.2 Bioavailability, absorption and distribution 13
2.1.2.3 Prevention of chronic diseases 14
2.1.3 Anthocyanins 15
2.1.3.1 Features of anthocyanins 15
2.1.3.2 Bioavailability, absorption and distribution 17
2.1.3.3 Prevention of chronic diseases 17
2.1.4 Betacyanins 19
2.1.4.1 Features of betacyanins 19
2.1.4.2 Bioavailability, absorption and distribution 21
2.1.4.3 Prevention of chronic diseases 22
2.2 Colouring agents 24
2.2.1 Type of colouring agents 25
2.3 Antioxidants 28
2.3.1 Bioactive compounds 29
2.4 Red-purple pitaya 31
2.4.1 Health benefits on consumption 33
2.5 Microalgae 34
2.5.1 Microalgae biorefinery 35
2.5.2 Docosahexaenoic acid 35
2.5.3 *Aurantiochytrium limacinum* SR21 36
2.5.4 Microalgae cultivation using waste feedstock 37

2.6 Industrial biotechnology 37

2.7 Extraction techniques 39

2.7.1 Conventional extraction technique 39

2.7.2 Novel extraction technique 39

2.7.2.1 Electricity treatment 40

2.7.2.2 Liquid biphasic system 42

CHAPTER 3: APPLICATION OF LIQUID BIPHASIC FLOTATION SYSTEM FOR BETACYANINS EXTRACTION FROM PEEL AND FLESH OF *HYLOCEREUS POLYRHIZUS* AND ANTIOXIDANT ACTIVITY EVALUATION… 48

3.1 Abstract 49

3.2 Introduction 50

3.3 Materials and methods 52

3.3.1 Materials 52

3.3.2 Apparatus 53

3.3.3 Processing of crude extract 53

3.3.4 Betacyanins extraction with LBF system 53

3.3.5 Analytical procedures 56

3.3.5.1 Colour characterisation 56

3.3.5.2 Determination of total betacyanins content (TBC) 56

3.3.5.3 Determination of total phenolic content (TPC) 57

3.3.5.4 Ferric reducing antioxidant power (FRAP) assay 57

3.3.5.5 Trolox equivalent antioxidant capacity (TEAC) assay 58

3.3.6 Calculations 58

3.3.7 Statistical analysis 59

3.4 Results and discussion 60

3.4.1 Effects of the types of alcohol and salt 60

3.4.2 Effects of the concentrations of alcohol and salt 65

3.4.3 Effects of the volumes of alcohol and salt 69

3.4.4 Effect of the addition of NaCl 73

3.4.5 Effect of the flotation time 75

3.4.6 Effect of the crude extract concentration 77

3.4.7 Effect of the type of crude extract 79

3.4.8 Colour characterisation 81

3.4.9 Antioxidant properties analysis 82

3.5 Concluding remarks 84

CHAPTER 4: APPLICATION OF LIQUID BIPHASIC PARTITIONING SYSTEM FOR BETACYANINS EXTRACTION FROM PEEL AND FLESH OF *HYLOCEREUS POLYRHIZUS* AND ANTIOXIDANT ACTIVITY EVALUATION…… **85**

4.1 Abstract 86

4.2 Introduction 87

4.3 Materials and methods 90

4.3.1 Materials 90

4.3.2 Processing of crude extract 90

4.3.3 Phase diagram of LBPS 91

4.3.4 Betacyanins extraction with ethanol/K_2HPO_4-based LBPS 91

4.3.5 Comparison study between LBPS and conventional solvent extractions 92

4.3.6 Analytical procedures 92

4.3.6.1 Colour characterisation 92

4.3.6.2 Determination of total betacyanins content (TBC) 93

4.3.6.3 Determination of total phenolic content (TPC) 93

4.3.6.4 Ferric reducing antioxidant power (FRAP) assay 94

4.3.6.5 Trolox equivalent antioxidant capacity (TEAC) assay 94

4.3.7 Calculations 95

4.3.8 Statistical analysis 96

4.4 Results and discussion 96

4.4.1 Phase diagram of alcohol/salt-based LBPS 96

4.4.2 Effects of concentrations of alcohol and salt 97

4.4.3 Effect of addition of NaCl 101

4.4.4 Effect of crude concentration 104

4.4.5 Betacyanins extraction efficiency between LBPS and conventional solvent extractions 107

4.4.6 Colour characterisation 108

4.4.7 Antioxidant properties analysis 109

4.5 Concluding remarks 112

CHAPTER 5: INTEGRATION PROCESS FOR BETACYANINS EXTRACTION FROM PEEL AND FLESH OF *HYLOCEREUS POLYRHIZUS*

USING LIQUID BIPHASIC ELECTRIC FLOTATION SYSTEM AND ANTIOXIDANT ACTIVITY EVALUATION 113

5.1 Abstract 114

5.2 Introduction 115

5.3 Materials and methods 118

5.3.1 Materials 118

5.3.2 Apparatus 118

5.3.3 Processing of crude extract 119

5.3.4 Betacyanins extraction with LBEF system 119

5.3.4.1 Comparison between LBF and LBEF system for betacyanins extraction 122

5.3.5 Analytical procedures 123

5.3.5.1 Colour characterisation 123

5.3.5.2 Determination of total betacyanins content (TBC) 123

5.3.5.3 Determination of total phenolic content (TPC) 124

5.3.5.4 Ferric reducing antioxidant power (FRAP) assay 124

5.3.5.5 Trolox equivalent antioxidant capacity (TEAC) assay 125

5.3.6 Calculations 125

5.3.7 Statistical analysis 126

5.4 Results and discussion 127

5.4.1 Comparison study of betacyanins extraction using LBF and LBEF system 127

5.4.2 Effect of the operation time in LBEF system 130

5.4.3 Effect of the voltage applied in LBEF system 134

5.4.4 Effect of the position of graphitic electrodes in LBEF system 136
5.4.5 Effect of the type of crude extract in LBEF system 137
5.4.6 Colour characterisation.. 137
5.4.7 Antioxidant properties analysis 138
5.5 Concluding remarks.. 141
CHAPTER 6: INTEGRATION PROCESS FOR BETACYANINS EXTRACTION FROM PEEL AND FLESH OF *HYLOCEREUS POLYRHIZUS* USING LIQUID BIPHASIC ELECTRIC PARTITIONING SYSTEM AND ANTIOXIDANT ACTIVITY EVALUATION... 142
6.1 Abstract... 143
6.2 Introduction 145
6.3 Materials and methods.. 147
6.3.1 Materials... 147
6.3.2 Apparatus.. 148
6.3.3 Processing of crude extract.. 148
6.3.4 Betacyanins extraction with LBEPS 149
6.3.4.1 Comparison between LBPS and LBEPS........................... 151
6.3.5 Comparison between optimised LBEPS and LBPS with optimised conditions of electricity treatment as post-treatment 152
6.3.6 Analytical procedures.. 152
6.3.6.1 Colour characterisation 152
6.3.6.2 Determination of total betacyanins content (TBC) 152
6.3.6.3 Determination of total phenolic content (TPC)................. 153

6.3.6.4 Ferric reducing antioxidant power (FRAP) assay 153

6.3.6.5 Trolox equivalent antioxidant capacity (TEAC) assay 154

6.3.7 Calculations 154

6.3.8 Statistical analysis 155

6.4 Results and discussion 156

6.4.1 Comparison study of betacyanins extraction using LBPS and LBEPS 156

6.4.2 Effect of the operation time in LBEPS 158

6.4.3 Effect of the voltage applied in LBEPS 163

6.4.4 Effect of the position of graphitic electrodes in LBEPS 165

6.4.5 Comparison study of betacyanins extraction using optimised LBEPS and LBPS with optimised conditions of electricity treatment as post-treatment 166

6.4.6 Colour characterisation.. 168

6.4.7 Antioxidant properties analysis 169

6.5 Concluding remarks 171

CHAPTER 7: INVESTIGATION OF BETACYANINS STABILITY FROM PEEL AND FLESH OF *HYLOCEREUS POLYRHIZUS* WITH FOOD ADDITIVES SUPPLEMENTATION AND PH TREATMENTS..................... 172

7.1 Abstract 173

7.2 Introduction 174

7.3 Materials and methods 177

7.3.1 Materials 177

7.3.2 Processing of crude extract ... 177

7.3.3 Betacyanins extraction with alcohol/salt-based LBPS ... 177

7.3.4 Stability study ... 178

7.3.4.1 Addition of food additives ... 179

7.3.4.2 pH treatments ... 179

7.3.5 Calculations ... 179

7.3.6 Kinetic analysis of betacyanins degradation rate ... 180

7.3.7 Statistical analysis ... 181

7.4 Results and discussion ... 181

7.4.1 Effects of addition of food additives ... 181

7.4.1.1 Addition of guar gum ... 184

7.4.1.2 Addition of ascorbic acid ... 188

7.4.2 Effect of pH treatments ... 192

7.4.3 Kinetic analysis of degradation of betacyanins ... 196

7.5 Concluding remarks ... 198

CHAPTER 8: DEVELOPMENT OF *AURANTIOCHYTRIUM LIMACINUM* SR21 WILD TYPE CULTIVATION USING SALT-RICH WASTE FEEDSTOCK FOR DOCOSAHEXAENOIC ACID PRODUCTION AND APPLICATION OF NATURAL COLOURANT IN FOOD PRODUCT ... 199

8.1 Abstract ... 200

8.2 Introduction ... 201

8.3 Materials and methods ... 203

8.3.1 Materials ... 203

8.3.2 Flask cultivation of *A. limacinum* SR21 WT 204

8.3.2.1 Replacement of KH_2PO_4 by K_2HPO_4-waste feedstock...... 205

8.3.3 Hard candy production 205

8.3.4 Analytical procedures.. 205

8.3.4.1 Analysis of microalga cell concentration........................... 205

8.3.4.2 Pre-treatment for analysis of lipid content......................... 206

8.3.4.3 Analysis of lipid content 207

8.3.4.4 Colour characterisation 208

8.3.5 Statistical analysis 208

8.4 Results and discussion... 208

8.4.1 Effects of number of moles of PO_4^{3-} on microalgal growth and lipid production.. 208

8.4.2 Effects of working concentration of phosphate salts on microalgal growth and lipid production 215

8.4.3 Hard candy production using natural plant colourants...................... 220

8.5 Concluding remarks... 221

CHAPTER 9: GENERAL CONCLUSIONS AND FUTURE PROSPECTIVE.. 222

References 225

Appendix 254

List of Figures

Figure 1-1: Flow chart illustrating the research works in this dissertation. 8

Figure 2-1: Chemical structures of some dietary carotenoids; (a) α-carotene, (b) β-carotene, (c) lycopene, (d) β-cryptoxanthin, (e) lutein and (f) zeaxanthin. 10

Figure 2-2: Chemical structure of anthocyanins. 15

Figure 2-3: Chemical structures of (a) betalains, (b) betacyanins, (c) betaxanthins, (d) betalamic acid (common building block of betalains) and (e) betanin (the most common betacyanins).. 20

Figure 2-4: Categories of colourants. 26

Figure 2-5: Various pitaya species; (a) Red skinned with red-purple flesh pitaya (*Hylocereus polyrhizus*), (b) Red skinned with white flesh pitaya (*Hylocereus undatus*), (c) Red skinned with white flesh pitaya (*Hylocereus undatus*) 31

Figure 2-6: Schematic diagram of different types of liquid biphasic system. 42

Figure 3-1: Schematic view of alcohol/salt-based LBF for betacyanins extraction from peel and flesh of red-purple pitaya. Flotation effect (gas bubbles) assisted in the upflow of betacyanins from aqueous bottom phase to alcohol-rich top phase...................... 54

Figure 3-2: Effect of varying ethanol concentrations in LBF composed of 10 mL of ethanol, 20 mL of 250 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings.

Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 66

Figure 3-3: Effect of varying K_2HPO_4 concentrations in LBF composed of 10 mL of 100% ethanol, 20 mL of K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 68

Figure 3-4: Effect of varying volumes of ethanol in LBF composed of 100% ethanol, 20 mL of 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 70

Figure 3-5: Effect of varying volumes of K_2HPO_4 in LBF composed of 10 mL of 100% ethanol, 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 72

Figure 3-6: Effect of addition of NaCl in LBF composed of 10 mL of 100% ethanol, 20 mL of 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15

min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 74

Figure 3-7: Effect of varying flotation time in LBF composed of 10 mL of 100% ethanol, 20 mL of 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Air flow rate is set at 20–30 cc/min. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 76

Figure 3-8: Effect of varying fresh crude extract (FE) concentrations of (a) peel and (b) flesh of red-purple pitaya in LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution for betacyanins extraction. Flotation time and air flow rate are set at 15 min and 20–30 cc/min. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 78

Figure 3-9: Comparison between different types of crude extract (fresh crude extract (FE) and dried crude extract (DE)) of (a) peel and (b) flesh of red-purple pitaya in LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution for betacyanins extraction. Flotation time and air flow rate are set at 15 min and 20–30 cc/min. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K. 80

Figure 3-10: Antioxidant properties analysis for peel and flesh extract of red-purple pitaya obtained using the optimised alcohol/salt-based LBF. Values are mean ± SD of

triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within the antioxidant properties assays. 83

Figure 4-1: Phase diagram of ethanol/K_2HPO_4-based LBPS. 97

Figure 4-2: Effects of varying concentrations of ethanol and K_2HPO_4 (%, w/w) in alcohol/salt-based LBPS with peel and flesh of red-purple pitaya, in which ethanol concentration was first optimised, and then followed by the optimisation of K_2HPO_4 concentration. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within the same category. (a) 20% of K_2HPO_4 with varying concentrations of ethanol in LBPS with the peel. (b) 27% of ethanol with varying concentrations of K_2HPO_4 in LBPS with the peel. (c) 20% of K_2HPO_4 with varying concentrations of ethanol in LBPS with the flesh. (d) 33% of ethanol with varying concentrations of K_2HPO_4 in LBPS with the flesh. .. 100

Figure 4-3: Effect of addition of NaCl in ethanol/K_2HPO_4-based LBPS with peel and flesh of red-purple pitaya. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within the same category. (a) Addition of 2% (w/w) of varying molarities of NaCl in 27% of ethanol/20% of K_2HPO_4-based LBPS with the peel. (b) Addition of varying concentrations (%, w/w) of 0.4 M NaCl in 27% of ethanol/20% of K_2HPO_4-based LBPS with the peel. (c) Addition of 2% (w/w) of varying molarities of NaCl in 33% of ethanol/20% of K_2HPO_4-based LBPS with the flesh. (d) Addition of varying concentrations (%, w/w) of 0.2 M NaCl in 33% of ethanol/20% of K_2HPO_4-based LBPS with the flesh. 103

Figure 4-4: Effect of varying concentrations of dried crude extract (DE) (%, w/w) added in ethanol/K_2HPO_4-based LBPS with (a) peel and (b) flesh of red-purple pitaya. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within the same category. 106

Figure 4-5: Comparison study of betacyanins extractions using the optimised ethanol/K_2HPO_4-based LBPS, 80% ethanol and purified water extraction technique. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within peel and flesh. 108

Figure 4-6: Antioxidant properties analysis, including antioxidant capacity assays (i.e. FRAP and TEAC) and antioxidant component assays (i.e. TPC and TBC) for peel and flesh extract of red-purple pitaya extracted using the optimised ethanol/K_2HPO_4-based LBPS. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within FRAP, TEAC, TPC and TBC. 110

Figure 5-1: (a) Schematic diagram of LBEF system for betacyanins extraction from peel and flesh of red-purple pitaya; the LBF apparatus connected to a regulated dual DC power supply using two graphitic electrodes (anode and cathode; one to detect current flow and another one to control voltage applied) in order to supply electricity continuously. (b) Experimental setup for the betacyanins extraction using LBEF system. 121

Figure 5-2: Comparison study of betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya using the optimised LBF system and LBEF system with initial

settings. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different (p < 0.05) using Tukey's test within C_t, E and K.. 129

Figure 5-3: Effects of several parameters in LBEF system for betacyanins extraction from peel of red-purple pitaya. OFAT approach was used in the optimisation study: (a) operation time, (b) voltage applied, (c) position of graphitic electrodes and (d) type of crude extract. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different (p < 0.05) using Tukey's test within C_t, E and K. 131

Figure 5-4: Effects of several parameters in LBEF system for betacyanins extraction from flesh of red-purple pitaya. OFAT approach was used in the optimisation study: (a) operation time, (b) voltage applied, (c) position of graphitic electrodes and (d) type of crude extract. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different (p < 0.05) using Tukey's test within C_t, E and K. 133

Figure 5-5: Antioxidant activity evaluation of peel and flesh extract of red-purple pitaya obtained from the optimised LBEF system. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different (p < 0.05) using Tukey's test within FRAP, TEAC, TPC and TBC. 140

Figure 6-1: (a) Schematic diagram of LBEPS for betacyanins extraction from peel and flesh of red-purple pitaya. (b) Experimental setup for the betacyanins extraction using LBEPS. 150

Figure 6-2: Comparison study of betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya using LBPS and LBEPS with initial settings. Values are mean ± SD

of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within C_t and K. 158

Figure 6-3: Effects of various parameters in LBEPS for betacyanins extraction from peel of red-purple pitaya. OFAT approach was applied in the optimisation study: (a) operation time (control: LBPS), (b) voltage applied and (c) position of graphitic electrodes. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within C_t and K. 161

Figure 6-4: Effects of various parameters in LBEPS for betacyanins extraction from flesh of red-purple pitaya. OFAT approach was applied in the optimization study: (a) operation time (control: LBPS), (b) voltage applied and (c) position of graphitic electrodes. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within C_t and K. 162

Figure 6-5: Effect of different voltages applied in LBEPS; comparison between (a) 4 V and (b) 5 V of voltage applied for the flesh and peel extract. 164

Figure 6-6: Comparison study of betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya using the optimised LBEPS and LBPS with the optimised conditions of electric system as post-treatment. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within C_t and K. 167

Figure 6-7: Antioxidant properties assessment of peel and flesh extract of red-purple pitaya obtained from the optimised LBEPS. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within FRAP, TEAC, TPC and TBC. 170

Figure 7-1: Betacyanins retentions (%) for control groups of (a) peel and (b) flesh extract of red-purple pitaya over a 10-days storage period. Values are mean ± SD of triplicate readings. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within day. 183

Figure 7-2: Betacyanins retentions (%) of (a) peel and (b) flesh extract of red-purple pitaya added with different concentrations of guar gum over a 10-days storage period. Values are mean ± SD of triplicate readings. I, II and III represent 0.1, 0.5 and 1.0% (w/v) of guar gum, respectively. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within day. 185

Figure 7-3: Betacyanins retentions (%) of (a) peel and (b) flesh extract of red-purple pitaya added with different concentrations of ascorbic acid over a 10-days storage period. Values are mean ± SD of triplicate readings. I, II and III represent 0.1, 0.5 and 1.0% (w/v) of ascorbic acid, respectively. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within day. 189

Figure 7-4: Betacyanins retentions (%) of (a) peel and (b) flesh extract of red-purple pitaya added with 0.5% (w/v) of ascorbic acid and with different pH treatments over a 10-days storage period. Values are mean ± SD of triplicate readings. I, II and III represent pH 3, 5 and 7, respectively. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within day. 193

Figure 8-1: Effect of different phosphate salts with varying number of moles of PO_4^{3-} on biomass concentration of *A. limacinum* SR21 WT cultivation; (a) 0.005% (w/v) of KH_2PO_4, (b) 20% (w/v) of K_2HPO_4-waste feedstock, (c) 20% (w/v) of K_2HPO_4 (AG). 1, 2 and 3 represent 1.282×10^{-4}, 2.564×10^{-4} and 5.128×10^{-4} mol/L of PO_4^{3-}, respectively. Values are mean ± SD (n = 3).. 211

Figure 8-2: Effect of working concentration of different phosphate salts on biomass concentration of *A. limacinum* SR21 WT cultivation; (a) 0.005% (w/v) of KH_2PO_4 and K_2HPO_4-waste feedstock, (b) different working concentrations of K_2HPO_4-waste feedstock. Values are mean ± SD (n = 3).. 216

Figure 8-3: Hard candy made with natural red colouring agents obtained from (a) peel and (b) flesh extract of red-purple pitaya... 220

List of Tables

Table 2-1: Dietary sources of plants that are rich in some carotenoids, such as α-carotene, β-carotene, lycopene, β-cryptoxanthin, lutein and zeaxanthin. 11

Table 2-2: Dietary sources of plants that are rich in anthocyanins. 16

Table 2-3: Dietary sources of plants that are rich in betacyanins. 21

Table 2-4: Nutritional facts and healthy functional of ripen fresh pitaya fruits......... 33

Table 3-1: The operating parameters for alcohol/salt-based LBF. 55

Table 3-2: LBF made of different combinations between alcohols and salts adopted for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. The system composed of 1 g of FE, 20 mL of 250 g/L salt solution, 10 mL of undiluted alcohol, 15 min of flotation time and 20–30 cc/min of air flow rate. 63

Table 3-3: The optimised conditions in LBF composed of alcohol and salt for betacyanins extraction from peel and flesh of red-purple pitaya. 81

Table 3-4: Colour characterisation for peel and flesh extract of red-purple pitaya. .. 81

Table 3-5: Correlation among the antioxidant properties assays, given correlation coefficient (r).. 84

Table 4-1: Colour characterisation for peel and flesh extract of red-purple pitaya extracted using the optimised ethanol/K_2HPO_4-based LBPS. 109

Table 4-2: Correlation among the antioxidant properties analysis, given correlation coefficient (r).. 111

Table 5-1: The operating parameters of LBEF system for betacyanins extraction from peel and flesh of red-purple pitaya. 122

Table 5-2: Colour characterisation of peel and flesh extract of red-purple pitaya obtained from the optimised LBEF system. 138

Table 5-3: Correlation study among antioxidant activities, i.e. FRAP, TEAC, TPC and TBC, given correlation coefficient (r). 141

Table 6-1: The operating parameters of LBEPS for betacyanins extraction from peel and flesh of red-purple pitaya. 151

Table 6-2: Colour characterisation of peel and flesh extract of red-purple pitaya obtained from the optimised LBEPS. 168

Table 6-3: Correlation study among the antioxidant properties assays, given correlation coefficient (r). 171

Table 7-1: Phase-forming components in alcohol/salt-based LBPS for peel and flesh of red-purple pitaya. 178

Table 7-2: Kinetic analysis of degradation of betacyanins in the extracts for all the stability treatments. 197

Table 8-1: Lipid analysis of *A. limacinum* SR21 WT cultivation using different phosphate salts with varying number of moles of PO_4^{3-}. 214

Table 8-2: Lipid analysis of *A. limacinum* SR21 WT cultivation using different phosphate salts (based on working concentration). 217

Table 8-3: Colour analysis of hard candy made with natural colourants obtained from the red-purple pitaya extract... 221

Table 9-1: Results of several extraction techniques on betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya in this dissertation...................................... 224

List of Abbreviations and Nomenclatures

a^*	:	Redness
A_{538}	:	Absorbance value at 538 nm
ABTS•	:	ABTS radical
AG	:	Analytical grade
ANOVA	:	Analysis of variance
AOC	:	Antioxidant capacity
ATPE	:	Aqueous two-phase extraction
ATPS	:	Aqueous two-phase system
b^*	:	Yellowness
BEs	:	Betanin equivalents
C^*	:	Chroma
C_b	:	Betacyanins concentration in salt-rich bottom phase
C_t	:	Betacyanins concentration in alcohol-rich top phase
CFR	:	Code of Federal Regulations
CVDs	:	Cardiovascular diseases
cyclo-DOPA	:	*cyclo*-3,4-dihydroxyphenylalanine
DC	:	Direct current
DE	:	Dried crude extract
DF	:	Dilution factor
DHA	:	Docosahexaenoic acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
E	:	Separation efficiency
EFSA	:	European Food Safety Authority

EUFIC	:	European Food Information Council
FAMEs	:	Fatty acid methyl esters
F-C	:	Folin-Ciocalteu
FDA	:	Food and Drug Administration
FE	:	Fresh crude extract
FID	:	Flame ionisation detector
FRAP	:	Ferric reducing antioxidant power
GAEs	:	Gallic acid equivalents
GC	:	Gas chromatography
G4	:	Grade 4
h°	:	Hue angle
HSD	:	Honestly significant difference
HVED	:	High voltage electrical discharges
ID	:	Internal diameter
K	:	Partition coefficient
k	:	Degradation rate constant
L	:	Path length of cuvette
L^*	:	Lightness
LBEF	:	Liquid biphasic electric flotation
LBEPS	:	Liquid biphasic electric partitioning system
LBF	:	Liquid biphasic flotation
LBPS	:	Liquid biphasic partitioning system
MW	:	Molecular weight
N_t	:	TBC after the stability treatment at day t
N_0	:	TBC before the stability treatment

N/A	:	Not applicable
NGIB	:	Next generation industrial biotechnology
OD	:	Optical density
OFAT	:	One-factor-at-a-time
ORAC	:	Oxygen radical absorbance capacity
PEF	:	Pulsed electric field
PO_4^{3-}	:	Phosphate ions
POME	:	Palm oil mill effluent
PUFAs	:	Polyunsaturated fatty acids
r	:	Correlation coefficient
R^2	:	Regression coefficient
ROS	:	Reactive oxygen species
RNS	:	Reactive nitrogen species
SD	:	Standard deviation
SS	:	Solvent sublation
t	:	Time
$t_{1/2}$	:	Half-life
TBC	:	Total betacyanins content
TBC_b	:	TBC in salt-rich bottom phase
TBC_{bi}	:	TBC in salt-rich bottom phase before the LBF process
TBC_f	:	TBC at the following day
TBC_i	:	TBC at day 0
TBC_t	:	TBC in alcohol-rich top phase
TEAC	:	Trolox equivalent antioxidant capacity
TEs	:	Trolox equivalents

TPC	:	Total phenolic content
UV-vis	:	Ultraviolet-visible
V	:	Volume
V_b	:	Volume of alcohol-rich top phase
V_r	:	Phase volume ratio
V_t	:	Volume of salt-rich bottom phase
W	:	Weight
WT	:	Wild type
ε	:	Molar extinction coefficient
4 °C D	:	4 °C dark storage
25 °C D	:	25 °C dark storage
25 °C L	:	25 °C daylight storage

List of Appendices

Appendix A: Gas chromatography analysis of fatty acid methyl ester standard mixture. ………………………………………………………... 254

CHAPTER 1: Introduction

1.1 Research background

With the rapid development of biotechnology in the past few decades, many researches have been conducted to elevate the efficiency of downstream processing. Particularly, the development of a green, simple, economically viable and high efficient bioseparation technology is a rapidly growing field in the biotechnology industries, especially in the field of food science (Chemat et al., 2017; Sankaran et al., 2018; Tang and Zhao, 2009; Xu et al., 2017).

In recent times, the arising of potential negative health impacts on the application of artificial food dyes, such as occurrence of intolerance and allergenic responses, have urged the search for alternative colourant sources. As a result, manufacturers and consumers are inclined toward the utilisation of natural ingredients as food dyes. Natural colourants are the safe and environmentally benign compounds offering health-promoting benefits due to the presence of antioxidants (Carocho et al., 2015; Gengatharan et al., 2016; Martins et al., 2016).

Natural red pigments are presently receiving immense attention in the food science industries, such as foods and pharmaceuticals, as to replace the artificial red dyes (Moreno et al., 2008). In particular, Aberoumand (2011) reported that the betalains are of growing interest in the food science processing. Betacyanins (a subgroup of betalains) are gaining higher interest in the food science processing as driven by their high tinctorial strength and healthy functional properties (Annie-Rose, 2016; Ciriminna et al., 2018; Leong et al., 2018c).

In addition, microalgal biotechnology applications are now a rapidly growing field. Especially, microalgae biorefinery is gaining global interest as an emerging biomass transformation approach. Generation of high value-added products in addition to biofuel through microalgae biorefinery have been developed. For example, microalgae biomass can be transformed into pigments, proteins, lipids, polyunsaturated fatty acids (PUFAs), carbohydrates, vitamins and antioxidants (Yen et al., 2013).

Taking the above into account, this research aims to extract betacyanins from peel and flesh of red-purple pitaya by employing non-conventional separation approaches. Extraction of betacyanins from various plant sources normally utilised conventional solid-liquid extraction techniques, and these extraction procedures are reported to have limitations, such as inefficiency, time-, energy- and cost-consuming as well as stability problems (Azmir et al., 2013; Ciriminna et al., 2018; Dai and Mumper, 2010; Leong et al., 2018a; Wang and Weller, 2006). On the other hand, liquid biphasic system, for instance, liquid biphasic partitioning system (LBPS) and liquid biphasic flotation (LBF) system, is an innovative and non-conventional separation technique. It is well-known as an easy, scalable, time-, cost- and energy-saving, effective as well as mild and green separation approach for many biotechnological and natural products nowadays (Albertsson, 1986; Hatti-Kaul, 2000; Show et al., 2013; Yau et al., 2015; Zimmermann et al., 2017).

Moreover, electricity treatment not only ameliorates the extraction efficiency of biomolecules but also acts as a green extraction technique. Therefore, we proposed an idea to integrate liquid biphasic system with electricity supply [i.e. liquid biphasic electric partitioning system (LBEPS) and liquid biphasic electric flotation (LBEF) system] in order to further enhance the liquid biphasic system for improving

biomolecules separation. Lastly, we will also demonstrate the applications of the stabilised betacyanins-containing extracts in food products preparation and salt-rich waste feedstock (i.e. unwanted solution from the liquid biphasic system) in microalgal cultivation for value-added products as to complete this research work.

1.2 Problem statement

The current scenario of seeking green, simple, economical and high efficient bioseparation approaches in the biotechnology industries, such as in the field of food science, has demanded the development of more innovative and non-conventional processing techniques. In particular, liquid biphasic system represents an effective non-conventional bioseparation technology that possesses various benefits like easiness of operation, scalable operation, time-, cost- and energy-saving as well as mild separation technique. It has been widely applied in the separation and purification of many biotechnological and natural products. Meanwhile, the high demand of natural colourants in the food science industries has urged the search of more natural pigments. The conventional extraction procedures generally exhibit difficulties. Therefore, the present research targets to improve the natural product separation by using a cost-effective and mild processing process.

1.3 Justification and novelty of the research

Liquid biphasic systems, such as LBF and LBPS, are effective separation techniques for many biotechnological and natural products. In this dissertation, several improvements and integration procedures have been applied on these systems to seek innovative substitute for the separation technology of biomolecules. In addition, red-purple pitaya can be used as a potential substitute for red beet as natural red colourant.

Natural red pigment extracted from red beet has been commercialised in the market as the oldest and the most abundant red food dye. However, there is a high demand for replacing the red colourant derived from red beet due to its major drawbacks, including formation of carcinogenic nitrosamines compound and presence of unpleasant earthy smell. In this case, both red-purple pitaya and red beet shared the same red pigment constituent. Unlike red beet, red-purple pitaya does not generate carcinogenic compound and unpleasant smell. Moreover, this study develops an effective way to produce high value-added products by microalgal cultivation using salt-rich waste feedstock. Also, natural plant pigment products were applied as natural colourants in hard candy production. These innovative and green ideas are interesting and useful in food industries, beside of creating new opportunities for microalgae and natural plant pigment applications.

1.4 Objectives

I. To apply LBF and LBPS for betacyanins extraction from red-purple pitaya

II. To integrate LBF and LBPS with electricity treatment for betacyanins extraction from red-purple pitaya

III. To investigate betacyanins stability from red-purple pitaya

IV. To develop applications on the stabilised betacyanins-containing extracts (alcohol-rich top phase) and salt-rich waste feedstock (salt-rich bottom phase)

1.5 Summary of the research

The aim of this research is to apply various liquid biphasic systems for the betacyanins extraction from red-purple pitaya. Both the peel and flesh part are used in the experiment as to fully utilise the whole fruit. Moreover, the stabilised betacyanins-

containing extracts are applied in food product whereas the salt-rich waste feedstock is utilised in microalgal culture.

Chapter 1 introduces the background of problem faced in downstream processing, particularly in the field of food science. It also introduces the potential outcomes of the solution along with the objectives of this research. This study demonstrates a diverse application of liquid biphasic systems in separation of betacyanins. The first two objectives are focussing on betacyanins extraction utilising different approaches of liquid biphasic system. Next, the stability of the betacyanins-containing extracts are further investigated. Last but not least, applications on the stabilised betacyanins-containing extracts in food products and the utilisation of salt-rich waste feedstock in microalgal cultivation are reported.

Chapter 2 discusses the background review of the research. This section covers a broad literature studies on several aspects, such as natural red pigments, colouring agents, antioxidants, microalgae, industrial biotechnology and extraction approaches on betalains (both the conventional and novel processing techniques).

Chapter 3 studies the betacyanins extraction from red-purple pitaya using LBF system composed of alcohol and salt. Various operating parameters, such as types/concentrations/volumes of alcohol and salt, addition of sodium chloride, flotation time and type/concentration of crude extract, were adopted to investigate the betacyanins extraction efficiency. In addition, colour characterisation and antioxidant properties analysis were evaluated on the peel and flesh extract obtained using the optimised LBF system.

Chapter 4 discusses the betacyanins extraction from red-purple pitaya by means of ethanol/K_2HPO_4-based LBPS. The operating parameters of the LBPS, such as concentrations of ethanol, K_2HPO_4, NaCl and crude of red-purple pitaya were optimised. Moreover, conventional solvent extractions using 80% (w/w) ethanol and purified water were conducted to compare the extraction efficiency of betacyanins with that of the LBPS. Lastly, colour characterisation and antioxidant properties analysis were carried out for the resulting extracts obtained from the optimised LBPS.

Chapter 5 develops the integrated process of LBF and electric system (LBEF system) for betacyanins extraction from red-purple pitaya. In this study, the betacyanins extraction using the optimised LBF system (previous experimental results obtained from Chapter 3 in this thesis) with electricity treatment was assessed with various operating parameters. Moreover, the betacyanins extraction using the optimised LBF and LBEF system with initial settings, colour characterisation and antioxidant activity were compared and assessed.

Chapter 6 reports the application of LBEPS for betacyanins extraction from red-purple pitaya. LBEPS is an integrated process of LBPS and electric system. The betacyanins extraction using LBEPS was first compared with that of LBPS (previous experimental results obtained from Chapter 4 in this thesis), and then followed by optimisation study on the LBEPS for the betacyanins extraction. In addition, comparison between the optimised LBEPS and the LBPS with the optimised conditions of electricity treatment (as post-treatment) as well as colour characterisation and antioxidant properties assessment were carried out.

Chapter 7 investigates the betacyanins stability from red-purple pitaya with several stability treatments over a 10-days storage period. The stability study was first

conducted using addition of two types of food additives (i.e. guar gum and ascorbic acid) as to compare their stabilising effects on betacyanins, in which due to their well-known food stabiliser feature. After that, pH treatments were conducted for the chosen food additive. In addition, the extracts were kept at different storage conditions. Last but not least, kinetic analysis of the degradation of betacyanins was conducted.

Chapter 8 presents the cultivation of *Aurantiochytrium limacinum* SR21 wild type using salt-rich waste feedstock obtained from our previously conducted experiment using liquid biphasic systems. 20% (w/v) of K_2HPO_4-waste feedstock was used as a replacement ingredient for 0.005% (w/v) of KH_2PO_4 in the flask cultivation. The KH_2PO_4 and K_2HPO_4-waste feedstock with different number of moles of phosphate ions (PO_4^{3-}) were first evaluated, followed by working concentration of the salts. Analyses of microalgal growth and lipid production, in particular DHA, were assessed. Subsequently, natural plant pigment products containing stabilised betacyanins (according to Chapter 7 in this thesis) were utilised as the natural red colourants for hard candy production.

To conclude, **Chapter 9** provides the general conclusions and future prospective of the research. It can be concluded that liquid biphasic systems are useful for separation of biomolecules, such as betacyanins.

1.6 Flow diagram of the research

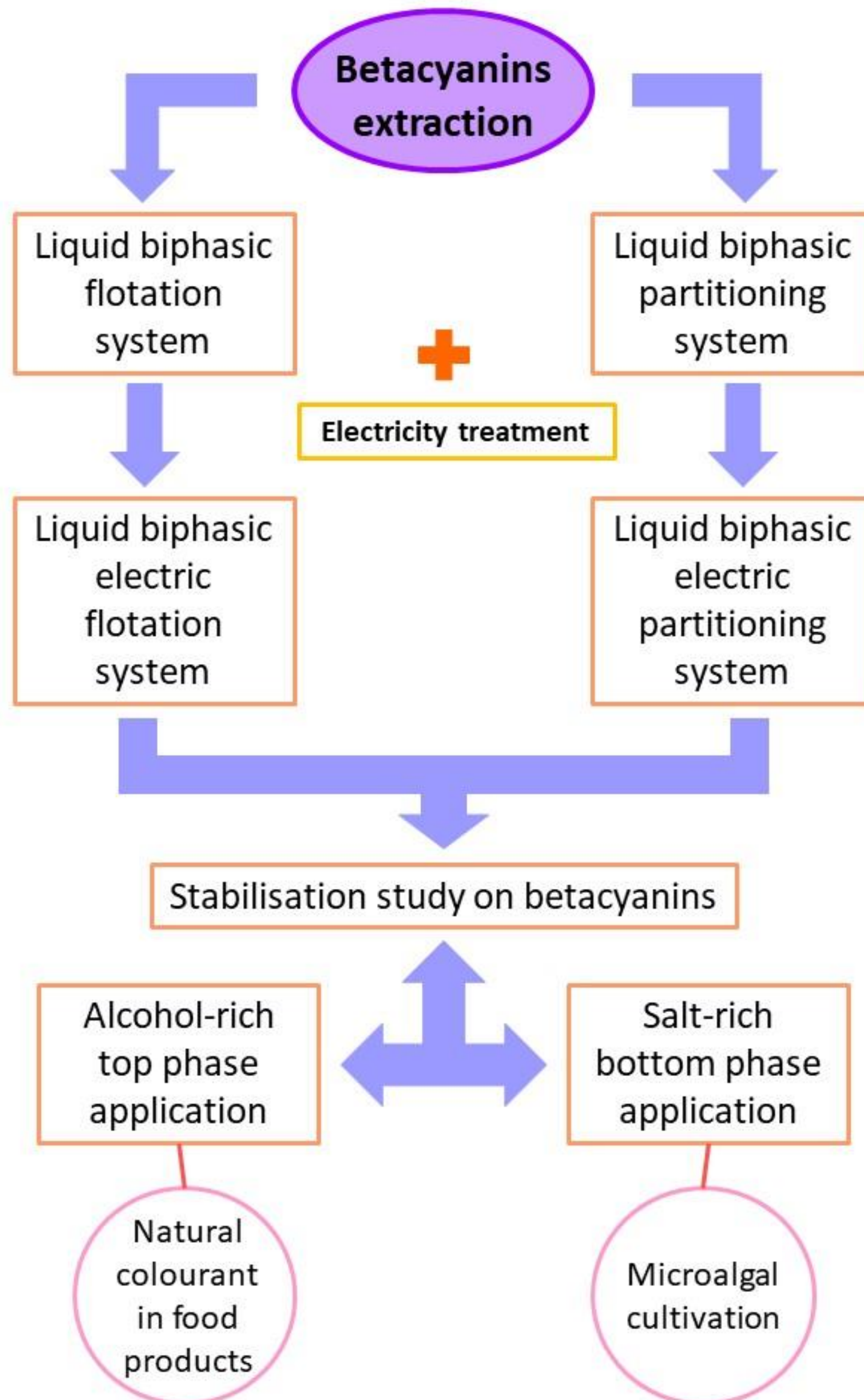


Figure 1-1: Flow chart illustrating the research works in this dissertation.

CHAPTER 2: Literature Review

2.1 Natural red pigments

Carotenoids (specifically lycopene), anthocyanins and betacyanins are phytochemicals responsible for red pigmentation in plants. These red pigments are from different classes of phytochemicals and displayed different characteristics. They possess antioxidant properties beneficial in the prevention of non-communicable diseases. Recently, natural colourants have attracted increasing attention from food industries as substitutes to artificial dyes, which have been discovered to cause negative impacts to human's health upon consumption. There is of growing interest in the natural red pigments in food science processing, due to their chronic diseases preventive effects. Stability of the natural pigments should be concerned, as it might affect the saturation of the colour.

2.1.1 Carotenoids

Carotenoids belong to the class of isoprenoid lipids (tetraterpernoids). According to the European Food Information Council (EUFIC), carotenoids are responsible for yellow, orange and red colour, and their colours formation mostly depend upon their conjugated carbon-carbon double bonds in the chemical structure. Carotenoids can only be synthesised by plants and microorganisms. They play the roles in the photosynthesis machinery and in the protection of plants against photo-damage (EUFIC, 2016; Khoo et al., 2011).

Carotenoids demonstrate health-promoting benefits, such as prevention of human diseases like cancers and age-related disorders. They are also a vital dietary source of vitamin A, and this is mainly contributed by α-carotene and β-carotene (i.e. vitamin A

precursors). The functions of vitamin A include regulation of hormone synthesis, improvement of immune system as well as regulation of skin's cell growth and differentiation. Although more than 600 types of carotenoids have been discovered from nature, but there are only around 40–50 types of carotenoids that can be absorbed and metabolised by human body (EUFIC, 2016; Krinsky and Johnson, 2005; Rao and Rao, 2007; Tapiero et al., 2004).

In particular, EUFIC (2016) reported that lycopene is the carotenoid largely responsible for the red pigmentation in fruits and vegetables. Other types of carotenoids, such as *α*-carotene, *β*-carotene and xanthophylls (e.g. lutein, zeaxanthin and cryptoxanthin), contribute to the yellow-orange colouration (Khoo et al., 2011). **Figure 2-1** shows the chemical structures of some dietary carotenoids, whereas examples of fruits and vegetables that are high in carotenoids are presented in **Table 2-1**.

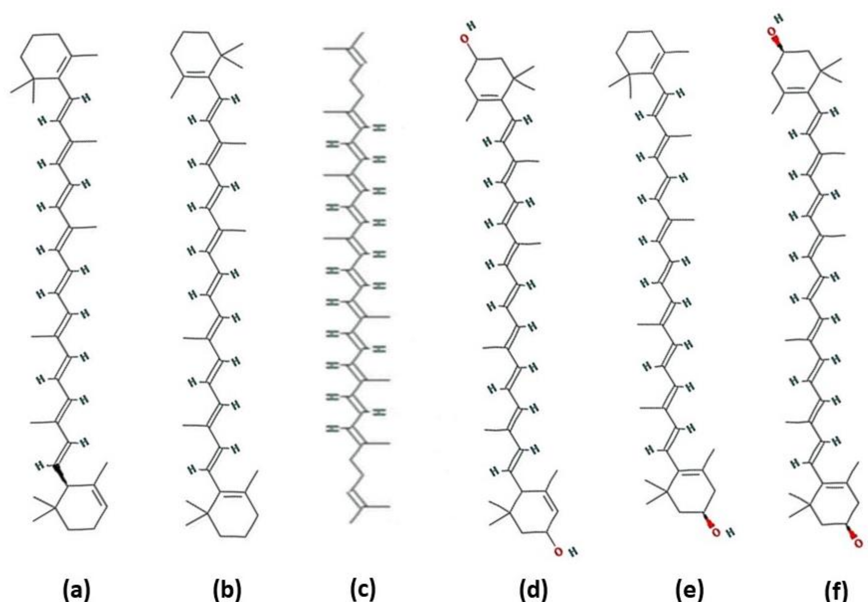


Figure 2-1: Chemical structures of some dietary carotenoids; (a) *α*-carotene, (b) *β*-carotene, (c) lycopene, (d) *β*-cryptoxanthin, (e) lutein and (f) zeaxanthin.

Table 2-1: Dietary sources of plants that are rich in some carotenoids, such as *α*-carotene, *β*-carotene, lycopene, *β*-cryptoxanthin, lutein and zeaxanthin.

Carotenoid	Colouration	Food sources	References
α-Carotene	Yellow-orange	Carrots	(Rao and Rao, 2007)
β-Carotene	Yellow-orange	Apricot, grapefruit, persimmon, pink guava, carrots, spinach, green collard, canteloupe, beet green, broccoli, tomato, yellow maize, sweet potato, mango, papaya, watermelon, pumpkin, green leafy vegetables (black nightshade and Mulla Thotakura)	(Khoo et al., 2011; Rao and Rao, 2007)
Lycopene	Red	Tomatoes, tomato-derived products (such as tomato juice, tomato sauce, tomato ketchup etc.), watermelon, red grapes, pepper, pink guava, pink grapefruit, papaya, apricot, red cabbage, asparagus	(Khoo et al., 2011; Kong et al., 2010; Krinsky and Johnson, 2005; Perveen et al., 2015; Roldán-Gutiérrez and Dolores Luque de Castro, 2007)
Lutein	Yellow-dark green	Spinach, green collard, beet green, broccoli, green peas, high concentration of egg yolks	(EUFIC, 2016; Rao and Rao, 2007)
β-Cryptoxanthin	Orange	Tangerine, papaya, persimmon, starfruit, chilli, maize, pepper	(Khoo et al., 2011; Rao and Rao, 2007)
Zeaxanthin	Yellow-green	Papaya, persimmon, pumpkin, maize, high concentration of egg yolks	(EUFIC, 2016; Rao and Rao, 2007)

2.1.2 Lycopene

2.1.2.1 Features of lycopene

Lycopene (as shown in **Figure 2-1c**), a naturally occurring red colour phytochemical (Khoo et al., 2011) synthesised by plants and microorganisms, belongs to the carotenoids family (Kong et al., 2010). It is a lipid-soluble antioxidant. Lycopene does not possess pro-vitamin A activity because its molecular structure lacks the terminal *β*-ionic ring as the basic structure for vitamin A. Lycopene is a highly unsaturated acyclic compound with an open straight chain hydrocarbon consisting of 11 conjugated and 2 unconjugated double bonds. The presence of many double bonds in the structure prompted the formation of different *cis* and *trans* isomeric forms of lycopene. Also, the red colouration of lycopene is manifested because of the presence of many double bonds that absorb more visible spectrum compared to other carotenoid members. These double bonds form the light absorbing chromophore and create a maximum absorption due to the higher wavelength value (Khoo et al., 2011; Perveen et al., 2015; Roldán-Gutiérrez and Dolores Luque de Castro, 2007; Viuda-Martos et al., 2014).

All *trans* isomeric forms of lycopene are present naturally. They are the most predominant isomers in fruits and vegetables (Khoo et al., 2011). However, *cis*-isomers of lycopene can be produced through mono- or poly-isomerisation by light and thermal energy as well as chemical reactions. Lycopene is known to be highly stable, but it can undergo oxidative-, thermal- and photo-degradation. According to Kong et al. (2010), lycopene is stable under the conditions of thermal processing and storage at low temperature (i.e. below 60 °C). In addition, 5-*cis* lycopene is the most

stable isomer and exhibits the highest antioxidant properties among the other isomers of lycopene (Honest et al., 2011).

Main sources of red lycopene are obtained from tomatoes and tomato by-products (Borguini and Ferraz Da Silva Torres, 2009; Perveen et al., 2015; Thakur et al., 1996; Viuda-Martos et al., 2014) as compared to the lycopene derived from other food sources, such as watermelon and red cabbage (Kong et al., 2010; Krinsky and Johnson, 2005). Rao and Rao (2007) reported that there is only 10–30% absorption rate of the dietary lycopene by humans from the consumed lycopene-containing foods, especially the tomatoes. The examples of red lycopene-containing fruits and vegetables are listed in **Table 2-1**.

2.1.2.2 Bioavailability, absorption and distribution

Since lycopene is a lipid-soluble compound, which is also similar to dietary fat, its absorption in human body is similar to other lipid-soluble compounds and dietary fat (Kong et al., 2010). Lycopene is dissolved and absorbed across gastrointestinal tract (i.e. stomach, duodenum and intestine) via chylomicron mediated mechanism supported by specific epithelial transporter (Viuda-Martos et al., 2014). The absorption of dietary lycopene is influenced by several factors. For instance, dietary lycopene is more efficiently dissolved in large intestine than in small intestine; but its absorption in the large intestine is insignificant because the large intestine does not absorb nutrients. Also, the intake of high fibres diet decreases the absorption of dietary lycopene in gastrointestinal tract (Kong et al., 2010). In addition, cooking procedures and temperatures, breakdown of dietary lycopene-containing foodstuffs, food processing etc. need to be carefully handled as they might affect the absorption of lycopene (Honest et al., 2011; Rao and Rao, 2007). Moreover, there are several studies

showing that the lycopene from tomatoes, especially tomato-based products, could be absorbed better by humans as compared to the lycopene derived from other food sources (Khoo et al., 2011; Perveen et al., 2015).

The absorbed lycopene is then distributed accordingly throughout the circulatory system into human organs and plasma. Liver, adrenal and reproductive tissues are found to be ten times higher lycopene concentration than other tissues and organs. Kong et al. (2010) reported a lycopene concentration sequence in human organs and tissues, which had been well studied and organised by many researchers. The highest lycopene concentration was in human testes, followed by an order of adrenal gland > liver > prostate > breast > pancreas > skin > colon > ovary > lung > stomach > kidney > fat tissue > cervix. Also, the concentration range was between 0.2–21.4 nmol/g of tissue (Honest et al., 2011; Rao and Rao, 2007; Viuda-Martos et al., 2014).

2.1.2.3 Prevention of chronic diseases

In accordance with Kong et al. (2010), lycopene efficiently acts as a singlet oxygen quencher and susceptibility to oxidative damage (Siddiqui et al., 2014; Viuda-Martos et al., 2014). Various types of cancers, such as prostate, breast, ovarian, cervical, liver, lung and other organ sites, have been reported to be prevented by the intake of lycopene and lycopene-based products (Perveen et al., 2015; Rao and Rao, 2007; Wootton-Beard and Ryan, 2011).

Other than that, the preventive roles of lycopene in other humans diseases include prevention of cardiovascular diseases (CVDs) (e.g. hypertension, heart disease and stroke) (Perveen et al., 2015; Rao and Rao, 2007; Takashima et al., 2012), prevention of bone diseases like osteoporosis (Viuda-Martos et al., 2014), promotion of healthy

lung and prevention of lung diseases like emphysema (Rao and Rao, 2007; Schünemann et al., 2002), prevention of neurodegenerative disorders (e.g. Alzheimer's disease, Parkinson's disease and vascular dementia) (Perveen et al., 2015; Viuda-Martos et al., 2014), prevention of infertility problem for men (Rao and Rao, 2007; Sabanegh, 2010) and promotion of foetal growth in pregnancy (Sharma et al., 2003).

2.1.3 Anthocyanins

2.1.3.1 Features of anthocyanins

Anthocyanins (**Figure 2-2**) found in plants have a role in providing red-purple colouration, nutritional and health benefits (Aberoumand, 2011; Horbowicz et al., 2008). There are wide sources of anthocyanins-containing crops exist on earth, and presented in **Table 2-2**. The formation of colour is dependent on pH and glycosylation patterns of anthocyanins. They are probably the most important phytochemicals after the chlorophyll (Fernandes et al., 2014; Kong, 2003). Stintzing and Carle (2004) reported that anthocyanins have an effect on masking the chlorophyll against high light intensity which then prevent the chloroplast from being photo-inhibition.

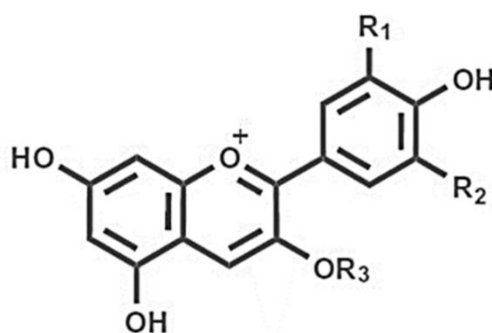


Figure 2-2: Chemical structure of anthocyanins.

Anthocyanins are water-soluble phytochemicals which are categorised under the group of flavonoids (i.e. class of phenolic compound) (Aberoumand, 2011; Dai and Mumper, 2010). Anthocyanins are less stable when compared to carotenoids. They are considered as highly reactive and sensitive molecules, and thus easily to be degraded (Robert et al., 1987). Their stability is mainly influenced by pH, arrangement of chemical structure, storage temperature, presence of light, metallic ions, oxygen, enzymes etc. Anthocyanins may be considered as glycosylated anthocyanidins because, in their chemical structures, sugars are usually attached to the 3-hydroxyl position of the anthocyanidin molecules or sometimes to the 5- or 7-flavylium position (Pina et al., 2012). Anthocyanins, together with other members of flavonoids, provide an important function in the resistance of crops to insects and pest attack in the nature ecosystems (Castañeda-Ovando et al., 2009; Fernandes et al., 2014; Li et al., 2012; Stintzing and Carle, 2004).

Table 2-2: Dietary sources of plants that are rich in anthocyanins.

Anthocyanins-containing crops	References
Different varieties of berries (e.g. strawberries, red and black raspberries, blueberries, blackberries, acai berries, mulberries, bilberries, noni berries, wolfberries, elderberries, boysenberries, red and blackcurrants etc.)	(Aberoumand, 2011; Cooke et al., 2005; Gopalan et al., 2012; Horbowicz et al., 2008)
Red and purple grapes, red wines, pomegranates, red cabbages, purple and black carrots, purple potatoes, purple corn, eggplant, radishes, curly kale, red bean, violet cauliflower, red lettuce, red onion	(Horbowicz et al., 2008; Norberto et al., 2013; Rodriguez-Amaya, 2016)
Sour cherries, sweet cherries, plums, blood oranges, apples	(Horbowicz et al., 2008; Sass-Kiss et al., 2005)
Tart cherries	(Kang et al., 2003)
Tulips, roses, orchids	(Costa et al., 2013)

2.1.3.2 Bioavailability, absorption and distribution

Anthocyanins are very reactive and sensitive to the extreme ranges of pH and temperature. Therefore, they are known to be the low bioavailability molecules. However, the native form of anthocyanins is easily absorbed and detected in plasma after digestion. Due to the low bioavailability, anthocyanins are generally detected in a tremendously low amount in human plasma after absorption. Generally, only 1% of the total anthocyanins intake will be detected (Fernandes et al., 2014; Norberto et al., 2013). The native structure of anthocyanins, such as cyanidin-3-glucoside and pelargonidin-3-glucoside possess a very fast metabolism and absorption rate when compared to other anthocyanins (Fernandes et al., 2014; Horbowicz et al., 2008; Pina et al., 2012).

The absorption and distribution of anthocyanins are occurred in gastrointestinal tract (Fernandes et al., 2014). Norberto et al. (2013) reported that the bioavailability and absorption of blueberry anthocyanins and also their nutrition and health-promoting effects upon consumption. Although blueberry anthocyanins have low bioavailability when compared to other blueberry polyphenols, but they can well retain in tissue plasma, and thus provide nutritional values and health-promoting benefits. Horbowicz et al. (2008) reported that individual's daily consumption of anthocyanins can be within the range of a few milligrams to hundreds milligrams, depending upon their requirements, nutritional habits and individual's preferences.

2.1.3.3 Prevention of chronic diseases

Several studies, including epidemiological studies and clinical testing, have been carried out to prove the pharmacological properties of anthocyanins and their benefits

to humans' health (de Pascual-Teresa and Sanchez-Ballesta, 2008; Giampieri et al., 2014). Both the anthocyanins and anthocyanidins have been found to exhibit a higher antioxidant activity than that of vitamin C and vitamin E (Clifford, 2000). They are claimed to be effective in scavenging free radicals like reactive oxygen species (ROS) and reactive nitrogen species (RNS) by donation of phenolic hydrogen atoms. By doing so, they are able to play a role as the inhibitors for mutagenesis and carcinogenesis (Castañeda-Ovando et al., 2009).

Prevention of cancers (e.g. colon, skin, liver and mammary cancer) by consumption of anthocyanins is indicated by an inhibition of anti-tumour activity reported in several articles (Cooke et al., 2005; de Pascual-Teresa and Sanchez-Ballesta, 2008; Gopalan et al., 2012; Horbowicz et al., 2008; Li et al., 2012; Stintzing and Carle, 2004; Wang and Stoner, 2008; Wang et al., 2015; Zhang et al., 2005). Anthocyanins are also effective in inhibiting lipoprotein oxidation and platelet aggregation, and therefore good in preventing of CVDs (Kong, 2003; Stintzing and Carle, 2004; Thompson et al., 2016).

Moreover, anthocyans and anthocyans-containing foods can reduce the risk of coronary heart disease (Cooke et al., 2005; Zhang et al., 2005). Prevention of neuronal illness (cognitive function/age-related disease) (Denise, 2014), diabetes, inflammation (Mizgier et al., 2016) as well as bacterial and viral infections (de Pascual-Teresa and Sanchez-Ballesta, 2008) are associated with the intake of anthocyanins too, thanks to their significant antioxidant activity (Castañeda-Ovando et al., 2009; Costa et al., 2013; Li et al., 2012; Pan et al., 2010).

2.1.4 Betacyanins

2.1.4.1 Features of betacyanins

Aside from lycopene and anthocyanins, betacyanins are also responsible for the red pigmentation in fruits and vegetables (Wrolstad and Culver, 2012). Betacyanins and anthocyanins are mutually exclusive (Khan and Giridhar, 2015), as they never co-exist in the same plants. It is either one or the other which will be responsible for the red colouration (Stintzing and Carle, 2004).

Betacyanins (**Figure 2-3b**) are water-soluble ammonium conjugates of betalamic acid with *cyclo*-3,4-dihydroxyphenylalanine (*cyclo*-DOPA). They contribute to a range of red to violet colouration in plants (Moreno et al., 2008). The most common betacyanins is betanin (betanidin 5-*O*-*β* glucoside) (Esatbeyoglu et al., 2015). Betacyanins are an important subgroup of betalains. Betalains, including red-violet betacyanins and orange-yellow betaxanthins, are water-soluble nitrogen-containing phytochemicals (Aberoumand, 2011; Wrolstad and Culver, 2012).

Formation of betacyanins occurred through the synthesis reaction of betalains from tyrosine by the condensation of betalamic acid with a derivative of DOPA. Betalamic acid is a central intermediate in the formation of all betalains (Esatbeyoglu et al., 2015; Khan and Giridhar, 2015; Pavokovic and Krsnik-Rasol, 2011; Stintzing and Carle, 2004). **Figure 2-3** shows the basic chemical structures of betalains (including betacyanins and betaxanthins), their common building block (betalamic acid) and betanin (the most common betacyanins).

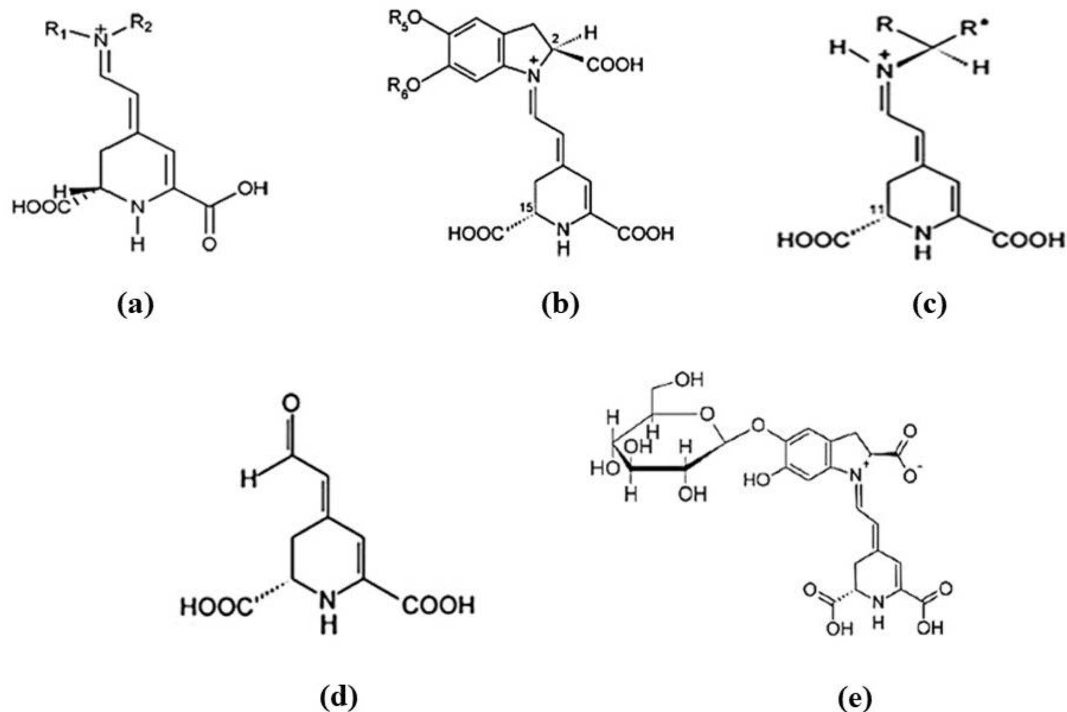


Figure 2-3: Chemical structures of (a) betalains, (b) betacyanins, (c) betaxanthins, (d) betalamic acid (common building block of betalains) and (e) betanin (the most common betacyanins).

Guaadaoui et al. (2014b) reported that the main edible sources of betalains are red beet, prickly pear (cactus), chard, amaranth grains or leaves, dragon fruit, ulluco tubers and djulis (a type of native cereal in Taiwan). European Food Safety Authority (EFSA) reported that betalains have a big potential role in food science application at present and in the future. This is because of their significant antioxidant capacity and health-promoting properties (Annie-Rose, 2016).

Betacyanins are known to be stable under low pH conditions (i.e. pH 3 to 7). Therefore, they are suitable for colouring of low acid foods (Agrawal, 2013; Delgado-Vargas et al., 2000). For example, Wrolstad and Culver (2012) reported that betacyanins at a pH of 6–7 makes them suitable as a colouring agents to be used in dairy products (i.e. yogurts and ice cream).

According to Pavokovic and Krsnik-Rasol (2011), betacyanins can be further divided into four subgroups, which are betanin, amaranthin, gomphrenin and 2-descarboxy-betanin. There are limited sources of betacyanins-containing crops exist on earth. This is due to the fact that betalains can only be found in angiosperms, in thirteen sub-order Chenopodineae within the Caryophyllales, and to some genera of the fungi Basidiomycetes as well as the mutual exclusiveness with anthocyanins. The important betacyanins-rich crops are red-purple pitaya and beetroot (Esatbeyoglu et al., 2015; Moreno et al., 2008; Stintzing and Carle, 2007; Stintzing et al., 2002). The examples of betacyanins-rich sources are presented in **Table 2-3**.

Table 2-3: Dietary sources of plants that are rich in betacyanins.

Betacyanins-containing crops		References
Family	Examples	
Chenopodiaceae	Red beet root, swiss chard, red goosefoot	(Aberoumand, 2011)
Cactaceae	Red-purple pitaya/red dragon fruit, cactus pear	(Esatbeyoglu et al., 2015; Moreno et al., 2008)
Amaranthaceae	Amaranth (leaf, grain)	(Esatbeyoglu et al., 2015; Moreno et al., 2008)
Portulacaceae	Moss rose	(Esatbeyoglu et al., 2015)
Aizoaceae	Ice plant	(Moreno et al., 2008; Stintzing and Carle, 2004)

2.1.4.2 Bioavailability, absorption and distribution

The stability of betacyanins could be influenced by temperature, pH, oxygen and light during storage and production stages. Nevertheless, there are also compounds that can act as betacyanins' stabilisers like antioxidants and chelating agents. For instance, the stability of betacyanins can be improved by a high concentration of betalains, a high degree of acylation and glucosylation, in a pH environment ranging

between 3 to 7, under dim light condition and at low temperature (Esatbeyoglu et al., 2015).

Esatbeyoglu et al. (2015) reported that oral bioavailability of betalains are similar to that of anthocyanins, and are estimated to be rather low. In particular, the absorption, metabolism and excretion of betalains occur in gastrointestinal tract, but the mechanism are yet to be fully understood and discovered. There is a need to further study and investigate this phenomenon (Clifford et al., 2015).

2.1.4.3 Prevention of chronic diseases

Betacyanins poses high antioxidant capabilities and anti-inflammatory properties (Tenore et al., 2012). They have the capability to stabilise free radicals due to their radicals scavenging properties, which play a role as electron donors (Clifford et al., 2015). Betacyanins also exhibit the inhibitory effects on lipid peroxidation and high oxygen radical absorbance capacity (ORAC) (Pavokovic and Krsnik-Rasol, 2011; Wu et al., 2006).

(a) ***Cancers***

According to Stintzing and Carle (2004), beetroot, a betacyanins-containing vegetable, has the ability to inhibit skin and lung cancer in mice. The beetroot is considered to be one of the top 10 most potent antioxidant-rich vegetables. It exhibits free radical scavenging properties as well as it can prevent active oxygen-induced and free radical mediated oxidation of biological molecules (Vulić et al., 2012; Vulic et al., 2013).

In addition, cancers preventing properties are also shown by the consumption of pitaya fruits (Dembitsky et al., 2011). Red pitaya, a fruit rich in betacyanins, is claimed to exhibit high antioxidant activity. Both peel and flesh part of the pitaya fruits contain a high amount of polyphenol (good source of antioxidants) and exhibit anti-proliferative effect on the growth of melanoma cancerous cells (Wu et al., 2006).

(b) ***Oxidative stress-related disorders***

Plants of the family of Amaranthaceae are reported to exhibit free radicals scavenging effects. These plants are known to be rich in betacyanins. In betacyanins, acylation mechanisms generally increase the antioxidant potential (Stintzing and Carle, 2004). Also, betalains from beetroot demonstrated free radicals scavenging and antioxidant activities (e.g. anti-microbial and cytotoxic activities). Red beets can effectively inhibit lipid peroxidation and heme decomposition. This suggests that they have a protective role against certain oxidative stress-related disorders, such as CVDs, cancers, aging and neurodegenerative disorders (Vulic et al., 2013).

Maigoda et al. (2016) studied the relationship between the consumption of red dragon fruit powder and the risk of getting obesity, by using rat as a model. Obesity might trigger other chronic diseases, such as CVDs, type II diabetes and cancers. Most of the chronic diseases are caused by the oxidative stress and inflammation. It is proven that the intake of red dragon fruit powder would decrease the levels of oxidative stress and inflammation, thereby reducing the chance of getting obesity.

Overall, there is not much research done on the function of betacyanins in prevention of chronic diseases. Since betacyanins are the potent antioxidants, they might be expected to exhibit the significant health-promoting effects. Therefore, it is

important to carry out more researches on their pharmacological properties, so as to enhance the usage of betacyanins and underutilised betacyanins-containing plants.

2.2 Colouring agents

In recent years, food industries are facing problems on the use of colouring matter on several applications, such as foodstuffs, cosmetics and nutraceuticals. Consumers are aware of the negative health and toxicological issues associated with the utilisation of artificial dyes (Robert et al., 1987). As a result, the manufactures tend to avoid the use of artificial colouring compounds and they incline toward the natural dyes which are mainly derived from natural resources (Aberoumand, 2011; Carocho et al., 2015; Downham and Collins, 2000; Giusti and Wrolstad, 2003; Wissgott and Bortlik, 1996).

Artificial colourants are known to cause allergenic and intolerance reactions in susceptible individuals. Also, it might trigger some chronic diseases like cancers and CVDs (Robert et al., 1987; Wissgott and Bortlik, 1996). In contrast, the use of natural dyes is known to be harmless to humans and to offer health-promoting effects. Also, it provides a clean label declaration as driven by its environmentally benign feature (Aberoumand, 2011; Carocho et al., 2015).

In food processing, an attractive and appealing appearance of food products is emphasised because the consumers largely assess the food quality based on the external appearance of food products. As such, colouring matters have been heavily applied to a variety of food products in market (Carocho et al., 2015). Three sets of colours, namely red, blue and yellow, are the basic colours that can be combined to form the secondary colours, such as green, purple and orange. The mixing of these

basic colours in different proportions results in the formation of other types of hues (Downham and Collins, 2000; Meurant, 1969).

Colours affect almost every moment of our lives. They play a significant role in food products, in which most of the times, the first impression is made based on their colours (Downham and Collins, 2000). For instance, the maturity's level of crops and the freshness of food products can be assessed based on the colour changes (Aberoumand, 2011). Due to the closely relationship between colours and consumers' expectation, the colouring matters have been widely applied in food products for following reasons (Carocho et al., 2015; Giusti and Wrolstad, 2003).

(a) Replacement of colour lost during the food processing
(b) Enhancement of colour in the present form of products, so as to improve their attractiveness and appearance
(c) Minimisation of batch-to-batch colours' variations
(d) Providing a colour to the final products

2.2.1 Type of colouring agents

According to Downham and Collins (2000), Aberoumand (2011), colourants can be divided into four groups, as illustrated in **Figure 2-4**. Both natural and nature-identical colourants are considered as naturally occurring colouring agents, which are mostly derived from plant pigments. In particular, phytochemicals (plant-based bioactive compounds) contribute to the colour of many plants. Phytochemicals, such as chlorophyll (class of tetrapyrrols), carotenoids (class of tetraterpernoids), anthocyanins (class of flavonoids) as well as betacyanins and betaxanthins (class of betalains) offer a huge range of colours (Aberoumand, 2011; Lancaster and Lister,

1997; Leong et al., 2018c). On the other hand, both synthetic and inorganic colourants are the artificial colouring compounds.

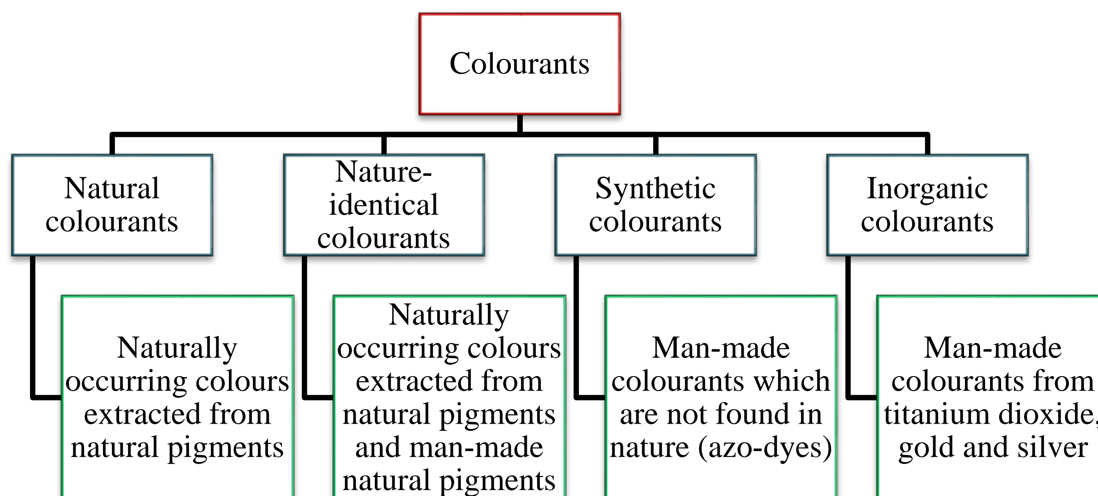


Figure 2-4: Categories of colourants.

For example, chlorophyll contributes mostly to the green colour (EUFIC, 2016) whereas carotenoids are responsible to the range of yellow-orange-red colour in many plants (Rao and Rao, 2007). Other than that, anthocyanins contribute to the red-purple-blue shade in many fruits and vegetables as well as some flowers (Bridle and Timberlake, 1997; Castañeda-Ovando et al., 2009; Wrolstad, 2004). Similarly, betacyanins confers a range of red-purple colour to many plants. On the other hand, betaxanthins contribute to the yellow-orange shade of plants (Aberoumand, 2011; Downham and Collins, 2000; Guaadaoui et al., 2014b).

Natural red plant pigments are presently receiving immense attention in the food science industries, such as foods and pharmaceuticals field, as to replace the artificial red dyes (Moreno et al., 2008). In particular, Aberoumand (2011) reported that betalains are of growing interest in the food science processing (Annie-Rose, 2016; Leong et al., 2018c).

A wide array of anthocyanins-containing extract has been used as food colouring agent. So far, there is only one dietary source of betalains, i.e. red beet, which has been extensively used in the food industry. There are a few chosen varieties of red beet provide natural red colourant, in which contributed by the red-purple betanin and its C15-isomer compound (isobetanin). A unique feature of betalains is their wide range of pH stability pH (3 to 7), which makes them suitable for a broad range applications in colouring low acid and neutral foods. Also, they can be used to substitute anthocyanins (Aberoumand, 2011).

Betanin is a compound mostly contributing to the formation of red colour in red beet. It has been in the market as the oldest and the most abundant red food dye (Schieber et al., 2001). Betanin has a colour code of E-162 in the European Union (Moreno et al., 2008) and of 73.40 in the chapter 21 of the Code of Federal Regulations (CFR) section of the Food and Drug Administration (FDA) in the USA. This natural red dye has been widely applied in various foodstuffs, such as ice cream, yoghurt, sausages etc. (Aberoumand, 2011).

Red beet contains geosmin that eventually form an unpleasant earthy smell. In addition, its high nitrate concentration is associated with the formation of nitrosamines (carcinogenic compound). These drawbacks limited the commercial potential of red beet as natural red colourant. As a consequence, there is a high demand for replacement of natural red colourant extracted from red beet in the market (Aberoumand, 2011).

Fruits from the family of Cactaceae, such as pitaya, have been recognised as a promising source of betalains. Red-purple pitaya (red skinned with purple-fleshed; *Hylocereus polyrhizus*) is rich in betacyanins. Additionally, it has a good smell and it does not produce any carcinogenic compounds. Hence, red-purple pitaya is known to

devoid of the mentioned drawbacks in red beet, and could be a good replacement of red beet (Aberoumand, 2011; Moreno et al., 2008).

2.3 Antioxidants

Antioxidants are known to be the bioactive compounds that have the capability to quench singlet oxygen species (i.e. first defence line mechanism against oxidative stress). They are also involved in the second defence line mechanism, which acts as a free radicals scavenger and chain initiation inhibitor or chain propagation disruptor (Podsędek, 2007). Antioxidants are able to fight against free radical species, such as ROS and RNS. Oxidative stress is mainly caused by the free radical species and it increases the risk of incidence of non-communicable diseases, such as cancers, CVDs, neurodegenerative disorders and general aging (Wang et al., 2011).

Dietary antioxidants can be divided into two groups, namely water-soluble (such as vitamin C and phenolic compounds) and lipid-soluble (such as vitamin E and carotenoids) antioxidants. Both of them have the capability to fight against oxidative stress by involving themselves in the first and second defence line mechanism. Thus, antioxidants protect cells against oxidative damage caused by the oxidative stress and free radical species (Podsędek, 2007).

A frequent consumption of dietary antioxidants, such as vitamin E (from food sources or supplements) is known to increase the longevity of human lifespan due to the prevention of age-associated diseases and fast aging, as well as the improved immune function in body (Meydani, 1999). Pérez-Jiménez et al. (2008) reported that the main dietary antioxidants are polyphenols, followed by vitamins and carotenoids.

Their appropriate daily intake should be about 1 g for polyphenols, 110 mg for antioxidant vitamins and 9.4 mg for carotenoids.

Antioxidant capacity (AOC) is a key parameter in the fields of food science and technology as well as nutritional studies (Pérez-Jiménez et al., 2008). It can be measured by various methods, for example, analyses of ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC). It is recommended to apply at least two different approaches to measure AOC due to the differences between each evaluation system and product characters (Müller et al., 2010).

Drying by means of high temperature and/or prolonged treatments cause a decrease in AOC and bioactive compounds. The losses of AOC and bioactive compounds could be minimised if the sample is freeze dried. Drying under vacuum is another option if freeze drying is not feasible, as long as the temperature is controlled and does not exceed 50–60 °C, depending upon the sample (Pérez-Jiménez et al., 2008).

2.3.1 Bioactive compounds

The term "bioactive" is comprised of two words: *bio-* and *–active*. In etymology, *bio-* from the Greek (βίο-) "*bios*" [bio-, -bio] refers as life while *–active* from the Latin "*activus*" refers as dynamic, full of energy, with energy, or involves an activity. Therefore, bioactive compound in this circumstance, means a substance involved in an activity which brings a form of life, a process, or a function (Guaadaoui et al., 2014a). In scientific point of view, the term "bioactive" is also termed as "biologically active". In other word, it is a substance that will demonstrate biological activity. On

the other hand, medical dictionaries defined bioactive compound as a substance (essential and non-essential compound) (Biesalski et al., 2009) that might trigger a response which in turn brings a beneficial effect to the humans body system (Guaadaoui et al., 2014a).

Moreover, bioactive compound is defined as a substance which demonstrates the capability or ability to interact with one or more component(s) of the living tissue by presenting a wide range of beneficial effects. It shows several bioactivities, such as anti-oxidant (Rufino et al., 2010), anti-inflammatory and anti-aging effect (Guaadaoui et al., 2014a; Guaadaoui et al., 2014b). In addition, bioactive compound is claimed to be a functional food due to its health-promoting functions like chronic diseases preventive role (Biesalski et al., 2009; Kris-Etherton et al., 2002; Martirosyan and Singh, 2015; Shashirekha et al., 2015; Weaver, 2014).

Bioactive compounds are gaining global interest in the food science processing due to their beneficial bioactivities. For instance, application of bioactive compounds in several fields, such as foods, pharmaceuticals, nutraceuticals, cosmetics, plant science, nano-bio science, modern pharmacology, geo-medicine etc. Consequently, bioactive compounds create a very promising prospect in research and development, for example, improving their salvage biosynthesis, understanding their bioavailability and health-promoting benefits on consumption (Biesalski et al., 2009; Guaadaoui et al., 2014a; Guaadaoui et al., 2014b; Kris-Etherton et al., 2002; Weaver, 2014).

Plant-based bioactive compounds or namely phytochemicals play the protective roles against external stress and pathogenic attack. Phytochemicals are categorised into phenolics, alkaloids, steroids, terpenes and saponins. Natural products and secondary metabolites extracted from living systems, notably from phytochemicals have

demonstrated a great potential in preventing the non-communicable and infectious diseases (Guaadaoui et al., 2014a; Guaadaoui et al., 2014b; Kris-Etherton et al., 2002; Shashirekha et al., 2015; Weaver, 2014).

2.4 Red-purple pitaya

Red-purple pitaya, or commonly known as red dragon fruit or red-purple pitahaya, has a scientific name of *Hylocereus polyrhizus*, as shown in **Figure 2-5a**. It also has a few nicknames, such as Queen or Lady of the Night, buah naga and Moonflower; due to its flowers only bloom at night time. The pitaya fruit is categorised under the Cactaceae family and the Cactoidea subfamily (i.e. fruit of the cactus species). Red-purple pitaya is a red-skinned fruit with red-purple flesh and black seed. It is an exotic yet underutilised berry fruit (Dembitsky et al., 2011).

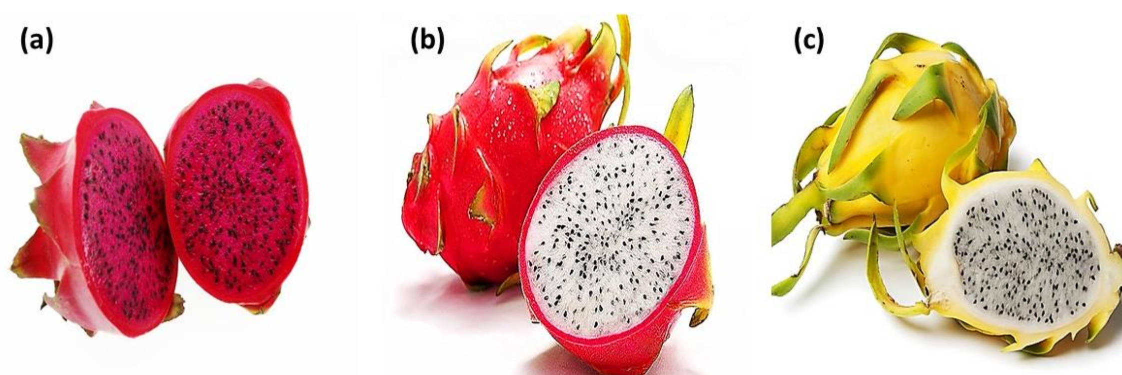


Figure 2-5: Various pitaya species; (a) Red skinned with red-purple flesh pitaya (*Hylocereus polyrhizus*), (b) Red skinned with white flesh pitaya (*Hylocereus undatus*), (c) Red skinned with white flesh pitaya (*Hylocereus undatus*)

The pitaya fruit is a seasonal fruit; it is only available in periods of April–May and September–November, depending on country. It is native to Mexico, Central and South American, but its cultivation is wide in nations, covering Southeast Asian countries like Malaysia, Taiwan, Indonesia, Vietnam, Israel and Thailand. Fruits of the

cactus species are easily to be cultivated due to their drought and heat resistance properties. They have strong roots system, and therefore are good at water conserving. One pitaya fruit weighs around 150–600 grams, and has a mildly sweet taste. It is best to be served cold. Fresh pitaya fruits will only last for several days: maximum of 14 days at 10 °C or 5 days at room temperature (Dembitsky et al., 2011; Nguyen, 2014; NutriNeat, 2018).

Other than red-purple pitaya, there are other species of pitaya fruits, such as *Hylocereus megalanthus* (yellow skinned fruit with white flesh) and *Hylocereus undatus* (red skinned fruit with white flesh) (see **Figure 2-5**). Nguyen (2014) reported that red-purple pitaya is of growing interest during last few years, particularly in Asian countries, due to its high nutritional values, significant health beneficial effects on consumption, high antioxidant capacity and other potential usages in food science processing (e.g. natural red dye) (Moreno et al., 2008).

The red-purple layer of the fruit is a good source of vitamins (such as vitamins B1, B2, B3 and C), minerals (e.g. potassium, sodium, calcium, iron and phosphorus), bioactive compounds (such as carbohydrate, glucose, protein, thiamine, phytoalbumin, niacin, pyridoxine, kobalamin, fat, flavonoids, phenolic compounds, polyphenol, carotene and betacyanins) and crude fibre (Nguyen, 2014). **Table 2-4** presents the nutritional facts and health-promoting benefits of ripen fresh pitaya fruits.

Table 2-4: Nutritional facts and healthy functional of ripen fresh pitaya fruits.

Nutritional facts per 100 g of serving ripen fresh pitaya fruits		Healthy functional
Moisture content	83.0 g	Enhance metabolic processes in humans' body
Protein	0.229 g	Enhance metabolic processes in humans' body
Carotene	0.012 g	Maintain or improve a healthy eyesight
Calcium	8.8 g	Reinforce bones, aid in the formation of healthy teeth
Phosphorus	36.1 mg	Aid in tissue formation
Iron	0.65 mg	Aid in blood production
Vitamin C	9.0 mg	Fast healing of bruises and cuts, improve immune system function
Riboflavin	0.045 mg	Regulate humans' growth and formation red blood cell, involve in energy production by regulate carbohydrates metabolism
Niacin	0.430 mg	Involve in energy production by regulate carbohydrates metabolism
Ashes	0.68 g	-

Source: NutriNeat (2018)

2.4.1 Health benefits on consumption

Frequent consumption of pitaya fruits could improve the overall digestive system, reduce bad cholesterol level as well as prevent constipation and cancers (Dembitsky et al., 2011; Nguyen, 2014). Both peel and flesh part of pitaya fruits contain high amount of polyphenol, which is a good source of antioxidants that have the ability to inhibit growth of melanoma cancerous cells in anti-proliferative study. In addition, the intake of these fruits promotes the anti-diabetic effect (Omidizadeh et al., 2014).

Maigoda et al. (2016) reported that the relationship between consumption of red dragon fruit powder with the decreasing risk of getting obesity using rat model. Obesity might trigger other chronic diseases, such as CVDs, type II diabetes and

cancers. Most of the chronic diseases are caused by oxidative stress and inflammation. This study showed the inhibition on oxidative stress and the decreasing inflammation rate, and thus reducing the chance of getting obesity (Maigoda et al., 2016).

2.5 Microalgae

Algae are known to be one of the oldest living organisms on earth, and have been recognised as a primitive plant (thallophytes). They are lacked of roots, stems and leaves, have unsterile covering of cells around the reproductive cells and possess chlorophyll a as their primary photosynthetic pigment. The algae structure is optimal for energy conversion. In addition, algae can easily adapt to prevailing environmental conditions and they are well grown in the long period, owing by their simple structure. Algae can be divided into three types that are auto-, hetero- and mixotrophic (auto- and heterotrophic). Autotrophic algae require only inorganic compounds (e.g. CO_2, salts and light energy) for growth whereas cultivation of heterotrophic algae require both the organic compounds and nutrients as the energy sources (Brennan and Owende, 2010; Lee, 2018).

Microalgae, both the prokaryotic cyanobacteria and the eukaryotic microalgae, are tremendously a diverse collection of organisms. They are important sources of various commercially high value-added products (Borowitzka, 2013; Brennan and Owende, 2010). There are various microalgae products; some are exclusive to microalgae (e.g. phycobiliproteins) while others can be synthesised from several sources like plants and microbial culture (such as polysaccharides, xanthophylls, β-carotene etc.). These microalgae products are mostly used as health food supplements, and the cultivation of unicellular green alga *Chlorella* and filamentous blue-green alga *Spirulina* are primarily for this purpose (Benemann et al., 1987).

Another crucial application of microalgae culture is for the wastewater treatment, as driven by the presence of bio-flocculation and chemical flocculation process during the cultivation (Benemann et al., 1987). Microalgae are known to be effective in biological treatment by removing nutrients (such as nitrogen, phosphorus etc.) and toxic metals, and as a result, they showed the good potential in wastewater treatment (Cai et al., 2013). Also, Xia and Murphy (2016) reported the benefits of microalga-based liquid digestate treatment that not only can greatly reduce the microalgal cultivation cost but also involve in the management of biogas by-products.

2.5.1 Microalgae biorefinery

Microalgal biotechnology applications are presently a rapidly growing field. In particular, microalgae biorefinery is gaining global interest as an emerging biomass transformation approach. The generation of high value-added products in addition to biofuel through microalgae biorefinery has been progressing well. For instance, microalgae biomass can be transformed into pigments, proteins, lipids, polyunsaturated fatty acids (PUFAs), carbohydrates, vitamins and antioxidants (Yen et al., 2013). These high value-added materials are subsequently applied in various commercial and industrial applications, such as foods, cosmetics, pharmaceuticals and nutraceuticals (Borowitzka, 2013; Brennan and Owende, 2010; Chew et al., 2017; Pulz and Gross, 2004; Spolaore et al., 2006; Vanthoor-Koopmans et al., 2013; Wang et al., 2017).

2.5.2 Docosahexaenoic acid

One of the major benefits of culturing microalgae is that they can accumulate high percentages of lipid in their bodies (approximately 20–50% of their total weight)

(Brennan and Owende, 2010). Among the microalgal lipids, docosahexaenoic acid (DHA) is a long chain PUFA that serve as a health food supplements (Borowitzka, 2013; Tan et al., 2016). DHA (22:6n-3) is classified as an important lipid in the omega-3 family because it is an essential nutrient for neurological and cognitive functions in humans (Bradbury, 2011; Calderon and Kim, 2004; Kawakita et al., 2006; Kim, 2007). A DHA-rich diet formulation is required for infants as DHA improves the growth and functional development of the brain in infants. DHA consumption is also important in adults as it aids in maintaining normal brain functionalities. Moreover, the sufficient DHA intake showed a preventive role in non-communicable diseases, such as diabetes, CVDs, neurodegenerative diseases, heart diseases and cancers (Bazan et al., 2011; Hashimoto, 2018; Horrocks and Yeo, 1999; Huang et al., 2012; Stillwell and Wassall, 2003).

2.5.3 *Aurantiochytrium limacinum* SR21

Aurantiochytrium limacinum SR21, or previously known as *Schizochytrium limacinum* SR21 (Yokoyama and Honda, 2007), is a highly oleaginous microalga strain and is reported as an excellent DHA producer. In addition, *Aurantiochytrium* species has a short cultivation time. Previous studies reported that the cultivation of *A. limacinum* SR21 under the optimised culture conditions produced the high concentrations of lipid, DHA and biomass. Usage of different carbon sources, such as glycerol, crude glycerol and glucose, have prompted for the higher lipid accumulation and microalgal growth in the cultivation of *A. limacinum* SR21 (Chi et al., 2007; Ethier et al., 2011; Gao et al., 2013; Huang et al., 2012; Li et al., 2015; Lung et al., 2016; Yokochi et al., 1998).

2.5.4 Microalgae cultivation using waste feedstock

Utilisation of waste feedstock in microalgae cultivation not only greatly reduces the cost of cultivation but also represents a good waste management. Dahiya et al. (2018) studied the food waste biorefinery and showed that the food waste can be incorporated into various bioprocesses to produce value-added bio-based products. In addition, Chen et al. (2018) reported the utilisation of anaerobic digestion products in microalgae cultivation. Microalgae can grow in the culture medium containing liquid and gaseous effluents of anaerobic digestion, and hence this creates an opportunity for waste biorefinery. Moreover, there are literatures on the usage of wastewaters resources from municipal, agricultural and industrial activities in microalgae cultivation. This approach not only reduced the cultivation cost but also ensure a sustainable management on waste feedstock (McGinn et al., 2011; Pittman et al., 2011).

For instance, several studies focused on the utilisation of biodiesel waste feedstock (i.e. crude glycerol) in the cultivation of *Schizochytrium limacinum* SR 21 for synthesis of value-added products, in particular DHA, by optimising the culture conditions (Chi et al., 2007; Ethier et al., 2011; Lung et al., 2016; Pyle et al., 2008). Also, Cheah et al. (2018) reported the application of palm oil mill effluent (POME) in the cultivation of *Chlorella sorokiniana*. This approach successfully achieved the higher microalgal biomass growth and lipid accumulation as well as POME remediation.

2.6 Industrial biotechnology

A sustainable production of value-added products from renewable sources and with the utilisation of enzymes and microorganisms by modern biotechnology is known as

industrial biotechnology or white biotechnology. It is also considered as the third wave of biotechnology, which is totally different from the (1) red (field of medical) and (2) green (field of agricultural) biotechnology. The industrial biotechnology has been receiving much attention globally over the past decades, driven mainly by the involvement in reducing of energy consumption along with the greenhouse gases emissions and waste generation (Tang and Zhao, 2009).

For example, Bhatia et al. (2018) studied the bioethanol production by utilising lignocellulosic biomass. It is a green approach on the fuels production that has received considerably interest as to enhance the sustainability in the terms of bioenergy. On the other hand, Chen and Jiang (2018) reported the use of next generation industrial biotechnology (NGIB) in the bioprocessing, in particular for extremophilic bacteria culture. NGIB is a new technology that allows bacterial cultivation under continuous and unsterile condition in plastic transparent bioreactors. Moreover, NGIB is known to devoid the difficulties of the current industrial biotechnology.

In recent times, industrial biotechnology tools including protein engineering and downstream bioprocessing have been employed for the sustainable production of value-added products in the food science processing industries. Meanwhile, the development of a green, simple, economically viable and effective separation technology is a rapidly growing field in the biotechnology industries (Chemat et al., 2017; Sankaran et al., 2018; Tang and Zhao, 2009; Xu et al., 2017).

2.7 Extraction techniques

2.7.1 Conventional extraction technique

Extraction of betalains from various plant sources is normally achieved using the conventional solid-liquid extraction approaches, such as maceration, infusion and Soxhlet extraction (Castellar et al., 2003; Celli and Brooks, 2017; Chong et al., 2014; Gengatharan et al., 2016; Hilou et al., 2013; Laqui-Vilca et al., 2018; Ramli et al., 2014; Ravichandran et al., 2013). They are generally achieved in water extraction; nevertheless, in most of the cases, 20–50% (v/v) of methanol/ethanol was utilised to achieve a complete extraction (Tiwari and Cullen, 2013).

These extraction procedures are reported to have limitations, for example, inefficiency, time-, energy- and cost-consuming, solvent contamination of the products, less eco-friendly, stability problems, batch-to-batch variations as well as lower yield of production (Azmir et al., 2013; Ciriminna et al., 2018; Dai and Mumper, 2010; Leong et al., 2018a; Wang and Weller, 2006). As a consequence, development of green, reliable, economically effective and high efficient separation techniques are deemed necessary as an alternative procedure for the betalains extraction. It is also now a rapidly growing field in the biotechnology and downstream processing industries (Chemat et al., 2017; Sankaran et al., 2018; Tang and Zhao, 2009).

2.7.2 Novel extraction technique

The drawbacks of conventional processing techniques demand the replacement of non-conventional and innovative extraction approaches in the downstream processing industries, especially in the field of food science applications. Various extraction techniques like ultrasound (sonication) (Laqui-Vilca et al., 2018; Ramli et al., 2014),

microwave (dielectric heating) (Bastos and Gonçalves, 2017), high pressure CO_2 (Ciriminna et al., 2018) and supercritical fluid processing as well as pulsed electric field (PEF) (Fincan et al., 2004) have been applied alongside the conventional extraction methods for the betalains extraction. These green techniques are proved to minimise the shortcomings of the conventional methods, provide a better extraction efficiency of betalains and reduce the processing time (Celli and Brooks, 2017; Chemat et al., 2017; Ciriminna et al., 2018; Laqui-Vilca et al., 2018; Xu et al., 2017).

In addition, aqueous two-phase extraction (ATPE) has been recently used for the separation, purification and concentration of betalains from plants (Chandrasekhar et al., 2015; Chethana et al., 2007; Santos et al., 2018). ATPE, or more commonly known as aqueous two-phase system (ATPS), is a type of liquid biphasic system, and has been reported as an effective bioseparation technique owing to the benefits of liquid biphasic system. The liquid biphasic system is well-known as an easy, scalable, time-, cost- and energy-saving, effective as well as mild and green separation approach for many biotechnological and natural products (Albertsson, 1986; Hatti-Kaul, 2000; Show et al., 2013; Yau et al., 2015; Zimmermann et al., 2017). Details of the liquid biphasic system will be discussed on the following sub-section (see Section 2.7.2.2).

2.7.2.1 Electricity treatment

Electricity treatment not only ameliorates extraction efficiency of biomolecules but also acts as a green extraction technique. It is considered as a type of non-thermal processing approach. Non-thermal processing methods improve the extraction yields by enhancing cell membrane permeabilisation with the use of several driving forces. These approaches improve the process efficiency by enhancing mass transfer of the

system and more eco-benign techniques (Boussetta and Vorobiev, 2014; Tiwari and Cullen, 2013).

For instance, PEF (a type of electricity treatment) is operated under electric potential difference as the driving force, and has been proved to be a promising tool for enhancing the extraction efficiency of bioactive compounds from various biological sources with the least degradation rate. It generates the high electrical field on sample situated between two electrodes and causes cell membrane disruption, and hence improves cell permeability (Azmir et al., 2013; Knorr, 2003; Tiwari and Cullen, 2013; Zimmermann et al., 1974).

A study conducted by Fincan et al. (2004) on the PEF treatment along with solid-liquid extraction of red beetroot pigment showed that the highest degree of extraction accomplished by PEF as compared with that of the treatments of freezing and mechanical pressing. Other than that, López et al. (2009) investigated the influence of PEF treatment to the grape pomace by evaluating various quality parameters and anthocyanins content of Cabernet Sauvignon wines. The utilisation of PEF treatment not only demonstrated that the freshly fermented wines model possessed stable colour intensity and visual properties but also the high total polyphenols index, tannins and anthocyanins content.

2.7.2.2 Liquid biphasic system

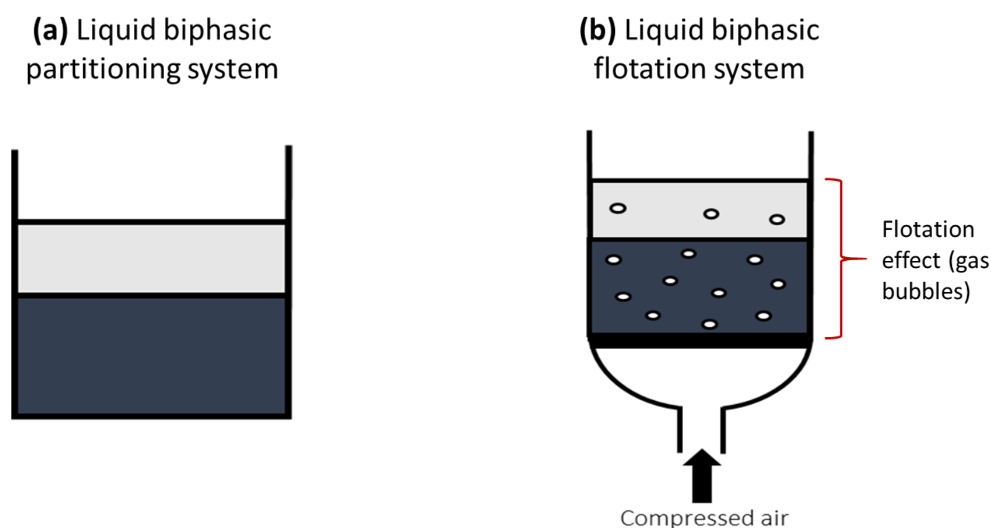


Figure 2-6: Schematic diagram of different types of liquid biphasic system.

(a) ***Liquid biphasic partitioning system***

Liquid biphasic partitioning system (LBPS) (see **Figure 2-6a**) is an innovative liquid biphasic system for separation of biomolecules as well as possesses the gifted advantages of liquid biphasic system. LBPS, or commonly known as ATPS (ATPS is a general terminology), is founded by Martinus Willem Beijerinck in 1896, where he observed and reported the phenomenon of two distinct phases formation by leaving a mixture of starch and gelatine to settle by gravity force. However, no further research was conducted to study on the phenomenon he had reported, until the real application of ATPS was discovered by Albertsson. Albertsson attempted the purification of chloroplast by ATPS, and successfully produced his first publication on the application of ATPS in the extraction and purification of biomolecules (Albertsson, 1986; Grilo et al., 2016; Hatti-Kaul, 2000; Iqbal et al., 2016).

This discovery was a major breakthrough in biomolecules separation as the conditions applied in ATPS is often attributed to high water content, ambient operating

conditions and the low toxicity of phase forming components, which are very suitable for extraction of biomolecules as compared to conventional solid-liquid extraction technique which uses a large amount of toxic organic solvents and harsh operating conditions (Iqbal et al., 2016). Upon its discovery, ATPS is mainly comprises of combinations between polymers and salts, such as polymer/polymer, polymer/salt and salt/salt. As the extraction takes place in ATPS, a two-phase solution is formed wherein the top phase is enriched with the targeted compound and the bottom phase is left with the unwanted compounds. As time passes, more developments in ATPS are in progress, such as the implementation of ionic liquids, alcohols and thermos separating polymers (Freire et al., 2012; González-Valdez et al., 2018; Hatti-Kaul, 2000; Lee et al., 2017; Li et al., 2010; Raja et al., 2011; Yau et al., 2015).

LBPS is a liquid-liquid fractionation method for separation and partition of biotechnological and natural products. It has been used in the field of biotechnology applications over the past half century (Hatti-Kaul, 2000). A wide range of biological materials, including plant and animal cells, microorganisms, fungi and their spores, virus, cell organelles (e.g. chloroplasts, mitochondria, membrane vesicles etc.), proteins, nucleic acids, antibodies, enzymes as well as natural compounds like phytochemicals (bioactive compounds) have been successfully separated and purified (Raja et al., 2011; Ratanapongleka, 2010; Rito-Palomares, 2004). LBPS is a technically simple, scalable, energy- and time-efficient, environmentally benign and mild bioseparation technique. In addition, it requires less solvent consumption during the separation process, and thus offers an attractive alternative that meets the requirements of the high demand in the industry processes (Feng et al., 2015; Hatti-Kaul, 2000; Khan et al., 2018; Santos et al., 2018; Yau et al., 2015; Zhang et al., 2016).

LBPS generally consists of polymer/polymer- and polymer/salt-based system. To form a polymer/salt-based LBPS, one must dissolve polymer and salt phases in water beyond their critical level which typically consists of 70–80% water content. One phase is enriched with the solute (targeted compound) while another phase remains with the unwanted compounds. Operation of LBPS begins with the gentle mixing of parent material containing the solute of interest along with the LBPS. When polymer/polymer or salt are mixed, these phases tend to aggregate and form two phases due to steric exclusion. The mixture is allowed to settle by gravitational force to complete the solute partition. Centrifugation can be used to hasten biphasic system formation, provided that they do not damage any biomolecules that is to be extracted (Albertsson, 1986; Hatti-Kaul, 2000; Iqbal et al., 2016).

Aside the polymer-based LPBS, alcohol/salt-based LBPS is usually formed by two immiscible phases consisting of short-chained aliphatic alcohols and usually, an inorganic salt; however, in order to achieve a greener alternative, an organic salt, such as citrates and acetates can be used to replace the inorganic salt. In comparison to the conventional polymer-based LBPS, the target compounds are easily recovered by removal of alcohol with evaporation, distillation or crystallisation. Aside from that, they have several advantages comparatively, such as faster segregation of phases, larger capacity in scale up, lower toxicity to the environment and a relatively inexpensive cost. These systems have been applied to the purification of biomolecules, including proteins, natural compounds, enzymes, acids, antibiotics and nucleic acids (Lo et al., 2015; Lo et al., 2018; Ooi et al., 2009; Zhang et al., 2013).

Various factors can be optimised in order to achieve a promising result on the partition of biomolecules in LBPS. These factors include phase-forming components

and their concentration, tie-lie length, pH, temperature, presence of neutral salts like sodium chloride and surface properties of biomolecules (Albertsson, 1986; Hatti-Kaul, 2000; Raja et al., 2011).

(b) ***Liquid biphasic flotation system***

Liquid biphasic flotation (LBF) system (see **Figure 2-6b**), or generally known as aqueous two-phase flotation (ATPF), is an integration process of ATPS and solvent sublation (SS) method (Show et al., 2011). LBF system is an innovative technology and is categorised under liquid biphasic system. This system integrates the principle of ATPS and mass transfer mode of SS (involves bubble adsorption). SS applies the mechanism of effective adsorption between surface of bubbles and surface-active material interaction in aqueous phase. The rising bubbles carrying the surface-active materials will release them at the organic solvent phase which is at the top column. SS has been successfully employed in the recovery of surface-active and hydrophobic compounds, and has showed the impressive separation efficiency of biomolecules (Lee et al., 2016; Mathiazakan et al., 2016; Sebba, 1959).

LBF system is highly biocompatible, with high enrichment factor, low environment impact, economical and easy to operate. Besides, the recovery of phase-forming compounds can be achieved easily. For example, in 2009, Bi and his colleagues proposed the ATPF method in separation and concentration of penicillin G from fermentation broth in polyethylene glycol/ammonium sulphate biphasic system. The authors showed that 97% of separation efficiency and 19 of concentration coefficient of penicillin G after the flotation process (Bi et al., 2009). Other than that, the LBF system has been widely applied in the separation of biomolecules, such as lipase,

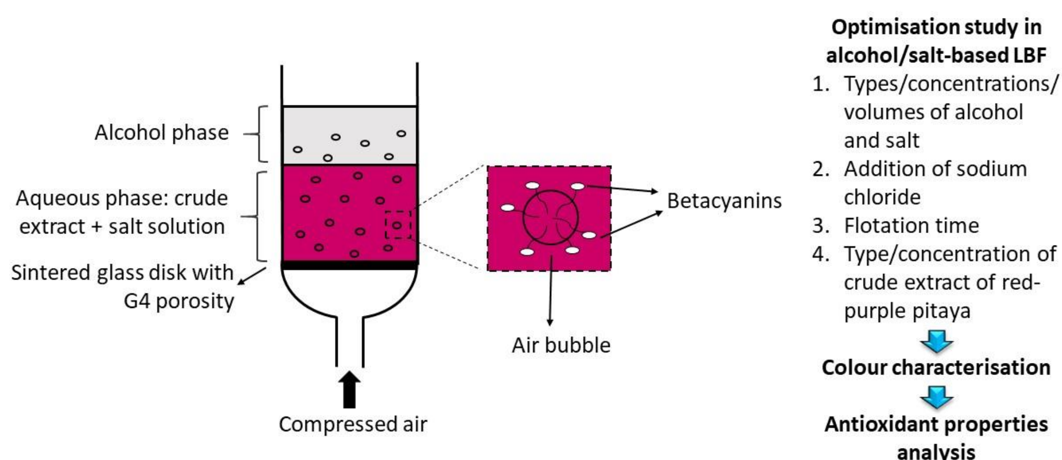
protein, natural products etc. (Bi et al., 2013; Leong et al., 2018a; Mathiazakan et al., 2016; Sankaran et al., 2018; Show et al., 2013).

Typically, a biphasic system consists of alcohol and salt solution, polymer and salt solution or polymer and polymer solution. For lower operational cost, alcohol/salt solution system is favoured due to the fact that alcohol is easier to be removed by evaporation. In LBF system, gas stream is introduced directly into the equipment. According to SS mass transfer mode, the gas stream acting as a carrier medium allows the surface active components (identified as hydrophobic in nature from the crude feed) to attach themselves on the air bubbles' surface in the salt solution. Thus, bubbles carrying the surface active components travel to the top phase containing alcohol, which is hydrophilic in nature (Phong et al., 2017). The ascending gas bubbles creates the flotation effect from aqueous phase to organic solvent phase. Gas stream is normally made of inert gas to prevent the unnecessary chemical reaction. Then, the accumulated surface-active material at the top phase will be recovered by evaporation method (Phong et al., 2017; Sankaran et al., 2018; Show et al., 2013).

LBF system is prepared by mixing the crude extract with aqueous phase, and then the solution in the bottom phase is transferred into the flotation column. Subsequently, a layer of aqueous polymer or organic solvent is added into the column as the top phase. The gases are bubbled into the bottom phase containing the aqueous salt solution and crude feedstock via a sintered glass disk located at the bottom of flotation cell. In particular, G4 porosity (5–10 µm pore size) of sintered glass disk is used in the LBF system as it is able to promote separation effectiveness (due to even bubbles size enter the column) in contrast to the bigger glass disk, i.e. G3 porosity with a pore size of 20–30 µm or the smaller sintered disk, i.e. G5 with pore size of 1–4 µm (Show et

al., 2013; Show et al., 2011). Finally, the bubbles rise to the top phase and mass transfer process takes place (SS mechanism). The efficiency of LBF system is dependent on a few variables, such as the type, concentration, volume, molecular weight of phase-forming components, size of gas bubbles, gas flow rate, flotation time, crude concentration and pH.

CHAPTER 3: Application of Liquid Biphasic Flotation System for Betacyanins Extraction from Peel and Flesh of *Hylocereus polyrhizus* and Antioxidant Activity Evaluation



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3.1 Abstract

This study investigated the use of liquid biphasic flotation (LBF) system which composed of alcohol and salt for betacyanins extraction from peel and flesh of red-purple pitaya (*Hylocereus polyrhizus*). Betacyanins have received a great deal of research attention owing to their promising antioxidant activity. The operating parameters of LBF, including types/concentrations/volumes of alcohol and salt, addition of sodium chloride, flotation time and type/concentration of crude extract, were adopted for the betacyanins extraction. Overall, the highest values of betacyanins concentration in alcohol-rich top phase (C_t), separation efficiency (E) and partition coefficient (K) of betacyanins that reveal a maximum betacyanins extraction from the peel (95.989%, 88.361% and 24.168, respectively) and flesh (95.488%, 94.886% and 21.195, respectively) were successfully achieved with the use of LBF system. In addition, both the peel and flesh extract of red-purple pitaya provided different hues and intensities of red colour, and their antioxidant properties were well retained.

Keywords: alcohol; betacyanins; *Hylocereus polyrhizus*; liquid biphasic flotation; salt

3.2 Introduction

Pitaya (*Hylocereus* sp.), or more commonly known as dragon fruit, is a type of underutilised fruits that is high in nutritional composition and antioxidant activity. It belongs to the family of Cactaceae. Red-purple pitaya (*Hylocereus polyrhizus*) is a red-skinned fruit with red-purple flesh and black seed, and it is rich in red-violet pigments known as betacyanins (Dembitsky et al., 2011). Betacyanins are phytochemicals that offer health-promoting effects, and they claimed to be effective in protect against degenerative diseases, such as cancers, cardiovascular diseases, heart diseases, diabetes and obesity (Leong et al., 2018c). Additionally, betacyanins demonstrated antioxidant properties like anti-inflammatory (Tenore et al., 2012), anti-radical scavenging (Clifford et al., 2015) and inhibitory effects on lipid peroxidation (Pavokovic and Krsnik-Rasol, 2011). They are an important subgroup of betalains (Aberoumand, 2011). The most common structure of betacyanins is betanin. Betalains are one of the natural colourants that have gained immense attention from the public. Recently, consumers are conscious of the possible toxicological aspects associated with the artificial colourants, and thus they incline towards the utilisation of natural ingredients in food processing (Martins et al., 2016). In addition, betalains have strong antioxidant activity and they are stable within a pH ranging from 3 to 7 (Esatbeyoglu et al., 2015; Moreno et al., 2008).

Betacyanins extraction from different sources with solvent extraction method, such as pitaya (Ramli et al., 2014; Stintzing et al., 2002), *Amaranth* (Chong et al., 2014), *Opuntia* (Castellar et al., 2006) and beetroot (de Azeredo et al., 2009) were reported. Conventional solvent extraction technique is commonly used in the extraction of phytochemicals, such as betacyanins, due to its simple procedure. However, it is an

expensive, time-consuming and not environmental friendly method which required large amount of solvent consumption (Azmir et al., 2013; Dai and Mumper, 2010; Zhang et al., 2013). Other alternative extraction method was proposed, for example, aqueous two-phase extraction (ATPE), which has been successfully applied for the separation and purification of betalains from beetroot (Chandrasekhar et al., 2015; Chethana et al., 2007). At present, development of a green food processing technique in food industry is deemed critically important (Chemat et al., 2017). Liquid biphasic flotation (LBF) system is a bubbles-assisted bioseparation technique conducted in a biphasic system. It is a new technology that integrates the principles of ATPE and solvent sublation (SS). ATPE is a liquid-liquid fractionation technique widely utilised for the purification of natural and biotechnological products, such as enzymes and protein (Yau et al., 2015). On the other hand, SS is based on the principle of adsorptive bubbles separation, in which the gas bubbles with substances adsorbed on their surface float up from one liquid phase to the other liquid phase (Sebba, 1959). Both ATPE and SS separation methods are proven to be technically simple, scalable and energy efficient. Hence, LBF inherits the advantages of ATPE and SS methods, for instance, high separation efficiency, high concentration coefficient, ease of operation and environmental friendliness (Bi et al., 2013; Mathiazakan et al., 2016; Phong et al., 2017; Sankaran et al., 2018; Show et al., 2013). In addition, LBF which developed by our research group, has successfully purify proteins from microalgae (Phong et al., 2017) and *Burkholderia cepacia* (Sankaran et al., 2018).

Therefore, the objective of this study was to investigate the betacyanins extraction from peel and flesh of red-purple pitaya with the utilisation of LBF system composed of alcohol and salt. Up-to-date, there are no reported studies exploring the applicability of LBF to the betacyanins extraction. The operating parameters of LBF, such as

types/concentrations/volumes of alcohol and salt, addition of sodium chloride, flotation time and type/concentration of crude extract, were adopted to investigate the betacyanins extraction efficiency from red-purple pitaya. Lastly, colour characterisation and antioxidant properties analysis were evaluated on the peel and flesh extract of red-purple pitaya obtained using the optimised LBF.

3.3 Materials and methods

3.3.1 Materials

Ethanol, methanol, 2-propanol, dipotassium hydrogen phosphate (K_2HPO_4), ammonium sulphate ($(NH_4)_2SO_4$), magnesium sulphate ($MgSO_4$), sodium carbonate (Na_2CO_3), sodium bicarbonate ($NaHCO_3$), tri-sodium citrate ($Na_3C_6H_5O_7$), sodium chloride (NaCl), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were purchased from R&M Chemicals (Selangor, Malaysia). 1-propanol, acetic acid (CH_3COOH) and sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$) were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was purchased from Fisher Scientific (Selangor, Malaysia). 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), Folin-Ciocalteu (F-C) reagent and gallic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA). All the above mentioned chemicals were of analytical grade (purity $> 95\%$). Ultrapure water produced from Milli-Q integral water purification system (Merck, Darmstadt, Germany) was used throughout this study. Red-purple pitaya was purchased from a local fruit stall at Semenyih, Selangor, Malaysia.

3.3.2 Apparatus

The apparatus used for LBF was a glass filter funnel (length: 20 cm, diameter: 2 cm) with a sintered glass disk (grade 4 (G4) porosity), and a bottom joint connected to the source of compressed air. Air bubbles were generated when the gas passed through the sintered glass disk (as shown in **Figure 3-1**).

3.3.3 Processing of crude extract

The red-purple pitaya was first cleaned under running tap water. After drying the fruit with tissue towel, its peel was removed using a knife. The peel and flesh of red-purple pitaya were then cut into small cubes before being blended into paste-like using a blender (Tefal Blendforce, Triple'Ax Technology 400 Watt, Malaysia). To prepare dried crude extract (DE), the fresh crude extract (FE) was freeze dried at -30 °C and 0.37 atm for 48 h using a freeze dryer (CHRIST Alpha 1-2 LDplus, Germany). Subsequently, the freeze-dried crude extract was ground into powder using a grinder (Tefal Blendforce, Triple'Ax Technology 400 Watt, Malaysia). The processing of the crude extract was carried out in a dim condition to minimise the loss of its pigment. Both FE and DE were then stored at -20 °C until further use.

3.3.4 Betacyanins extraction with LBF system

LBF composed of alcohol and salt was adopted for the extraction of betacyanins from peel and flesh of red-purple pitaya. The operating parameters of LBF, including types/concentrations/volumes of alcohol and salt, addition of NaCl, flotation time and type/concentration of crude extract, were used in the optimisation study for the betacyanins extraction (**Table 3-1**). The effects of the LBF parameters on the betacyanins extraction from aqueous phase (salt-rich bottom phase) to alcohol-rich top

phase in LBF process were studied using one-factor-at-a-time (OFAT) approach. For initial setting, 1 g of FE (peel or flesh of red-purple pitaya) was mixed with 20 mL of 250 g/L (w/w) salt solution. Then, 10 mL of 100% (w/w) alcohol (i.e. undiluted alcohol) was added slowly. The flotation time and air flow rate were set at 15 min and 20–30 cc/min, respectively. To avoid disturbance to the equilibrium of two-phase system, the air flow rate was kept constant throughout the experiment. The experiment was conducted at room temperature (25 ± 1 °C). **Figure 3-1** illustrates the schematic diagram of betacyanins extraction from red-purple pitaya using alcohol/salt-based LBF.

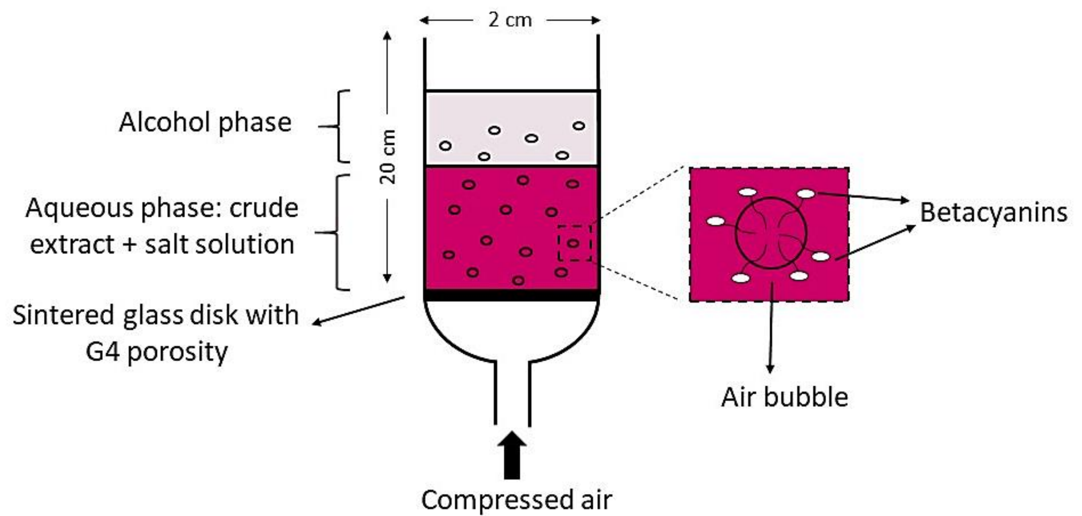


Figure 3-1: Schematic view of alcohol/salt-based LBF for betacyanins extraction from peel and flesh of red-purple pitaya. Flotation effect (gas bubbles) assisted in the upflow of betacyanins from aqueous bottom phase to alcohol-rich top phase.

Table 3-1: The operating parameters for alcohol/salt-based LBF.

No.	Operating parameter	Initial setting	Variables	Unit	Justification
1.	Type of alcohol	Ethanol	Methanol, 1-propanol, 2-propanol	N/A	First parameter; in order to optimise for concentration and volume of alcohol
2.	Type of salt solution	K_2HPO_4	$(NH_4)_2SO_4$, $MgSO_4$, Na_2CO_3, $Na_3C_6H_5O_7$	N/A	First parameter; in order to optimise for concentration and volume of salt solution
3.	Concentration of alcohol	100	50, 60, 70, 80, 90	% (w/w)	Undiluted alcohol (assume as 100%)
4.	Concentration of salt solution	250	150, 200, 300, 350	g/L (w/w)	(Mathiazakan et al., 2016)
5.	Volume of alcohol	10	2.5, 5, 15, 20	mL	
6.	Volume of salt solution	20	10, 15, 25, 30	mL	
7.	Addition of NaCl solution	3 mL of varying molarities	0.2, 0.4, 0.6, 0.8, 1.0	Molarity	10% of the total working volume in LBF
8.	Flotation time	15	5, 10, 20, 25	min	
9.	Concentration of FE	1	0.5, 2, 3	g	
10.	Type of crude extract	N/A	Fresh crude extract (FE), dried crude extract (DE)	g	

N/A: Not applicable

3.3.5 Analytical procedures

3.3.5.1 Colour characterisation

The colour parameters of L^*, a^* and b^* of the peel and flesh extract of red-purple pitaya were measured using a colorimeter (Lovibond LC 100, model RM 200, The Tintometer Ltd, United Kingdom). L^*, a^* and b^* are lightness, redness and yellowness, respectively. The hue angle (h°) and chroma (C^*) were calculated according to equations (3-1) and (3-2), respectively.

$$h^\circ = \tan^{-1}\frac{b^*}{a^*} \qquad (3\text{-}1)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad (3\text{-}2)$$

3.3.5.2 Determination of total betacyanins content (TBC)

The TBC in the crude extract was analysed using a UV-vis spectrophotometer (UV-1800, Shimadzu, Japan) at 538 nm. The TBC was expressed as mg of betanin equivalents (BEs) per 100 g of crude extract, and was calculated according to equation (3-3) (Ramli et al., 2014):

$$TBC = \frac{A_{538} \times MW \times V \times DF}{\varepsilon \times L \times W} \times 100 \qquad (3\text{-}3)$$

Where A_{538} = absorbance value at 538 nm, MW = molecular weight of betanin (550 $g.mol^{-1}$), V = volume of sample (mL), DF = dilution factor, ε = molar extinction coefficient of betanin (65000 $L.mol^{-1}.cm^{-1}$), L = path length of cuvette (1 cm), W = weight of crude extract (g)

3.3.5.3 Determination of total phenolic content (TPC)

The TPC was analysed based on Folin-Ciocalteu (F-C) method as described in literatures (Hajimahmoodi et al., 2013; Singleton et al., 1999). A diluted F-C reagent was prepared by mixing 10 mL of F-C reagent with 90 mL of purified water. 100 μL of sample or gallic acid solution (calibration curve) was mixed with 500 μL of diluted F-C reagent in a test tube. The mixture was then incubated for 5 min at room temperature under dark condition. After that, 2 mL of $NaHCO_3$ solution (60 g/L) was added to the mixture. The mixture was shake gently, and was then kept under dark condition for 90 min at room temperature. The absorbance value of the mixture was measured at 725 nm using a UV-vis spectrophotometer. The TPC was expressed as mg of gallic acid equivalents (GAEs) per 100 g of crude extract.

3.3.5.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to method as described in literatures (Benzie and Strain, 1996; Fu et al., 2011). Acetate buffer (300 mmol/L, pH 3.6) was first prepared by mixing 3.1 g of $C_2H_3NaO_2 \cdot 3H_2O$ with 16 mL of acetic acid per litre of purified water. FRAP reagent was prepared freshly by mixing 10 mL of 10 mmol/L TPTZ solution (0.0031 g of TPTZ in 1 mL of 40 mmol/L HCl) and 10 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution (0.0054 g/mL) for every 100 mL of 300 mmol/L acetate buffer. The FRAP reagent was preheated to 37 °C before use. The FRAP assay was conducted by mixing 100 μL of sample or $FeSO_4 \cdot 7H_2O$ solution (calibration curve), 300 μL of purified water, and 3 mL of FRAP reagent in a test tube. Subsequently, the mixture was incubated for 4 min at 37 °C. The absorbance value of mixture was measured at 593 nm using a UV-vis spectrophotometer. The result was expressed as μmol of Fe(II) per g of crude extract.

3.3.5.5 Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay was carried out based on ABTS radical (ABTS•) method as described in literatures (Fu et al., 2011; Re et al., 1999). ABTS• stock solution was first prepared by mixing 7 mmol/L of ABTS solution and 2.45 mmol/L of $K_2O_8S_2$ solution at v:v ratio of 1:1, and was then incubated at room temperature under dark condition for 12–16 h. Next, the ABTS• stock solution was diluted with ethanol to reach an absorbance value of 0.70 ± 0.05 at 734 nm, and was incubated at 30 °C. To conduct the TEAC assay, 100 µL of sample or trolox solution (calibration curve) or ethanol (control) was mixed with 3.8 mL of diluted ABTS• solution. After 6 min of incubation at 30 °C, the absorbance value of the mixture was measured at 734 nm using a UV-vis spectrophotometer. The result was expressed as µmol of trolox equivalents (TEs) per g of crude extract. The percentage of scavenging on ABTS• was calculated using equation (3-4) (Olajuyigbe and Afolayan, 2011):

$$\text{Percentage of scavenging (\%)} = \frac{\text{control} - \text{sample or standard}}{\text{control}} \times 100 \qquad (3\text{-}4)$$

3.3.6 Calculations

Partition coefficient (K) of betacyanins in alcohol/salt-based LBF was calculated according to equation (3-5) (Chandrasekhar et al., 2015):

$$\text{K} = \frac{\text{TBC}_t}{\text{TBC}_b} \qquad (3\text{-}5)$$

Where TBC_t and TBC_b are TBC in the alcohol-rich top phase and salt-rich bottom phase at equilibrium, respectively.

Betacyanins concentrations (%) in alcohol-rich top phase (C_t) and salt-rich bottom phase (C_b) were calculated according to equations (3-6) and (3-7), respectively:

$$C_t(\%) = \frac{\text{TBC in top phase}}{\text{TBC in crude extract}} \times 100 = \frac{TBC_t}{TBC_t + TBC_b} \times 100 \quad (3\text{-}6)$$

$$C_b(\%) = \frac{\text{TBC in bottom phase}}{\text{TBC in crude extract}} \times 100 = \frac{TBC_b}{TBC_t + TBC_b} \times 100 \quad (3\text{-}7)$$

The betacyanins extraction from salt-rich bottom phase to alcohol-rich top phase was evaluated using separation efficiency (E). E (%) of betacyanins in alcohol/salt-based LBF was calculated according to equation (3-8):

$$E(\%) = \left(1 - \frac{TBC_b}{TBC_{bi}}\right) \times 100 \quad (3\text{-}8)$$

Where TBC_{bi} and TBC_b are TBC in salt-rich bottom phase collected before and after the LBF process, respectively.

3.3.7 Statistical analysis

Triplicate experimental readings were recorded and were used in the statistical analysis, and the values were expressed as mean ± standard deviation (SD) (n = 3). The experiment was repeated three times in order to further verify the results. The data analysis was performed by IBM SPSS statistics program (SPSS version 23.0 for window, IBM Corporation, Armonk, New York, United States). The data were subjected to one-way ANOVA, and the mean differences were compared using Tukey HSD post-hoc multiple comparisons test. The data were considered statistically significant difference where $p < 0.05$. The relationships among the antioxidant properties assays were analysed using Pearson's correlation test.

3.4 Results and discussion

3.4.1 Effects of the types of alcohol and salt

The LBF made of different combinations between alcohols (ethanol, methanol, 1-propanol and 2-propanol) and salts [K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4$, Na_2CO_3 and $Na_3C_6H_5O_7$] were used to study the betacyanins extraction efficiency from peel and flesh of red-purple pitaya (**Table 3-2**). In this experiment, the salt solution was first used to extract betacyanins from the red-purple pitaya. Flotation effect (gas bubbles) assisted in the upflow of betacyanins from aqueous phase (salt-rich bottom phase) to alcohol-rich top phase. The betacyanins adsorbed on the surfaces of the gas bubbles were carried up to the alcohol-rich top phase as the bubbles rose through the column. Alcohol is an effective hydrophilic organic solvent in the extraction of plant polyphenols (Dai and Mumper, 2010), and therefore it acts as a further extractive solvent for betacyanins in the LBF.

From our observations, methanol was unable to form a two-phase system with all the tested salts. Phong et al. (2017) also reported that no two-phase formation was observed in methanol/salt-based LBF for protein recovery from wet microalgae. This is due to the high evaporation rate of methanol and the presence low salt concentration (Phong et al., 2017). High evaporation rate of methanol prompted a rapid loss of the solvent from the top phase, and thereby restricted the betacyanins extraction in the alcohol-rich top phase. Additionally, a low salt concentration influenced the system equilibrium, and thus reduced the tendency of the formation of a biphasic system. Although the air flow was low and the presence of high volumetric ratios, the natural movement of the flotation process (gas bubbles) also causes a mixture between the two phases and disturbed the phase equilibrium.

Previous studies reported that ethanol was difficult to form a two-phase system with salt (Mathiazakan et al., 2016; Ooi et al., 2009; Phong et al., 2017). However, our observations showed a two-phase formation between ethanol and K_2HPO_4, and most probably could be due to the presence of salting-out effect between the undiluted ethanol and 250 g/L of K_2HPO_4 solution (Grundl et al., 2017). Salting-out is an effect based on the electrolyte-non electrolyte interaction, in which the non-electrolyte could be less soluble at high salt concentrations (Grundl et al., 2017). In this case, the high concentration of K_2HPO_4 provided a higher tendency to form a biphasic system with ethanol, besides might be assisted by their hydrophobic and hydrophilic interaction. Moreover, oxidation of betacyanins was observed when the crude extract (peel and flesh of red-purple pitaya) was added to Na_2CO_3 solution with pH 11. The oxidation of betacyanins was indicated by the change of colour of aqueous phase from red-purple to yellow-brown. Betacyanins are sensitive pigments that can be easily oxidised in high alkalinity environment, and it is only stable within a pH ranging from 3 to 7. Oxidation causes betacyanins to lose their nature properties, such as colour and antioxidant properties (Esatbeyoglu et al., 2015; Khan and Giridhar, 2015). In this study, the total betacyanins content was calculated based on betanin equivalents, and according to Wybraniec and Michałowski (2011), oxidation of betanin caused the formation of monodecarboxylated and monodehydrogenated betanin, and therefore, we suggest that the colour changed was due to the presence of these compounds.

The LBF with peel of red-purple pitaya which composed of 1-propanol + $Na_3C_6H_5O_7$, 1-propanol + K_2HPO_4 and 2-propanol + all the tested salts showed a negative separation efficiency (E) (**Table 3-2a**). Likewise, the LBF with flesh of red-purple pitaya composed of 1-propanol + all the tested salts except $MgSO_4$, 2-propanol + $Na_3C_6H_5O_7$ and 2-propanol + $(NH_4)_2SO_4$ showed a similar result (negative E) (**Table**

3-2b). The negative value of separation efficiency showed that there was an improved betacyanins extraction after the LBF process, but the extracted betacyanins were mostly remained in the aqueous bottom phase. The flotation effect did not rose much betacyanins from the aqueous bottom phase to the alcohol-rich top phase. Overall, LBF composed of ethanol and K_2HPO_4 showed the highest betacyanins concentration in alcohol-rich top phase (C_t), E, and partition coefficient (K) of betacyanins from the peel (79.532 ± 0.406%, 65.361 ± 0.307% and 3.887 ± 0.097, respectively) and flesh (71.160 ± 0.256%, 73.797 ± 0.734% and 2.468 ± 0.031, respectively). Hence, ethanol/K_2HPO_4-based LBF was used for further studies.

Table 3-2: LBF made of different combinations between alcohols and salts adopted for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. The system composed of 1 g of FE, 20 mL of 250 g/L salt solution, 10 mL of undiluted alcohol, 15 min of flotation time and 20–30 cc/min of air flow rate.

(a)

Type of salt solution	Type of alcohol	Two-phase formation in LBF	Betacyanins concentration (%)		Separation efficiency (E) (%)	Partition coefficient (K)
			Top phase (C_t)	Bottom phase (C_b)		
$Na_3C_6H_5O_7$	Ethanol	No				
	Methanol	No				
	1-Propanol	Yes	2.816 ± 1.932[e,f]	97.184 ± 1.932[b,c]	-111.020 ± 11.031[c,d]	0.029 ± 0.021[c,d]
	2-Propanol	Yes	9.954 ± 1.204[c]	90.046 ± 1.204[e]	-154.861 ± 43.165[d]	0.111 ± 0.015[b,c]
$(NH_4)_2SO_4$	Ethanol	No				
	Methanol	No				
	1-Propanol	Yes	0[g]	100[a]	28.217 ± 2.794[a]	0[d]
	2-Propanol	Yes	2.130 ± 0.507[f,g]	97.87 ± 0.507[a,b]	-118.348 ± 3.039[c,d]	0.022 ± 0.005[c,d]
K_2HPO_4	**Ethanol**	**Yes**	**79.532 ± 0.406[a]**	**20.468 ± 0.406[g]**	**65.361 ± 0.307[a]**	**3.887 ± 0.097[a]**
	Methanol	No				
	1-Propanol	Yes	4.604 ± 0.264[d,e]	95.396 ± 0.264[c,d]	-92.233 ± 3.526[b,c]	0.048 ± 0.003[c,d]
	2-Propanol	Yes	13.668 ± 0.627[b]	86.332 ± 0.627[f]	-57.257 ± 12.714[b]	0.158 ± 0.008[b]
$MgSO_4$	Ethanol	No				
	Methanol	No				
	1-Propanol	Yes	0[g]	100[a]	36.227 ± 6.861[a]	0[d]
	2-Propanol	Yes	5.317 ± 0.702[d]	94.683 ± 0.702[d]	-53.246 ± 7.371[b]	0.056 ± 0.008[c,d]
Na_2CO_3	Ethanol					
	Methanol		Oxidation of betacyanins occurred before the LBF process.			
	1-Propanol					
	2-Propanol					

Values are mean ± SD of triplicate readings.

Values in a column followed by different letter(s) are significantly different ($p < 0.05$) using Tukey's test.

Table 3-2 continued, (b)

Type of salt solution	Type of alcohol	Two-phase formation in LBF	Betacyanins concentration (%)		Separation efficiency (E) (%)	Partition coefficient (K)
			Top phase (C_t)	Bottom phase (C_b)		
$Na_3C_6H_5O_7$	Ethanol	No				
	Methanol	No				
	1-Propanol	Yes	2.247 ± 0.331[d,e]	97.753 ± 0.331[b,c]	-30.206 ± 0.567[f]	0.023 ± 0.003[d,e]
	2-Propanol	Yes	5.094 ± 0.241[c]	94.906 ± 0.241[d]	-8.099 ± 1.690[e]	0.054 ± 0.003[c,d]
$(NH_4)_2SO_4$	Ethanol	No				
	Methanol	No				
	1-Propanol	Yes	1.688 ± 0.190[e]	98.312 ± 0.190[b]	-7.823 ± 1.585[e]	0.017 ± 0.002[e]
	2-Propanol	Yes	3.089 ± 0.247[d]	96.911 ± 0.247[c]	-6.291 ± 1.685[e]	0.032 ± 0.003[c,d,e]
K_2HPO_4	**Ethanol**	**Yes**	**71.160 ± 0.256[a]**	**28.840 ± 0.256[f]**	**73.797 ± 0.734[a]**	**2.468 ± 0.031[a]**
	Methanol	No				
	1-Propanol	Yes	0[f]	100[a]	-8.281 ± 1.067[e]	0[e]
	2-Propanol	Yes	5.903 ± 0.694[c]	94.097 ± 0.694[d]	33.478 ± 1.213[b]	0.063 ± 0.008[c]
$MgSO_4$	Ethanol	No				
	Methanol	No				
	1-Propanol	Yes	0[f]	100[a]	11.316 ± 3.184[d]	0[e]
	2-Propanol	Yes	24.445 ± 0.623[b]	75.555 ± 0.623[e]	25.076 ± 1.601[c]	0.324 ± 0.011[b]
Na_2CO_3	Ethanol	Oxidation of betacyanins occurred before the LBF process.				
	Methanol					
	1-Propanol					
	2-Propanol					

Values are mean ± SD of triplicate readings.
Values in a column followed by different letter(s) are significantly different ($p < 0.05$) using Tukey's test.

3.4.2 Effects of the concentrations of alcohol and salt

The LBF composed of 250 g/L of K_2HPO_4 solution with varying concentrations of ethanol ranging from 50 to 100% (w/w) were used in the study of betacyanins extraction from red-purple pitaya, as shown in **Figure 3-2**. According to our findings, 50 and 60% ethanol with 250 g/L of K_2HPO_4 solution were unable to form a two-phase system. This could be due to the lower concentration of ethanol has weaker hydrophobic interaction with the higher concentration of K_2HPO_4 solution, and thereby they showed a lower tendency to form a biphasic system (Mathiazakan et al., 2016). An increase concentration of ethanol from 70 to 100% increased the values of C_t, E and K of betacyanins from the peel and flesh of red-purple pitaya (**Figure 3-2a,b**). Collectively, LBF composed of 100% ethanol and 250 g/L of K_2HPO_4 solution showed the highest values of C_t, E and K of betacyanins from the peel (79.532 ± 0.406%, 65.361 ± 0.307% and 3.887 ± 0.097, respectively) and flesh (71.160 ± 0.256%, 73.797 ± 0.734% and 2.468 ± 0.031, respectively). This could be due to the increasing concentration of alcohol increase the extraction efficiency of polyphenols like betacyanins (Dai and Mumper, 2010).

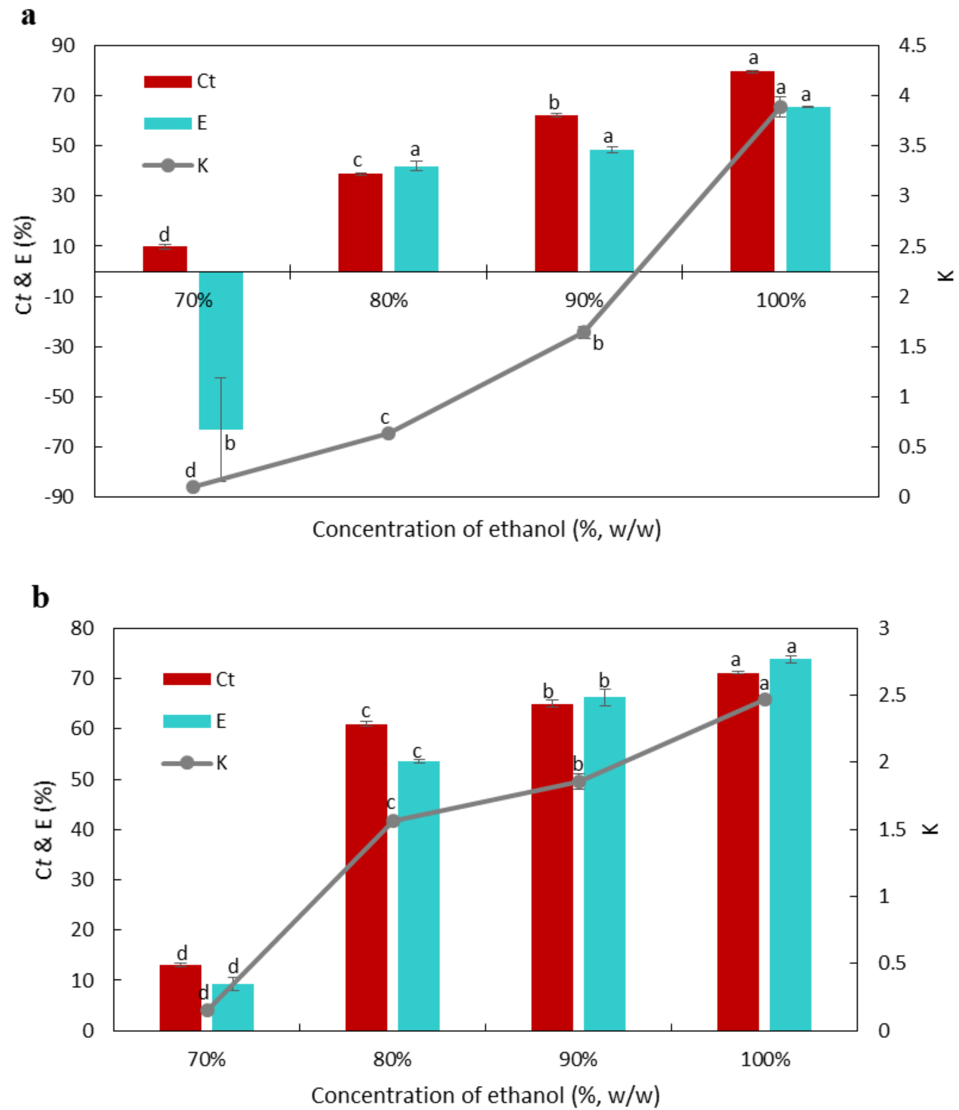


Figure 3-2: Effect of varying ethanol concentrations in LBF composed of 10 mL of ethanol, 20 mL of 250 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

Subsequently, the LBF composed of 100% ethanol with varying concentrations of K_2HPO_4 solution ranging from 150 to 350 g/L were adopted for the betacyanins extraction from red-purple pitaya (**Figure 3-3**). Based on our results, no two-phase formation was observed in LBF composed of 100% ethanol and 150 g/L of K_2HPO_4 solution with the peel, whereas the LBF composed the same constituents with the flesh showed a two-phase system. It could be explained by the characteristics of FE of peel and flesh of red-purple pitaya. They have different water content that would disturb the equilibrium of the LBF system; i.e. the FE of the peel contains less water content than that of the FE of the flesh (Dembitsky et al., 2011). From **Figure 3-3**, the highest C_t, E and K of betacyanins were noted at LBF composed of 200 g/L of K_2HPO_4 solution and 100% ethanol for the peel (95.989 ± 0.479%, 88.361 ± 1.708% and 24.168 ± 2.949, respectively) and flesh (95.488 ± 0.213%, 94.887 ± 0.060% and 21.195 ± 1.030, respectively). Beyond the K_2HPO_4 concentration at 200 g/L, the betacyanins extraction efficiency was found to be decreased. This situation might be due to higher than or lesser than the 200 g/L of K_2HPO_4 concentration reduce the solubility of betacyanins in the aqueous bottom phase, and thus decreased its extraction efficiency. Hence, this restricted the upflow of betacyanins to alcohol-rich top phase for further extraction process. Theoretically, the addition of salt concentration in the bottom phase reduces the solubility of the analyte, and consequently, increases the yield or concentration values at the top phase, partition coefficient and separation efficiency as seen in literatures (Bi et al., 2009; Lu et al., 2016; Padilha et al., 2017; Xia et al., 2016). However, too much salt concentration can damage the properties of the natural pigments, such as betacyanins, due to their highly sensitivity feature (Khan and Giridhar, 2015).

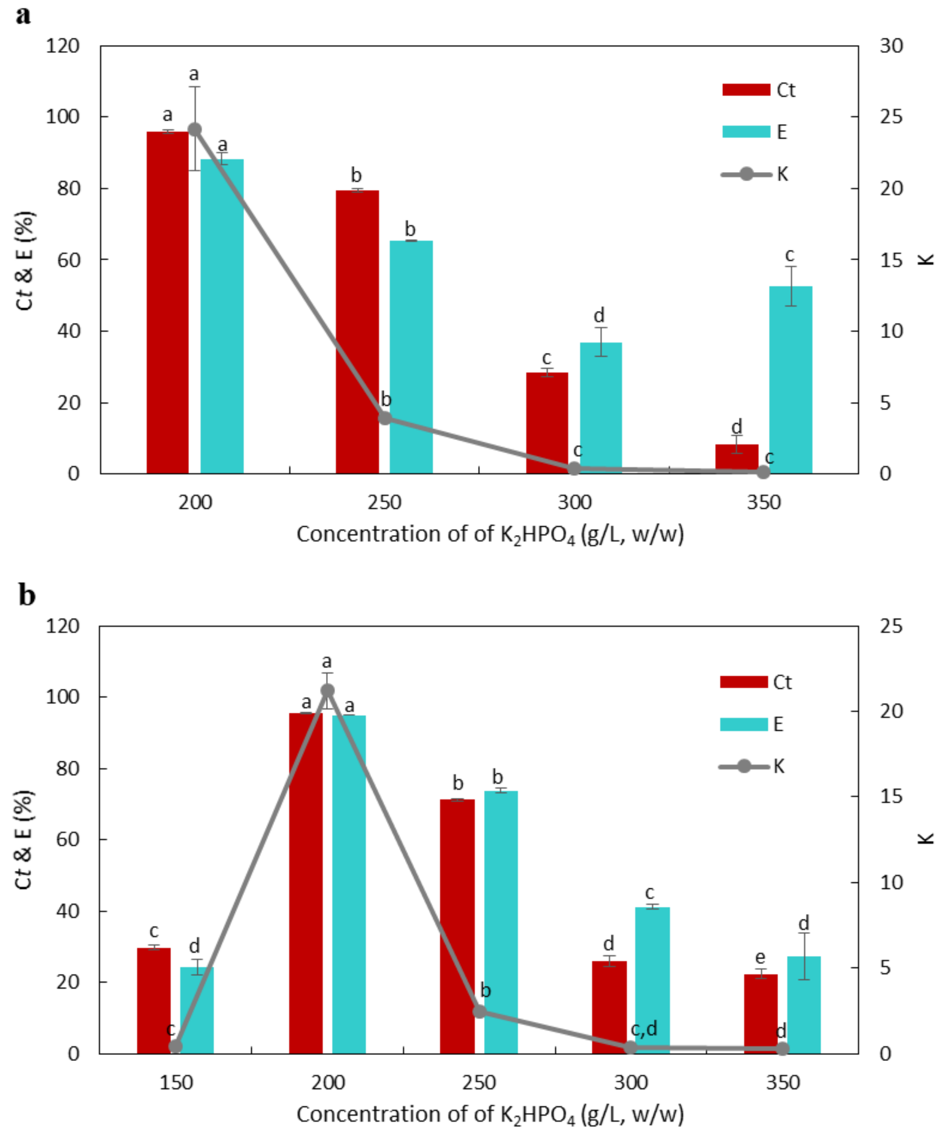


Figure 3-3: Effect of varying K_2HPO_4 concentrations in LBF composed of 10 mL of 100% ethanol, 20 mL of K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different (p < 0.05) using Tukey's test within C_t, E and K.

3.4.3 Effects of the volumes of alcohol and salt

Based on the previous results, the LBF composed of 100% ethanol and 200 g/L of K_2HPO_4 solution was used in the studies of varying volumes of alcohol (2.5 to 20 mL) and salt solution (10 to 30 mL) for the betacyanins extraction from red-purple pitaya. According to our results, the LBF composed of 2.5 and 5 mL of 100% ethanol with 20 mL of 200 g/L K_2HPO_4 solution were unable to form a biphasic system. Smaller volume of the alcohol (≤ 5 mL) showed a weaker hydrophobic interaction with the salt solution, and thus reveal in a lower tendency of biphasic system formation. Additionally, the smaller volume of the alcohol cannot covered the entire working volume of the LBF system (30 mL). This is due to its rapid evaporation rate, and thus the extracting volume of the alcohol-rich top phase for betacyanins extraction was limited. As depicted in **Figure 3-4**, an increase in the volume of ethanol from 10 to 20 mL decreased the values of C_t, E and K of betacyanins. Our results reveal that the LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution provided the highest C_t, E and K of betacyanins for the betacyanins extraction from peel (95.989 ± 0.479%, 88.361 ± 1.708% and 24.168 ± 2.949, respectively) and flesh (95.488 ± 0.213%, 94.886 ± 0.060% and 21.195 ± 1.030, respectively) of red-purple pitaya.

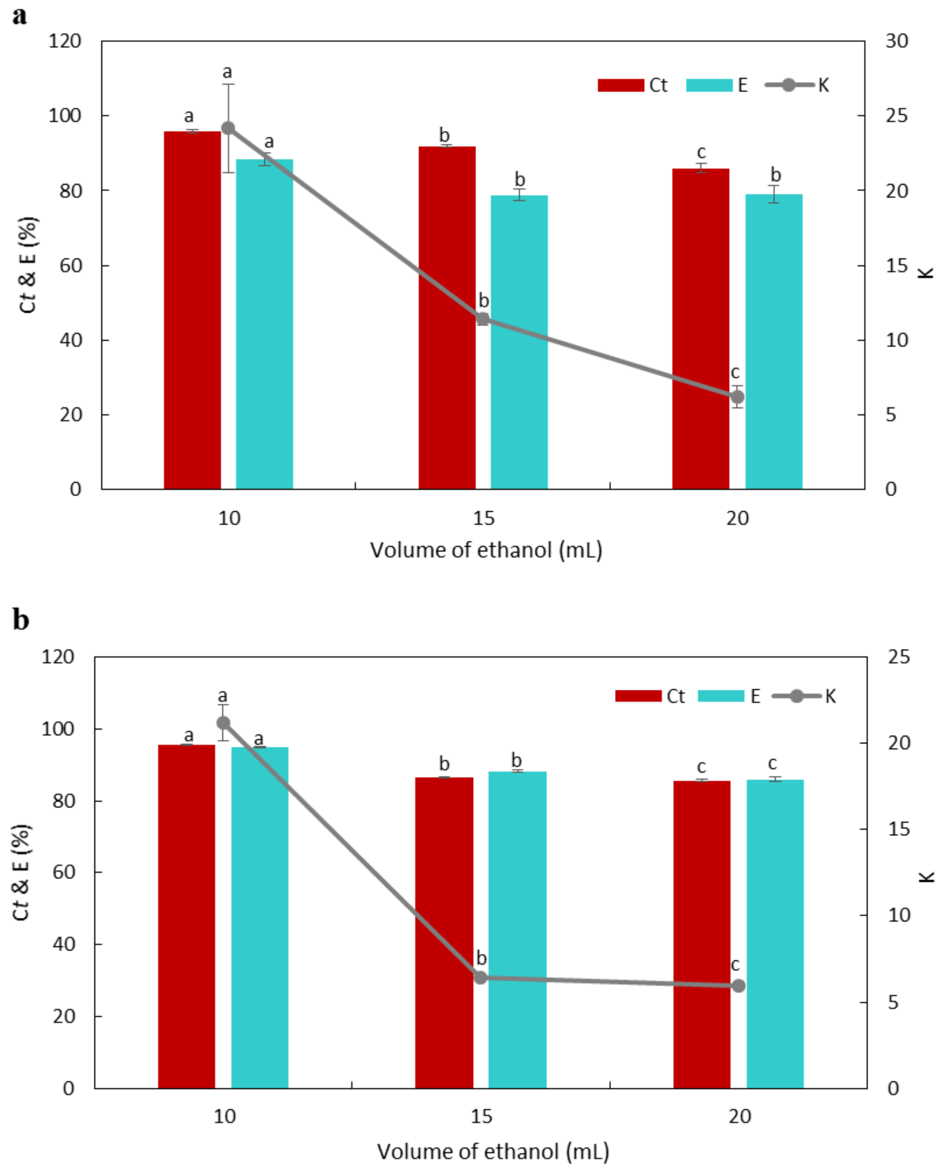


Figure 3-4: Effect of varying volumes of ethanol in LBF composed of 100% ethanol, 20 mL of 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

In the study of the betacyanins extraction using LBF with varying volumes of 200 g/L K_2HPO_4 solution, the LBF composed of 10 mL of 200 g/L K_2HPO_4 solution and 10 mL of 100% ethanol was not conducted. This is because the v:v ratio of the alcohol to salt solution is 1:1, in which its betacyanins extraction had been evaluated earlier (i.e. 20 mL of 200 g/L K_2HPO_4 solution and 20 mL of undiluted ethanol), and the result was not good and not promising as well. An increase in the volume of K_2HPO_4 solution from 10 to 15 mL prompted an increasing values of C_t, E and K of betacyanins from the peel and flesh of red-purple pitaya. A further increase in the volume of K_2HPO_4 until 30 mL decreased the betacyanins extraction. Collectively, the highest values of C_t, E and K of betacyanins for the peel (95.989 ± 0.479%, 88.361 ± 1.708% and 24.168 ± 2.949, respectively) and flesh (95.488 ± 0.213%, 94.886 ± 0.060% and 21.195 ± 1.030, respectively) with LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution infer an optimum betacyanins extraction (**Figure 3-5**).

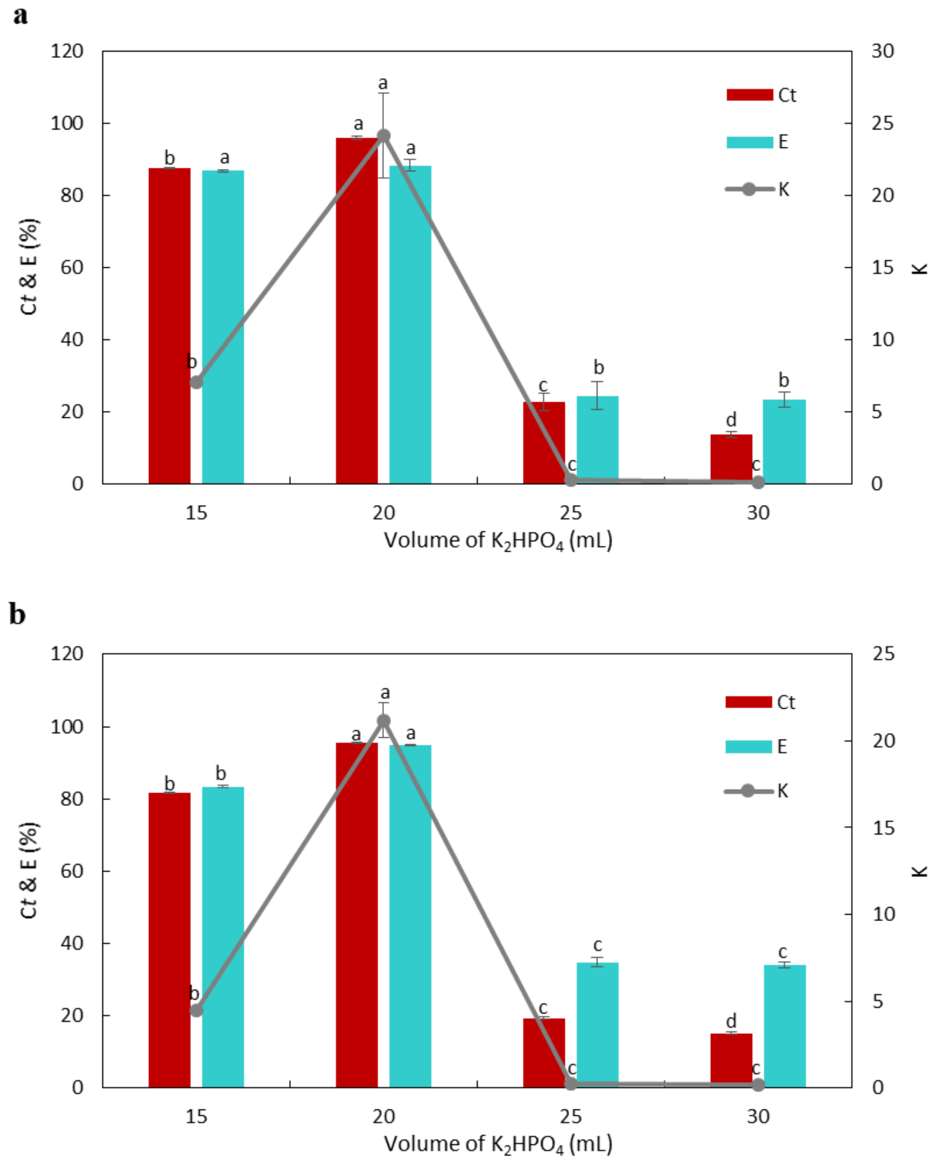
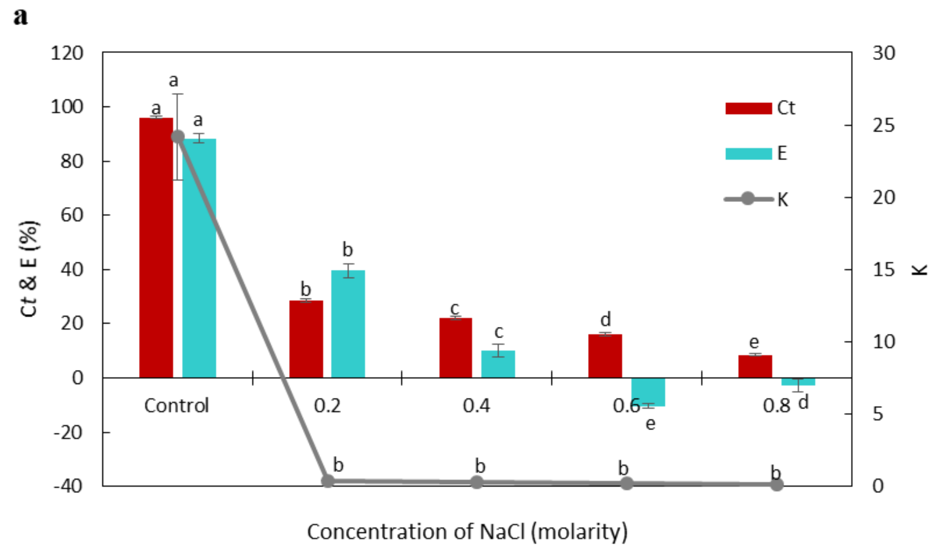


Figure 3-5: Effect of varying volumes of K_2HPO_4 in LBF composed of 10 mL of 100% ethanol, 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

3.4.4 Effect of the addition of NaCl

The optimised LBF composed of ethanol and K_2HPO_4 was used in the study of the effect of addition of NaCl for the betacyanins extraction from peel and flesh of red-purple pitaya (**Figure 3-6**). In this case, 3 mL of varying molarities of NaCl solution ranging from 0.2 to 1.0 M were tested for their betacyanins extraction efficiency. Previous studies reported that addition of NaCl could improve the separation and partition efficiency of lipase protein with alcohol/salt-based aqueous two-phase system (Ooi et al., 2009). A change in the water solvent structure and hydrophobic interaction in the biphasic system due to the addition of neutral salts, such as NaCl, caused the greater partitioning of the sample compound to another liquid phase (Albertsson, 1986). However, our findings showed no improvement on the betacyanins extraction efficiency after the addition of NaCl, yet worsen the situation. Also, addition of 1.0 M NaCl to the LBF with the peel disturbed the biphasic system formation, while this was not observed on the LBF with the flesh and 1.0 M NaCl additive. This could be due to the different characteristics of the peel and flesh of red-purple pitaya (as mentioned in the section 3.4.2). According to our findings, the LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution without the addition of NaCl (i.e. control) showed the highest C_t, E and K of betacyanins for the peel (95.989 ± 0.479%, 88.361 ± 1.708% and 24.168 ± 2.949, respectively) and flesh (95.488 ± 0.213%, 94.886 ± 0.060% and 21.195 ± 1.030, respectively). Addition of varying molarities of NaCl significantly reduced the betacyanins extraction, which indicated by the lower values of C_t, E and K of betacyanins for both peel and flesh in the optimised LBF. This could be due to the addition of NaCl influenced the system equilibrium, and thus decreased the betacyanins extraction efficiency.



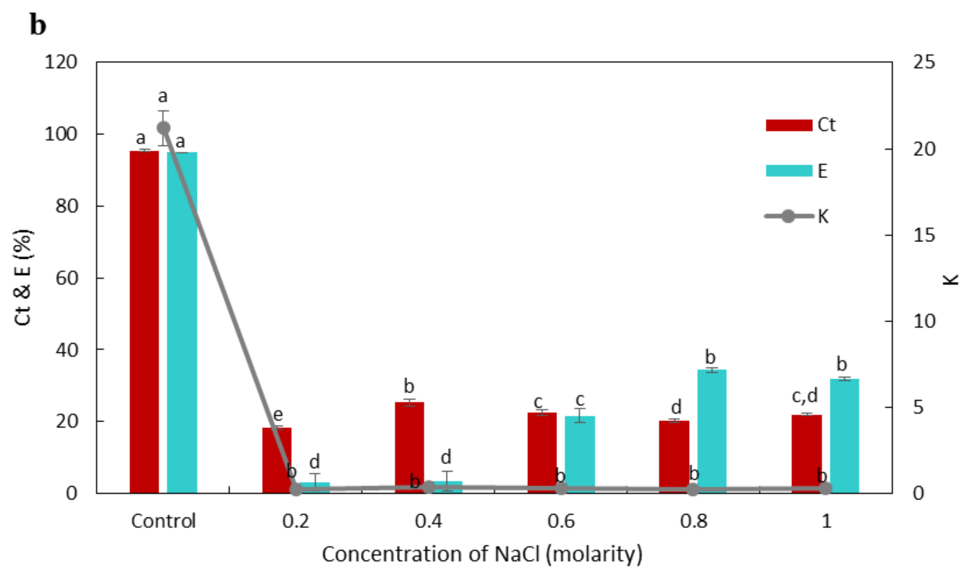


Figure 3-6: Effect of addition of NaCl in LBF composed of 10 mL of 100% ethanol, 20 mL of 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Flotation time and air flow rate are set at 15 min and 20–30 cc/min, respectively. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

3.4.5 Effect of the flotation time

The duration of flotation effect was varied from 5–25 min in the LBF consisting of 1 g of FE (peel or flesh), 20 mL of 200 g/L K_2HPO_4 solution and 10 mL of 100% ethanol to study their betacyanins extraction efficiency. The extraction efficiency of betacyanins increase as the time increased from 5 to 15 min, which then slightly decreased for the subsequent flotation time up to 25 min. Collectively, the highest C_t, E and K of betacyanins from peel (95.989 ± 0.479%, 88.361 ± 1.708% and 24.168 ± 2.949, respectively) and flesh (95.488 ± 0.213%, 94.886 ± 0.060% and 21.195 ± 1.030, respectively) of red-purple pitaya reveal a maximum betacyanins extraction with the LBF at 15 min of flotation time, as shown in **Figure 3-7**. Hence, 15 min of flotation time was chosen in LBF process, and further extending in the flotation period was deemed unnecessary.

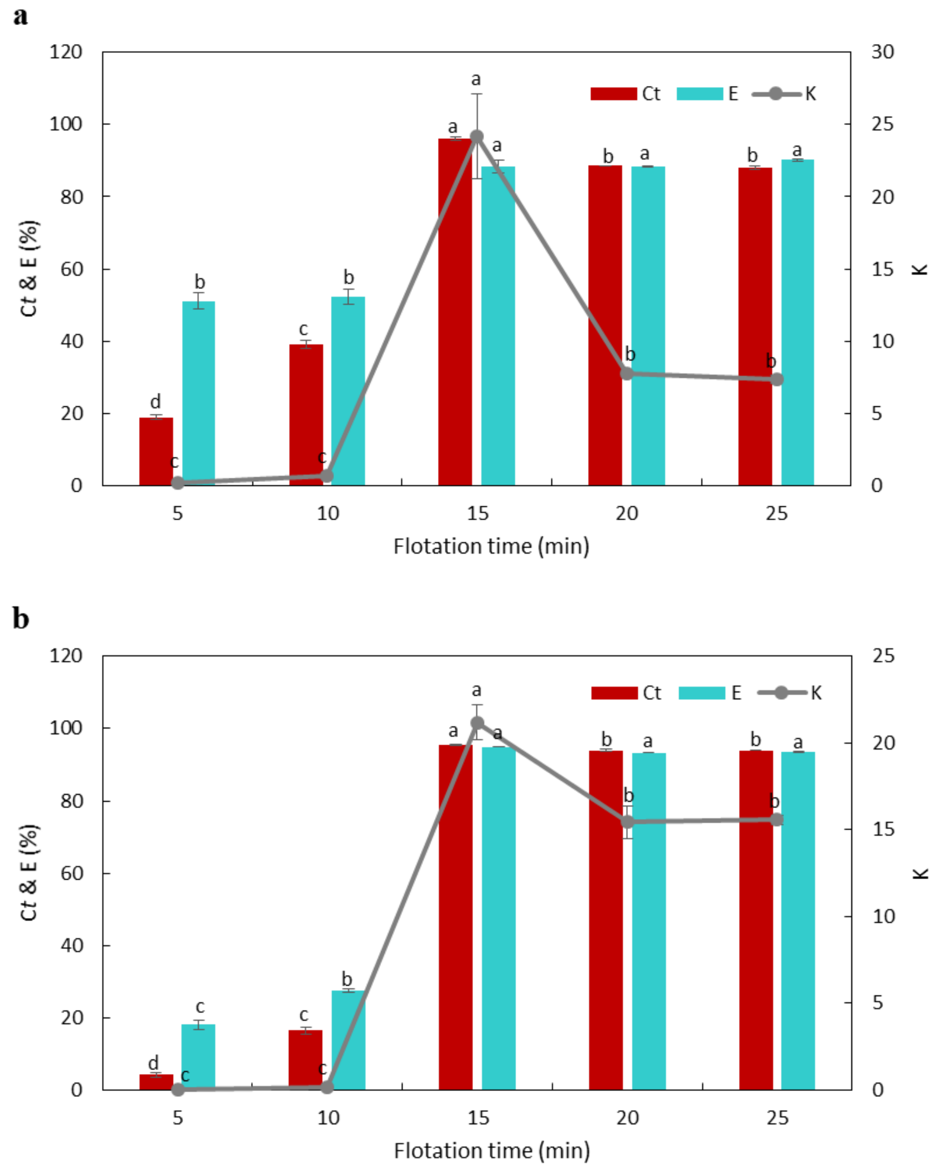


Figure 3-7: Effect of varying flotation time in LBF composed of 10 mL of 100% ethanol, 20 mL of 200 g/L K_2HPO_4 solution and 1 g of FE for betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya. Air flow rate is set at 20–30 cc/min. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

3.4.6 Effect of the crude extract concentration

The LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution with varying FE concentrations ranging from 0.5 to 3 g were evaluated for their betacyanins extraction from the peel and flesh of red-purple pitaya. In this case, 3 g of FE of red-purple pitaya disturbed the formation of a biphasic system in the LBF. This could be due to the overloaded of crude extract affect the system equilibrium, and thus infer in a lower tendency of a biphasic system formation. Previous studies reported that overloaded of crude resulted in the formation of a homogenous one-phase system (Mathiazakan et al., 2016; Phong et al., 2017). As depicted in **Figure 3-8a**, an increase in the FE concentration of the peel from 0.5 to 2 g increased the betacyanins extraction efficiency, with the highest C_t at 98.330 ± 0.094%, E at 98.364 ± 0.076% and K of betacyanins at 58.994 ± 3.405 noted at 2 g of FE. On the other hand, 0.5 g of FE of the flesh showed the highest values of C_t (97.511 ± 0.487%), E (99.414 ± 0.194%) and K of betacyanins (40.264 ± 8.417). A further increase in the FE concentration of the flesh until 2 g showed reduction in the betacyanins extraction rate (**Figure 3-8b**). From our results, the betacyanins concentration in alcohol-rich top phase (C_t) in the LBF with the peel composed of 1 and 2 g as well as in the LBF with the flesh composed of 0.5 and 1 g showed not much difference (< 2.5%). Therefore, 1 g of FE of peel and flesh were chosen to be used in the LBF, and for further studies. This could be compared easily due to the same FE concentration.

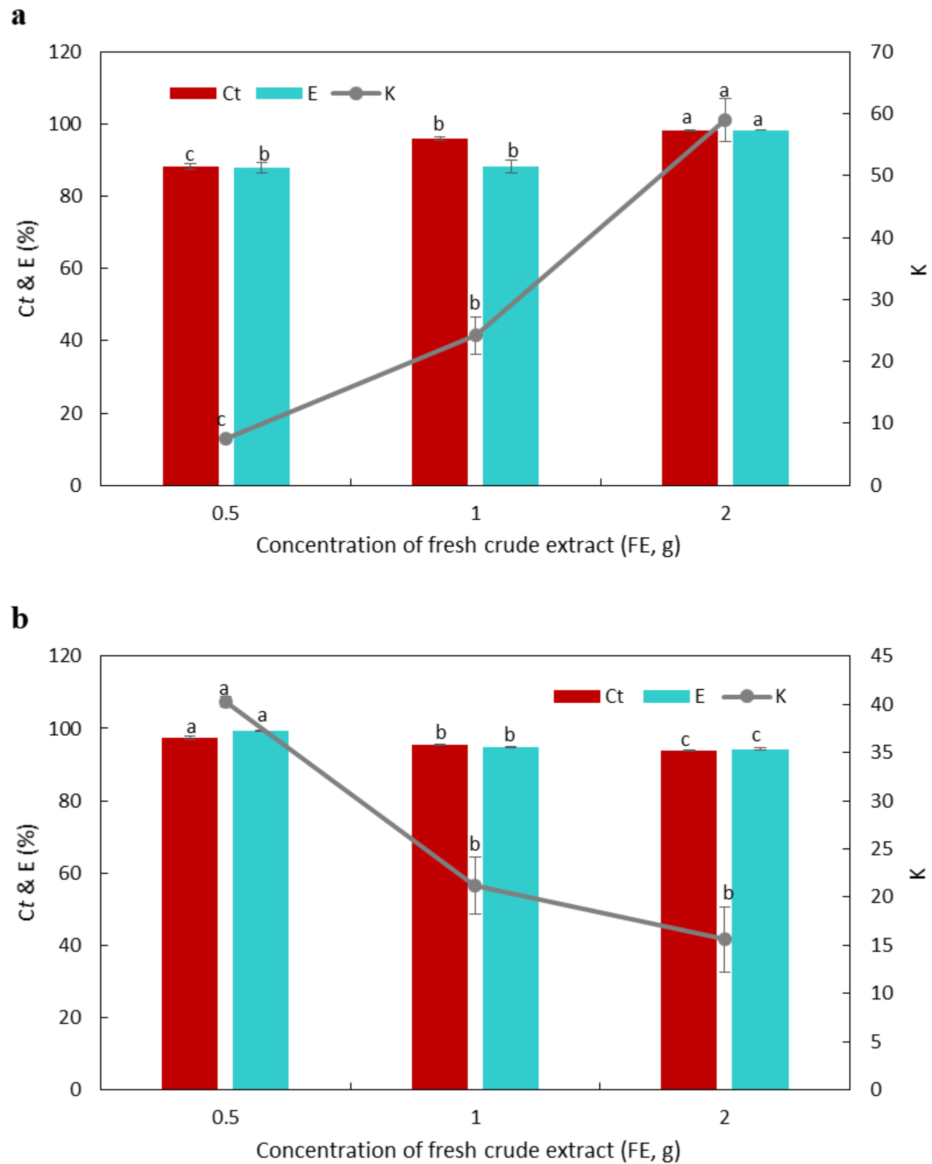


Figure 3-8: Effect of varying fresh crude extract (FE) concentrations of (a) peel and (b) flesh of red-purple pitaya in LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution for betacyanins extraction. Flotation time and air flow rate are set at 15 min and 20–30 cc/min. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

3.4.7 Effect of the type of crude extract

Based on the previous results, the optimised LBF composed of ethanol, K_2HPO_4 and 1 g of FE was used in the study of the effect of different types of crude extract for the betacyanins extraction from peel and flesh of red-purple pitaya. Dried crude extract (DE) was used to compare with the fresh crude extract (FE) of the red-purple pitaya. Our observations showed that 1 g of DE of the red-purple pitaya formed a gel-like with the aqueous bottom phase, and thereby difficult in the analysis for betacyanins content. This could be due to the presence of a higher amount of pectin in the dried crude extract. Therefore, 1 g of FE and 0.1 g of DE of both peel and flesh of red-purple pitaya was utilised for the betacyanins extraction using the optimised LBF. As depicted in **Figure 3-9**, the FE of the peel and flesh showed the highest values of C_t and K that infer a maximum betacyanins extraction. There was an approximately increment of 6% for C_t obtained from the FE of both the peel and flesh compared to that of from the DE. On the other hand, a roughly of 2.6-folds increment in the K of betacyanins from the FE of both the peel and flesh was noted compared to that of from the DE. All the E values obtained from the FE and DE of both the peel and flesh did not vary much among them, and they were in the range of 88 to 95%. Dried crude extract of both the peel and flesh of red-purple pitaya contain more pectin compared to that of the fresh crude extract. Pectin promotes a more rapid degradation of betacyanins (Chethana et al., 2007), and therefore reduced the presence of betacyanins for further extraction process. **Table 3-3** summarises all the optimised conditions in the alcohol/salt-based LBF with the peel and flesh of red-purple pitaya that achieved a maximum betacyanins extraction.

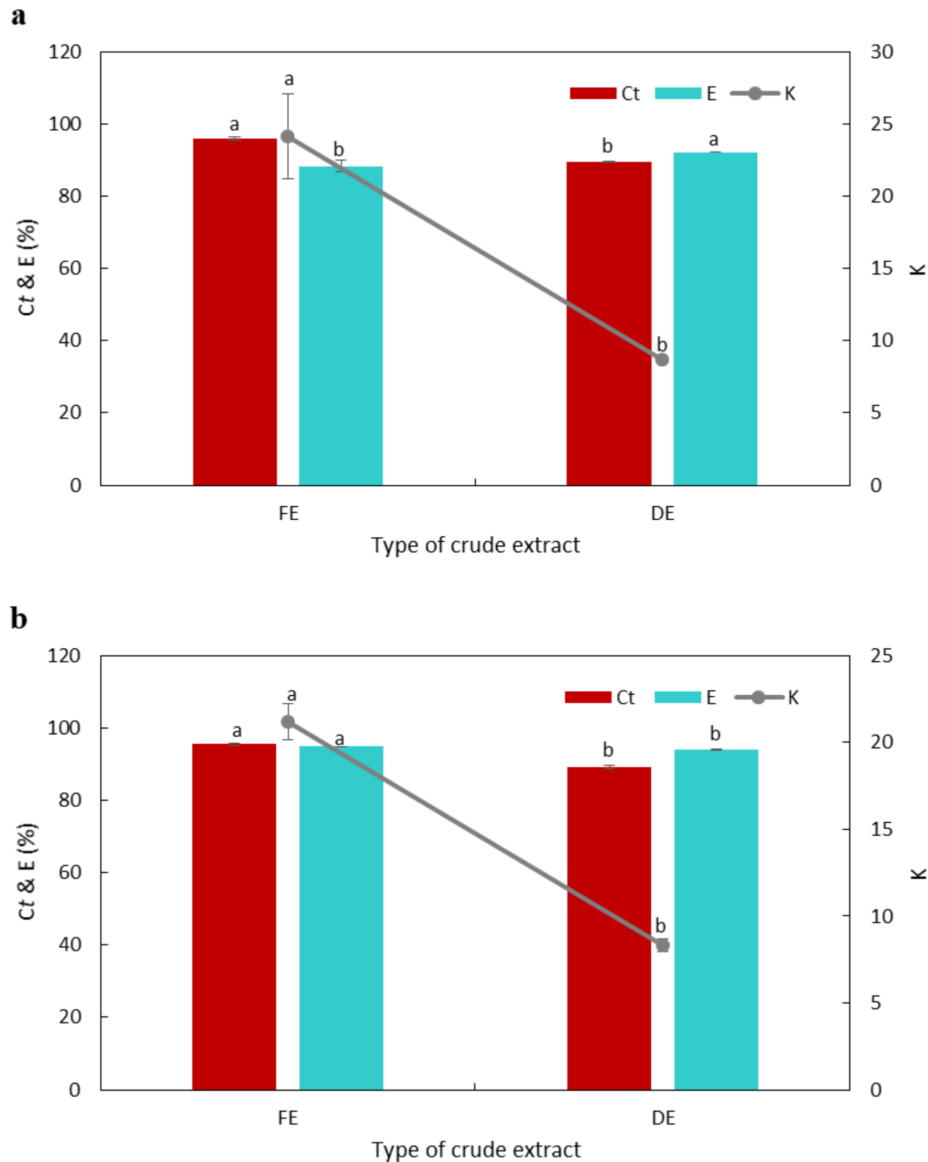


Figure 3-9: Comparison between different types of crude extract (fresh crude extract (FE) and dried crude extract (DE)) of (a) peel and (b) flesh of red-purple pitaya in LBF composed of 10 mL of 100% ethanol and 20 mL of 200 g/L K_2HPO_4 solution for betacyanins extraction. Flotation time and air flow rate are set at 15 min and 20–30 cc/min. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

Table 3-3: The optimised conditions in LBF composed of alcohol and salt for betacyanins extraction from peel and flesh of red-purple pitaya.

Crude extract	Alcohol	Salt	Flotation time (min)	Air flow rate (cc/min)	Betacyanins concentration in alcohol-rich top phase (C_t, %)	Separation efficiency (E, %)	Partition coefficient (K)
1 g of FE of peel	10 mL of 100% ethanol	20 mL of 200 g/L K_2HPO_4 solution	15	20–30 (constant throughout the experiment)	95.989 ± 0.479	88.361 ± 1.708	24.168 ± 2.949
1 g of FE of flesh					95.488 ± 0.213	94.886 ± 0.060	21.195 ± 1.030

3.4.8 Colour characterisation

The peel and flesh extract of red-purple pitaya obtained through the optimised ethanol/K_2HPO_4-based LBF (alcohol-rich top phase) were evaluated for their colour characterisation, and the results are given in **Table 3-4**. Our results reveal that both the peel and flesh extract provided a similar range of red colour, i.e. in red-bluish (positive a^* and negative b^*), but they were presented in different hues, lightness, saturations and intensities. The flesh extract showed a higher degree of redness and bluish compared to that of the peel extract. This concludes that both the peel and flesh extract could be applied as natural red colourant in different hues and intensities, and thus replace the artificial colourants.

Table 3-4: Colour characterisation for peel and flesh extract of red-purple pitaya.

Colour parameter	Peel extract	Flesh extract
Lightness (L^*)	46.400 ± 0.100	25.800 ± 0.173
Redness (a^*)	13.100 ± 0.100	33.900 ± 0.100
Yellowness (b^*)	-4.100 ± 0.000	-30.233 ± 0.231
Chroma (C^*)	13.7333 ± 0.152	45.433 ± 0.153
Hue angle (h°)	342.500 ± 0.100	318.267 ± 0.252

Values are mean ± SD of triplicate readings.

3.4.9 Antioxidant properties analysis

The peel and flesh extract of red-purple pitaya extracted with the optimised LBF (alcohol-rich top phase) were used in the antioxidant properties analysis (**Figure 3-10**). The FRAP and TEAC assay were used to evaluate their antioxidant capacity in reducing of an oxidant probe that indicated through a colour change of the mixture. The oxidant probes used in the FRAP and the TEAC were TPTZ and ABTS radical cation, respectively. According to Dai and Mumper (2010), both FRAP and TEAC are electron transfer-based assays, and well known as the standardised methodologies in evaluation of antioxidant capability. Evaluation on the antioxidant activity is crucial as it determines the effectiveness of the antioxidant effects. Based on our results, the peel and flesh extract of red-purple pitaya showed 13.286 ± 0.214 and 15.414 ± 0.391 μmol Fe(ll)/g crude extract, respectively, (calibration curve equation: A_{593} = 0.0007([$FeSO_4 \cdot 7H2O$]) + 0.124; R^2 = 0.9986) in the FRAP assay. On the other hand, the peel and flesh extract recorded 19.653 ± 0.077 and 19.862 ± 0.189 μmol TEs/g crude extract, respectively, (calibration curve equation: Percentage of scavenging (%) = 0.0966([trolox]) + 2.8333; R^2 = 0.9946) in the TEAC assay. Collectively, the peel and flesh of red-purple pitaya showed closely values of FRAP and TEAC.

The TPC and TBC assay were used to evaluate the presence of polyphenols and betacyanins, respectively. In this case, the peel and flesh extract of red-purple pitaya showed 53.056 ± 1.203, 56.381 ± 2.919 mg GAEs/100 g crude extract, respectively (calibration curve equation: A725 = 0.0036([gallic acid]) + 0.0086; R^2 = 0.9995) for the polyphenols determination. In addition, the peel extract showed 7.566 ± 0.580 mg BEs/100 g crude extract, whereas the flesh extract provided 23.005 ± 1.161 mg BEs/100 g crude extract for the betacyanins determination. Both the peel and flesh part

of red-purple pitaya were proved to contain antioxidant activity, and our results were higher compared to the previous studies reported on antioxidant activity of pitaya fruit (Fu et al., 2011).

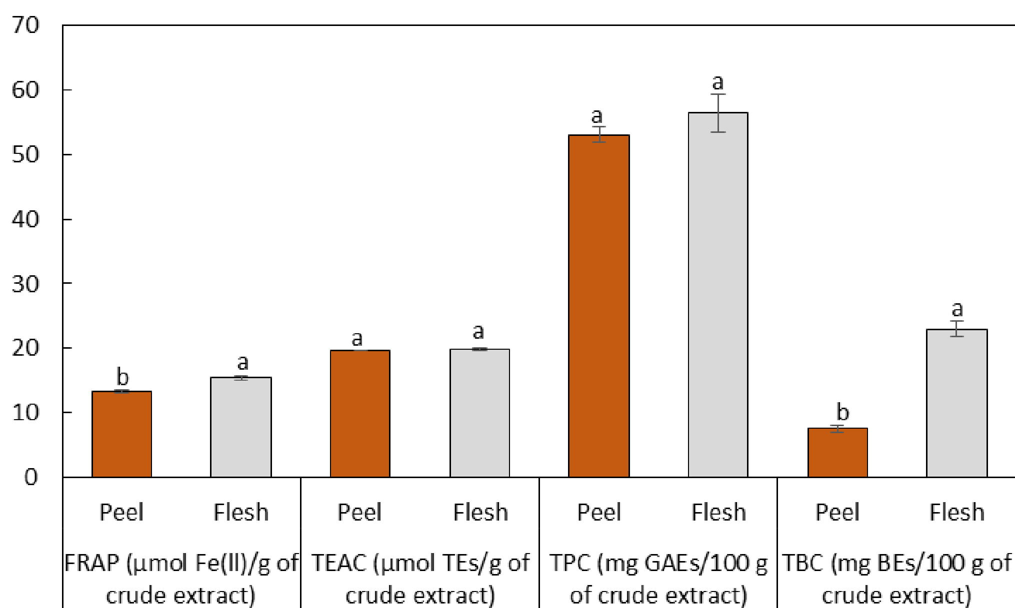


Figure 3-10: Antioxidant properties analysis for peel and flesh extract of red-purple pitaya obtained using the optimised alcohol/salt-based LBF. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant different (*p* < 0.05) using Tukey's test within the antioxidant properties assays.

Pearson's correlation test was performed to study the relationships among the antioxidant properties assays. FRAP, TEAC, TPC and TBC have relationship among each other. The FRAP and TBC showed a significant strong positive correlation (r = 0.989). Other antioxidant properties assays showed a non-significant moderate positive correlation, except TEAC and TPC. The TEAC and TPC showed a non-significant weak negative correlation (r = -0.093, $p = 0.860$). This infer that the higher the TEAC, the smaller the TPC. The closer the correlation coefficient (r) value to 1, the stronger the correlation between them (**Table 3-5**).

Table 3-5: Correlation among the antioxidant properties assays, given correlation coefficient (r).

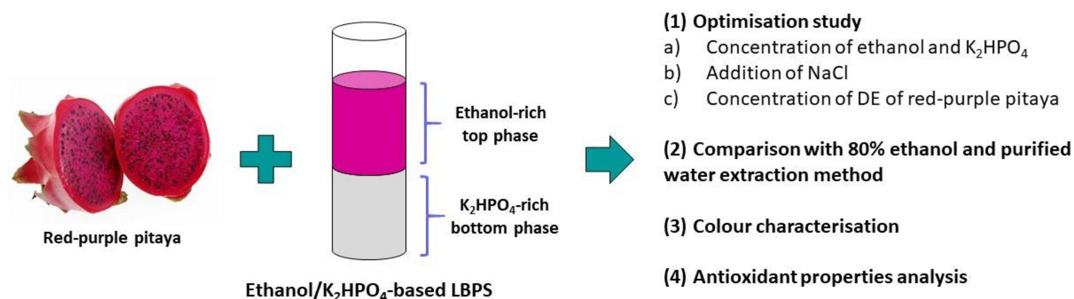
	TEAC	TPC	TBC
FRAP	0.787	0.528	0.989*
TEAC		-0.093	0.718
TPC			0.619

* Indicated significantly different at $p < 0.01$ (2-tailed).

3.5 Concluding remarks

LBF system offers a green bioseparation technology for the betacyanins extraction from peel and flesh of red-purple pitaya. A satisfactory betacyanins extraction from the peel and flesh were successfully achieved with the LBF composed of 20 mL of 200 g/L K_2HPO_4 solution, 10 mL of 100% ethanol, 1 g of FE and 15 min of flotation time. Our results reveal that both the peel and flesh extract of red-purple pitaya provided different hues, lightness, intensities and saturations of red colour, as well as their antioxidant activity were well retained. In addition, this study fully utilised fruit by-product (i.e. peel of red-purple pitaya), and both the peel and flesh extract of red-purple pitaya could be adopted as the potential replacement for artificial colourants. This is due to their food safe properties and the presence of antioxidants. Furthermore, stability of the betacyanins could further investigate since they are sensitive pigments. A stabilised betacyanins are believed to be beneficial for the food science industry because they are a good source of antioxidants.

CHAPTER 4: Application of Liquid Biphasic Partitioning System for Betacyanins Extraction from Peel and Flesh of *Hylocereus polyrhizus* and Antioxidant Activity Evaluation



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4.1 Abstract

Betacyanins have gained increasing interest as natural food dye because of their health-promoting effects. This study investigated the betacyanins extraction from peel and flesh of red-purple pitaya using ethanol/K_2HPO_4-based liquid biphasic partitioning system (LBPS) by optimised concentrations of ethanol, K_2HPO_4, NaCl and crude. Overall, the highest values of betacyanins concentration in alcohol-rich top phase (C_t), phase volume ratio (V_r) and partition coefficient (K) of betacyanins that represented an optimum betacyanins extraction from the peel (98.080%, 1.667 and 51.097, respectively) and flesh (96.256%, 2.167 and 25.764, respectively) were achieved. Lastly, conventional solvent extractions, colour characterisation and antioxidant activity evaluation were conducted.

Keywords: antioxidant activity; betacyanins; liquid biphasic partitioning system; red-purple pitaya

4.2 Introduction

Betacyanins are red-violet pigments, which belong to the class of betalains. They are water soluble ammonium conjugates of betalamic acid with *cyclo*-3,4-dihydroxyphenylalanine (*cyclo*-DOPA) (Khan and Giridhar, 2015). The most simple and commonly found structure of betacyanins is betanin (Esatbeyoglu et al., 2015). Being a plant-based bioactive compound (phytochemical), betacyanins are promising source of antioxidants (Dembitsky et al., 2011). Aside from the high antioxidant activity, betacyanins also exhibit anti-inflammatory and anti-radicals scavenging properties. Hence, they can play a protective role against chronic diseases, such as cancers, obesity, diabetes, heart diseases and cardiovascular diseases (Leong et al., 2018c). At present, betalains are of growing interest in food processing not only as natural colourants, but also as an antioxidants additive (Aberoumand, 2011). Additionally, they show wide applications in colouring of mildly acidic to neutral foodstuffs due to their pH stability ranging from 3 to 7 (Moreno et al., 2008). Nowadays, consumers are concerned about the use of synthetic dyes in the food processing because of the arising of negative health issues and toxicological aspects that are associated with the synthetic dyes (Carocho et al., 2015).

Natural red pigment extracted from red beet (i.e. betanin) has been commercialised in the market as the oldest and the most abundant red dye for food applications (Moreno et al., 2008). However, there is a high demand for replacing the red colourant derived from red beet due to its major drawbacks, including formation of carcinogenic nitrosamines compound and presence of unpleasant earthy smell. In this case, red-purple pitaya could be used as a potential substitute for red beet. Red-purple pitaya, or more commonly known as red dragon fruit (*Hylocereus polyrhizus*), is a species of

pitaya having red skin with red-purple flesh. Both red-purple pitaya and red beet shared the same red pigment constituent. Unlike red beet, red-purple pitaya does not generates carcinogenic compound and unpleasant smell (Aberoumand, 2011; Esatbeyoglu et al., 2015; Moreno et al., 2008). Red-purple pitaya belongs to the Cactaceae family, and is categorised under the subfamily Cactoidea. It is a type of underutilised fruit that is rich in nutritional values and antioxidants. Also, red-purple pitaya is easily found in Malaysia (Dembitsky et al., 2011).

Extraction process of betacyanins from different sources, including pitaya (Ding et al., 2009; Ramli et al., 2014; Wybraniec and Mizrahi, 2002), *Amaranth* (Chong et al., 2014), *Opuntia* fruits (Castellar et al., 2006; Castellar et al., 2003) and beetroot (de Azeredo et al., 2009; Ravichandran et al., 2013) were reported, but they were mostly conducted using conventional solvent extraction method. Conventional solvent extraction technique, which is normally applied in the extraction of phytochemicals and natural products, has the downsides of being time-consuming, costly and not environmentally benign due to large amount of solvent consumption (Azmir et al., 2013; Dai and Mumper, 2010; Feng et al., 2015; Tan et al., 2017; Zhang et al., 2013). Therefore, the use of a simple, cost-effective and green technique in the extraction of phytochemicals and natural products is much appreciable. Liquid biphasic partitioning system (LBPS) is a liquid-liquid fractionation method for separation and partition of biotechnological and natural products. Generally, the targeted compounds will be partitioned to the top phase. LBPS is a technically simple, scalable, energy-efficient, environmentally benign and mild bioseparation technique. In addition, it requires less solvent consumption during the separation process, and thus offers an attractive alternative that meets the requirements of the high demand in the industry processes

(Feng et al., 2015; Lin et al., 2012; Santos et al., 2018; Yau et al., 2015; Zhang et al., 2016).

The objective of the present study was to extract betacyanins from peel and flesh of red-purple pitaya using a simple yet effective green bioseparation technique, i.e. alcohol/salt-based LBPS. Up to date, alcohol/salt-based LBPS is conducted for betacyanins extraction from red-purple pitaya for the first time. Alcohol is an effective solvent in the extraction of phytochemicals, especially plant polyphenols (Dai and Mumper, 2010). This is because alcohol could break down pectin in the fruits; pectin promotes a more rapid degradation process of betalains (Chethana et al., 2007). Also, salt solution, such as dipotassium hydrogen phosphate (K_2HPO_4) and sodium chloride (NaCl) could enhance the extraction and partition of targeted products (e.g. biotechnological and natural products) in the separation process (Ooi et al., 2009; Yau et al., 2015; Zhang et al., 2013). In accordance with Zhang et al. (2013), the combination of ethanol and K_2HPO_4 represents a food-safe combination to be used in aqueous biphasic system for separation of natural products. Therefore, this study utilised the combination of ethanol and K_2HPO_4 in alcohol/salt-based LBPS for the betacyanins extraction. The operating parameters of the LBPS, such as concentrations of ethanol, K_2HPO_4, NaCl and crude of red-purple pitaya were optimised. Moreover, conventional solvent extractions using 80% (w/w) ethanol and purified water were conducted to compare the extraction efficiency of betacyanins with that of the LBPS. Lastly, colour characterisation and antioxidant properties analysis were carried out for the resulting extracts (alcohol-rich top phase) of red-purple pitaya obtained from ethanol/K_2HPO_4-based LBPS.

4.3 Materials and methods

4.3.1 Materials

Absolute ethanol, dipotassium hydrogen phosphate (K_2HPO_4), sodium chloride ($NaCl$), sodium bicarbonate ($NaHCO_3$), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were purchased from R&M Chemicals (Selangor, Malaysia). Acetic acid (CH_3COOH) and sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$) were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was purchased from Fisher Scientific (Selangor, Malaysia). 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), Folin-Ciocalteu (F-C) reagent and gallic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA). All of the chemicals used were of analytical grade (purity > 95%). Ultrapure water produced from a Milli-Q integral water purification system (Merck, Darmstadt, Germany) was used throughout the experiment. Red-purple pitaya was purchased from a local fruit stall at Semenyih, Selangor, Malaysia.

4.3.2 Processing of crude extract

The processing of crude extract was conducted in a dim light condition in order to minimise the loss of its pigment. The red-purple pitaya was first cleaned under running tap water, and was then dried with tissue towel. Subsequently, the peel of red-purple pitaya was removed from the flesh. After that, both the peel and flesh of red-purple pitaya were cut into thin pieces, and were stored at -80 °C for 24 h. To prepare dried crude extract (DE), the sample was freeze-dried at -30 °C and 0.37 atm for 48 h using

a freeze dryer (CHRIST Alpha 1-2 LDplus, Germany). The freeze-dried crude extract was ground into powder using a grinder (Tefal Blendforce, Triple'Ax Technology 400 Watt, Malaysia), and was kept at -20 °C until further use.

4.3.3 Phase diagram of LBPS

The binodal curve, which separates the one-phase area from the two-phase area in a phase diagram of ethanol and K_2HPO_4, was estimated using cloud-point method as described by Albertsson (1986). In brief, LBPS was prepared by mixing ethanol and K_2HPO_4 solution in a centrifugal tube until the first sign of turbidity was observed, indicating that formation of two phases could eventually occur. Purified water was then added dropwise under gentle mixing, until the turbidity disappeared. The cloud points were estimated by measuring the total weight of the purified water added. The concentrations of the phase-forming components (i.e. ethanol and K_2HPO_4) in the LBPS were calculated. The binodal curve was then plotted at varying the ethanol and K_2HPO_4 concentration (Ooi et al., 2009).

4.3.4 Betacyanins extraction with ethanol/K_2HPO_4-based LBPS

The LBPS was prepared in a 15 mL graduated centrifugal tube by weighing an appropriate amount (%, w/w) of ethanol and K_2HPO_4 with 1% (w/w) of DE of the red-purple pitaya. Purified water was then added to the system to obtain a final mass of 10 g. After thorough mixing by gentle agitation, the mixture was centrifuged at 3000 rpm for 20 min to induce a phase separation. The volumes of the top and bottom phase were then measured, followed by the collection of sample from both phases for analysis of total betacyanins content (TBC). Several operating parameters of LBPS, such as concentrations of ethanol and K_2HPO_4, addition of NaCl and DE concentration

of red-purple pitaya were optimised for the betacyanins extraction using one-factor-at-a-time (OFAT) approach.

4.3.5 Comparison study between LBPS and conventional solvent extractions

Betacyanins extraction using conventional solvent extraction method was conducted based on method as described in literatures (Ramli et al., 2014; Wybraniec and Mizrahi, 2002). 1 g of DE of peel and flesh of red-purple pitaya was mixed with 50 mL and 25 mL of 80% (w/w) ethanol, respectively. Subsequently, the mixture was centrifuged at 3000 rpm for 20 min for the purpose of obtaining the liquid extract. The liquid extracts of peel and flesh of red-purple pitaya were collected, and were evaluated for their TBC. These procedures were repeated by replacing 80% ethanol with purified water.

4.3.6 Analytical procedures

4.3.6.1 Colour characterisation

The colour parameters, i.e. L^*, a^* and b^* of liquid extracts of the peel and flesh of red-purple pitaya were measured using a colorimeter (Lovibond LC 100, model RM 200, The Tintometer Ltd, United Kingdom). L^*, a^* and b^* are lightness, redness and yellowness, respectively. The hue angle (h°) and chroma (C^*) were calculated according to equations (4-1) and (4-2), respectively.

$$h^\circ = \tan^{-1}\frac{b^*}{a^*} \quad (4\text{-}1)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (4\text{-}2)$$

4.3.6.2 Determination of total betacyanins content (TBC)

The TBC in the crude extract was analysed using a UV-vis spectrophotometer (UV-1800, Shimadzu, Japan) at 538 nm. The TBC was expressed as mg of betanin equivalents (BEs) per 100 g of crude extract, and was calculated according to equation (4-3) (Ramli et al., 2014).

$$\mathrm{TBC} = \frac{A_{538} \times MW \times V \times DF}{\varepsilon \times L \times W} \times 100 \qquad (4\text{-}3)$$

Where A_{538} = absorbance value at 538 nm, MW = molecular weight of betanin (550 $g.mol^{-1}$), V = volume of sample (mL), DF = dilution factor, ε = molar extinction coefficient of betanin (65000 $L.mol^{-1}.cm^{-1}$), L = path length of cuvette (1 cm), W = weight of crude extract (g)

4.3.6.3 Determination of total phenolic content (TPC)

The TPC was evaluated based on Folin-Ciocalteu (F-C) method as described in literatures (Fu et al., 2011; Hajimahmoodi et al., 2013; Singleton et al., 1999). In brief, a diluted F-C reagent was prepared by mixing 10 mL of F-C reagent with 90 mL of purified water. 100 µL of sample or gallic acid solution (calibration curve) was mixed with 500 µL of diluted F-C reagent in a test tube. The mixture was incubated for 5 min at room temperature under dark condition. After that, 2 mL of $NaHCO_3$ solution (60 g/L) was added to the mixture. The mixture was shake gently, and was then kept under dark condition for 90 min at room temperature. The absorbance value of the mixture was measured at 725 nm using a UV-vis spectrophotometer. The TPC was expressed as mg of gallic acid equivalents (GAEs) per 100 g of crude extract.

4.3.6.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted based on method as described in literatures (Benzie and Strain, 1996; Fu et al., 2011). Acetate buffer (300 mmol/L, pH 3.6) was first prepared by mixing 3.1 g of $C_2H_3NaO_2·3H_2O$ with 16 mL of acetic acid per litre of purified water. FRAP reagent was prepared freshly by mixing 10 mL of 10 mmol/L TPTZ (0.0031 g/mL of 40 mmol/L HCl) and 10 mL of 20 mmol/L $FeCl_3·6H_2O$ (0.0054 g/mL) for every 100 mL of 300 mmol/L acetate buffer, and was preheated to 37 °C before use. The FRAP assay was carried out by mixing 100 μL of sample or $FeSO_4·7H_2O$ solution (calibration curve), 300 μL of purified water and 3 mL of FRAP reagent in a test tube. The mixture was then incubated for 4 min at 37 °C. The absorbance value of the mixture was measured at 593 nm using a UV-vis spectrophotometer. The result was expressed as μmol of Fe(II) per g of crude extract.

4.3.6.5 Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay was conducted based on ABTS radical (ABTS•) method as described in literatures (Fu et al., 2011; Re et al., 1999). ABTS• stock solution was first prepared by mixing 7 mmol/L of ABTS with 2.45 mmol/L of $K_2O_8S_2$ at v:v proportion of 1:1, and was then incubated at room temperature under dark condition for 12–16 h. Then, the ABTS• stock solution was diluted with ethanol as to reach an absorbance value of 0.70 ± 0.05 at 734 nm, and was incubated at 30 °C. The TEAC assay was carried out by mixing 100 μL of sample or trolox solution (calibration curve) or ethanol (control) with 3.8 mL of diluted ABTS• solution. The absorbance value of the mixture was measured at 734 nm using a UV-vis spectrophotometer after 6 min of incubation at 30 °C. The result was expressed as μmol of trolox equivalents (TEs) per

g of crude extract. The percentage of scavenging on ABTS• was calculated using equation (4-4) (Olajuyigbe and Afolayan, 2011).

$$\text{Percentage of scavenging (\%)} = \frac{\text{control} - \text{sample or standard}}{\text{control}} \times 100 \qquad (4\text{-}4)$$

4.3.7 Calculations

Partition coefficient (K) of betacyanins in alcohol/salt-based LBPS was calculated according to equation (4-5) (Leong et al., 2018a).

$$\mathrm{K} = \frac{\mathrm{TBC}_t}{\mathrm{TBC}_b} \qquad (4\text{-}5)$$

Where TBC_t and TBC_b are TBC in the alcohol-rich top phase and salt-rich bottom phase at equilibrium, respectively.

Phase volume ratio (V_r) is defined as ratio of the volume of alcohol-rich top phase to the volume of the salt-rich bottom phase at equilibrium, and was calculated according to equation (4-6) (Chandrasekhar et al., 2015).

$$\mathrm{V}_r = \frac{\mathrm{V}_t}{\mathrm{V}_b} \qquad (4\text{-}6)$$

Where V_t and V_b are volumes of the alcohol-rich top phase and salt-rich bottom phase at equilibrium, respectively.

Betacyanins concentrations (%) in alcohol-rich top phase (C_t) and salt-rich bottom phase (C_b) were calculated according to equations (4-7) and (4-8), respectively (Leong et al., 2018a).

$$\mathrm{C}_t(\%) = \frac{\text{TBC in top phase}}{\text{TBC in crude extract}} \times 100 = \frac{\mathrm{TBC}_t}{\mathrm{TBC}_t + \mathrm{TBC}_b} \times 100 \qquad (4\text{-}7)$$

$$C_b(\%) = \frac{\text{TBC in bottom phase}}{\text{TBC in crude extract}} \times 100 = \frac{\text{TBC}_b}{\text{TBC}_t + \text{TBC}_b} \times 100 \qquad (4\text{-}8)$$

4.3.8 Statistical analysis

Triplicate experimental readings were recorded and were used in statistical analysis, and the values were expressed as mean ± standard deviation (SD) (n = 3). The statistical analysis was performed by IBM SPSS statistics program (SPSS version 23.0 for window, IBM Corporation, Armonk, New York, United States). The data were subjected to one-way ANOVA, and the mean differences were compared using Tukey HSD post-hoc multiple comparisons test. Pearson's correlation test was carried out to study the relationships among the antioxidant properties assays. The data were considered to be statistically significant difference where $p < 0.05$.

4.4 Results and discussion

4.4.1 Phase diagram of alcohol/salt-based LBPS

The phase diagram of ethanol/K_2HPO_4-based LBPS was estimated to delineate the potential working area of LBPS composed of ethanol and K_2HPO_4 by determining the appropriate ratio of phase-forming components in the biphasic system at equilibrium, as shown in **Figure 4-1**. The experimental binodal data were well fitted with the calculated binodal data, using Merchuk equation [$Y = a \exp(bX^{0.5} - cX^3)$] (Merchuk et al., 1998), $[EtOH] = 0.608 \exp((-2.720 \times [K_2HPO_4]^{0.5}) - (10.700 \times [K_2HPO_4]^{3}))$ with a $R^2 = 0.991$; where [EtOH] and [K_2HPO_4] are the weight fraction percentages of ethanol and K_2HPO, and 0.608, -2.720 and 10.700 are the constants obtained by the regression of the experimental binodal data. As depicted in **Figure 4-1**, the binodal curve divided the phase diagram into two regions; a two-phase forming area (i.e. above the curve) and a homogenous one-phase forming area (i.e. at and below the curve).

From our observations, the binodal curve of ethanol/K_2HPO_4 system was far away from the origin, which showed a similar trend as reported by Ooi et al. (2009). This is due to the high polarity of ethanol greatly improved the solubility and miscibility of K_2HPO_4 in the system. Ethanol-rich top phase and K_2HPO_4-rich bottom phase were observed in the biphasic formation. Also, an increase in the concentration of ethanol was coupled with a lower concentration of K_2HPO_4 in the biphasic formation.

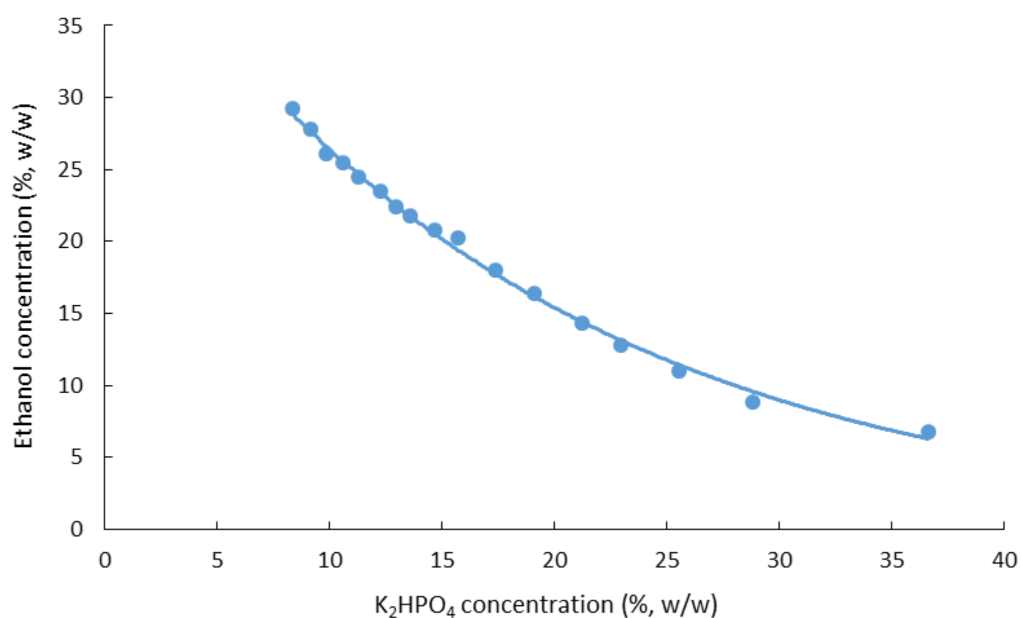


Figure 4-1: Phase diagram of ethanol/K_2HPO_4-based LBPS.

4.4.2 Effects of concentrations of alcohol and salt

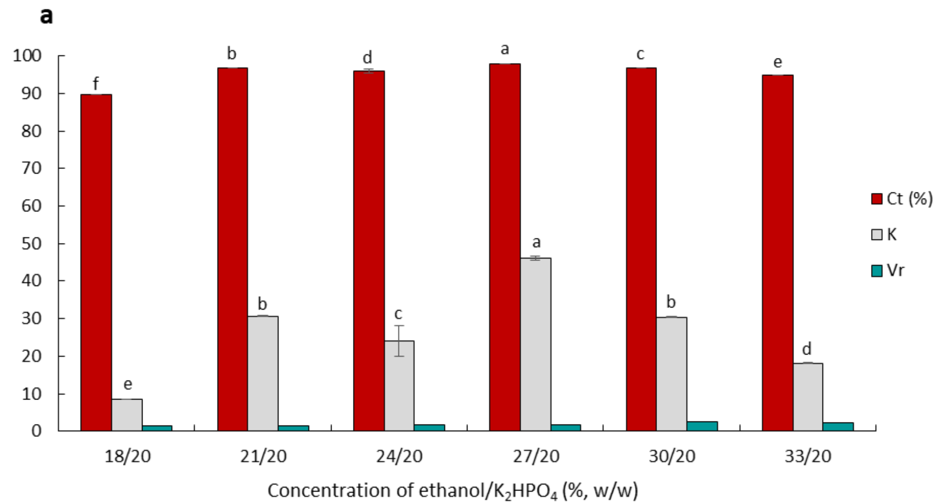
Varying concentrations of ethanol and K_2HPO_4 were used to investigate the extraction efficiency of betacyanins from peel and flesh of red-purple pitaya in the alcohol/salt-based LBPS, as shown in **Figure 4-2**. The chosen concentrations of the ethanol and K_2HPO_4 in the investigation were predetermined based on the binodal curve plotted on the phase diagram of ethanol/K_2HPO_4-based LBPS (**Figure 4-1**). In

this process, the concentration of ethanol was first optimised, and then followed by the optimisation of concentration of K_2HPO_4.

In alcohol/salt-based LBPS composed of 20% (w/w) K_2HPO_4, an increase in the concentration of ethanol from 18% to 27% (w/w) increased the betacyanins concentration in alcohol-rich top phase (C_t) from peel of red-purple pitaya from 89.598 ± 0.008% to 97.874 ± 0.024%. A further increase in the concentration of ethanol until 33% (w/w) resulted in a reduction of the C_t from the peel. Likewise, a similar trend was observed on the phase separation of ethanol/K_2HPO_4-based LBPS (which was indicated by phase volume ratio (V_r) and partition coefficient (K) of betacyanins) from the peel in the LBPS. A better phase separation with a V_r at 1.833 and a K of betacyanins at 46.052 ± 0.535 were achieved for the LBPS composed of 27% (w/w) ethanol and 20% (w/w) K_2HPO_4 (**Figure 4-2a**). Subsequently, LBPS composed of 27% (w/w) ethanol was used in the study with varying concentrations of K_2HPO_4, as shown in **Figure 4-2b**. An increased concentration of K_2HPO_4 from 10% to 25% (w/w) did not improve the C_t, V_r and K of betacyanins from peel of red-purple pitaya in LBPS. The LBPS composed of 27% (w/w) ethanol and 10% (w/w) K_2HPO_4 showed higher values for C_t, V_r and K of betacyanins at 96.425 ± 0.002%, 16.000 and 26.971 ± 0.014, respectively. However, its betacyanins extraction efficiency was not good as compared to the LBPS with 27% (w/w) ethanol and 20% (w/w) K_2HPO_4, as indicated by its smaller values of C_t and K of betacyanins. Hence, LBPS composed of 27% (w/w) ethanol and 20% (w/w) K_2HPO_4 with the peel of red-purple pitaya was used for further studies.

Meanwhile, LBPS with flesh of red-purple pitaya composed of 20% (w/w) K_2HPO_4 was fixed, and the parameter of varying concentrations of ethanol was evaluated

(**Figure 4-2c**). Increasing the concentration of ethanol from 18% to 33% (w/w) increased the values of C_t, V_r and K of betacyanins from the flesh in LBPS. The LBPS composed of 33% (w/w) ethanol and 20% (w/w) K_2HPO_4 showed higher values for C_t, V_r and K of betacyanins at 92.499 ± 0.028%, 2.167 and 12.331 ± 0.049, respectively. After that, LBPS with flesh of red-purple pitaya composed of 33% (w/w) ethanol was fixed to study the effect of varying concentrations of K_2HPO_4, as depicted in **Figure 4-2d**. From our observations, an increase in the concentration of K_2HPO_4 from 10% to 13% (w/w) improved the betacyanins extraction efficiency from the flesh. A further increase in the concentration of K_2HPO_4 until 25% (w/w) showed no improvement on the betacyanins extraction efficiency. The LBPS composed of 33% (w/w) ethanol and 13% (w/w) K_2HPO_4 resulted in higher values for C_t, V_r and K of betacyanins at 90.356 ± 0.999%, 3.875 and 9.439 ± 1.023, respectively. However, the LBPS composed of 33% (w/w) ethanol and 20% (w/w) K_2HPO_4 with the flesh of red-purple pitaya was chosen for further studies. This is due to its better betacyanins extraction efficiency (higher values of C_t and K of betacyanins) as compared to the LBPS composed of 33% (w/w) ethanol and 10% (w/w) K_2HPO_4 with the flesh of red-purple pitaya.



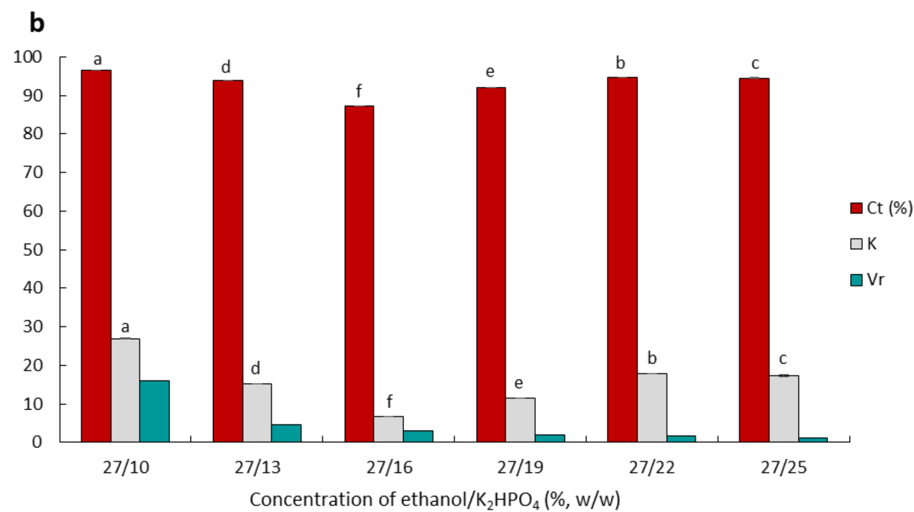


Figure 4-2: Effects of varying concentrations of ethanol and K_2HPO_4 (%, w/w) in alcohol/salt-based LBPS with peel and flesh of red-purple pitaya, in which ethanol concentration was first optimised, and then followed by the optimisation of K_2HPO_4 concentration. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference (*$p < 0.05$*) using Tukey's test within the same category. (a) 20% of K_2HPO_4 with varying concentrations of ethanol in LBPS with the peel. (b) 27% of ethanol with varying concentrations of K_2HPO_4 in LBPS with the peel. (c) 20% of K_2HPO_4 with varying concentrations of ethanol in LBPS with the flesh. (d) 33% of ethanol with varying concentrations of K_2HPO_4 in LBPS with the flesh.

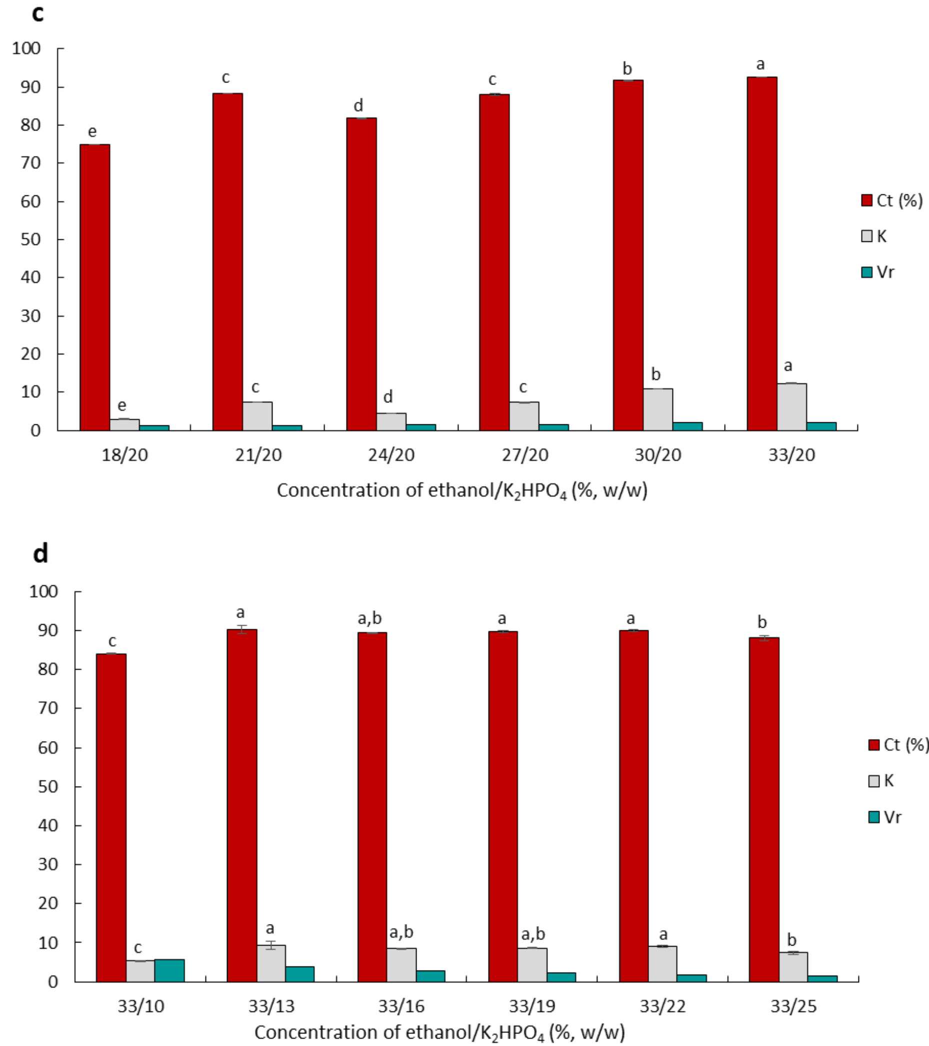


Figure 4-2 continued.

4.4.3 Effect of addition of NaCl

The effect of addition of NaCl in the ethanol/K_2HPO_4-based LBPS with peel and flesh of red-purple pitaya is shown in **Figure 4-3**. According to our findings, addition of 2% (w/w) of 0.4 M NaCl and 2% (w/w) of 0.2 M NaCl in the ethanol/K_2HPO_4-based LBPS with the peel (**Figure 4-3a**) and flesh (**Figure 4-3c**), respectively, showed an optimum betacyanins extraction. The values of C_t, V_r and K of betacyanins in the LBPS with the peel were 98.080 ± 0.051%, 1.667 and 51.097 ± 1.354, respectively, whereas the LBPS with the flesh showed a C_t at 96.256 ± 0.207%, a V_r at 2.167 and a

K of betacyanins at 25.764 ± 1.525. As compared to the control groups for both LBPS with the peel and flesh of red-purple pitaya, their partitioning behaviours of betacyanins to the alcohol-rich top phase were improved, as indicated by their K value. LBPS with the peel and 2% (w/w) of 0.4 M NaCl additive showed an increment of approximately 5% from 46.052 ± 0.535 to 51.097 ± 1.354, while there was an approximately two-fold increment from 12.331 ± 0.049 to 25.764 ± 1.525 in the LBPS with the flesh and 2% (w/w) of 0.2 M NaCl additive. An enhancement in the partition of betacyanins to alcohol-rich top phase caused an increase in the betacyanins concentration in alcohol-rich top phase.

Subsequently, the chosen NaCl molarity in the LBPS with peel (0.4 M) and flesh (0.2 M) of red-purple pitaya were further studied with addition of varying concentrations from 2–10% (w/w), as shown in **Figure 4-3b,d**. Our results reveal that addition of 2% (w/w) of 0.4 M and 0.2 M NaCl in both LBPS with the peel and flesh, respectively, were showed to exhibit the maximum partition efficiency of betacyanins. A further increase in the concentration of chosen molarity of NaCl until 10% (w/w) did not show any improvement on the partitioning behaviour of betacyanins, as well as the betacyanins extraction, yet worsen their condition. This could be due to the reason that the high concentration of NaCl in the LBPS has influenced the system equilibrium. Therefore, a decrease in the partitioning behaviour of betacyanins and betacyanins concentration in the alcohol-rich top phase were observed. According to Ooi et al. (2009), addition of NaCl could improve the partitioning of lipase to the alcohol-rich top phase in alcohol/salt-based aqueous two-phase system. Addition of neutral salts like NaCl can affect the water solvent structure and hydrophobic interactions, and thus influence the partitioning behaviour of the loaded sample. This

is attributed by the generation of electrical potential difference between the phases in the biphasic system (Albertsson, 1986; Hatti-Kaul, 2000).

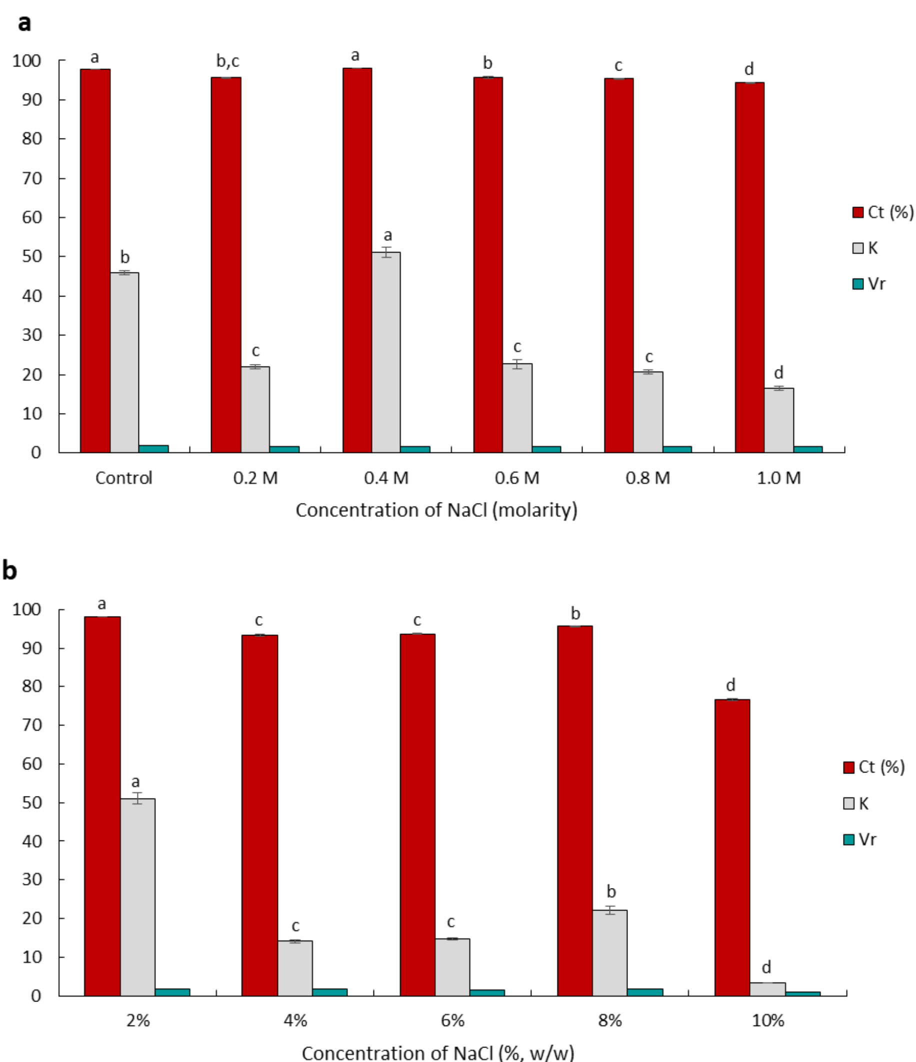


Figure 4-3: Effect of addition of NaCl in ethanol/K_2HPO_4-based LBPS with peel and flesh of red-purple pitaya. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference (*p* < 0.05) using Tukey's test within the same category. (a) Addition of 2% (w/w) of varying molarities of NaCl in 27% of ethanol/20% of K_2HPO_4-based LBPS with the peel. (b) Addition of varying concentrations (%, w/w) of 0.4 M NaCl in 27% of ethanol/20% of K_2HPO_4-based LBPS with the peel. (c) Addition of 2% (w/w) of varying molarities of NaCl in 33% of ethanol/20% of K_2HPO_4-based LBPS with the flesh. (d) Addition of varying concentrations (%, w/w) of 0.2 M NaCl in 33% of ethanol/20% of K_2HPO_4-based LBPS with the flesh.

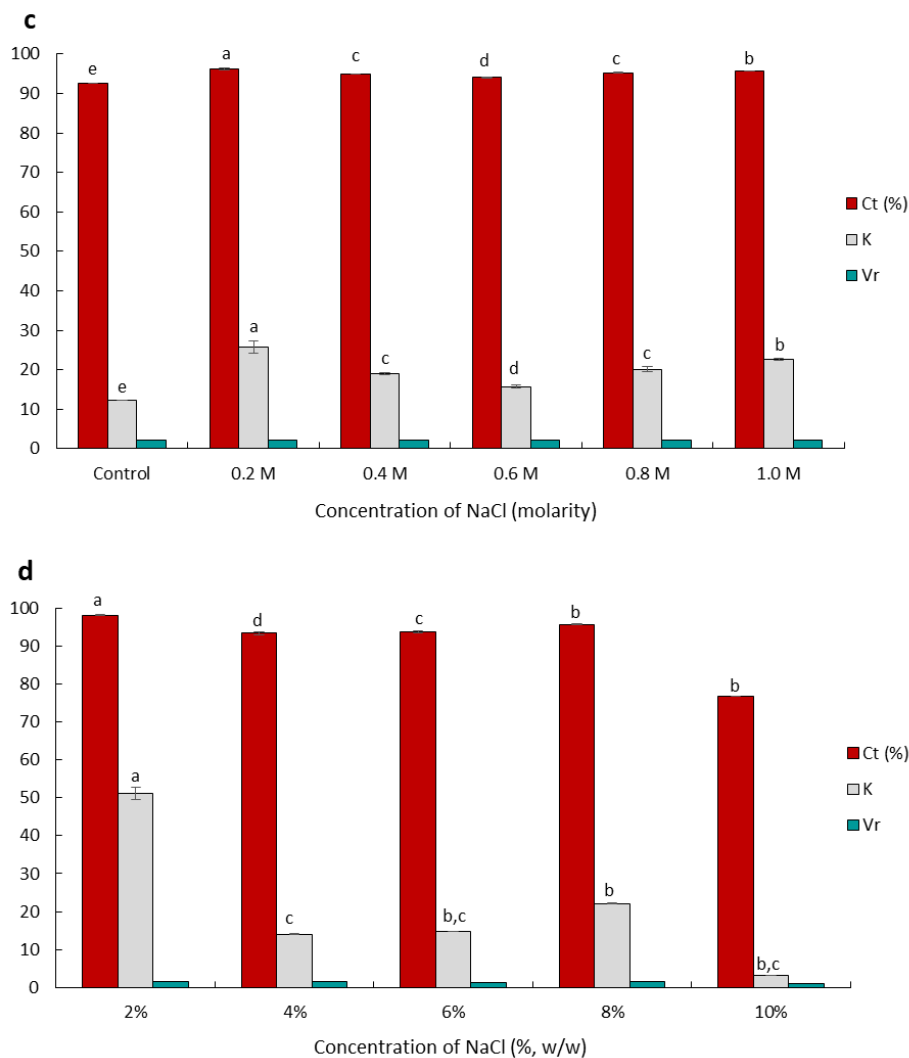


Figure 4-3 continued.

4.4.4 Effect of crude concentration

Based on the previous results, the optimised ethanol/K_2HPO_4-based LBPS with peel and flesh of red-purple pitaya were further studied with the addition of varying dried crude extract (DE) concentrations of red-purple pitaya, i.e. 1%, 5%, 7.5%, and 10.0% (w/w), as depicted in **Figure 4-4**. From our observations, 10% (w/w) of DE in the 10 g LBPS formed a gel-like mixture, and this could be most probably due to the overloading of the crude extract in the biphasic system. The gel-like mixture was due to the presence of large amount of pectin, and the amount of ethanol present in the

sample was insufficient to break down the pectin. As reported by Chethana et al. (2007), pectin promotes a more rapid degradation of betalains, and thereby reduces the betacyanins extraction. As shown in **Figure 4-4a,b**, 1% (w/w) of DE of the peel and flesh in the 10 g LBPS showed the maximum betacyanins extraction. The highest values of C_t, V_r and K of betacyanins in the LBPS with the peel were 98.080 ± 0.051%, 1.667 and 51.097 ± 1.354, respectively, whereas the LBPS with the flesh was noticed to show the highest values of C_t at 96.256 ± 0.207%, V_r at 2.167 and K of betacyanins at 25.764 ± 1.525. A further increase in the DE concentration of the peel and flesh of red-purple pitaya decreased the betacyanins extraction. This could be due to the overloaded crude extract in the LBPS that has influenced the system equilibrium, and thus affect the extraction efficiency of betacyanins. As mentioned earlier, pectin promotes a more rapid degradation of betacyanins, and thereby reduces the betacyanins concentration from the crude extract in the presence of a high amount of pectin in the extract. Overall, the optimum concentrations (%, w/w) of phase-forming components in the 10 g alcohol/salt-based LBPS for the betacyanins extraction from the peel and flesh of red-purple pitaya were optimised as such, 27% of ethanol + 20% of K_2HPO_4 + 50% of purified water + 2% of 0.4 M NaCl + 1% of DE and 33% of ethanol + 20% of K_2HPO_4 + 44% of purified water + 2% of 0.2 M NaCl + 1% of DE, respectively.

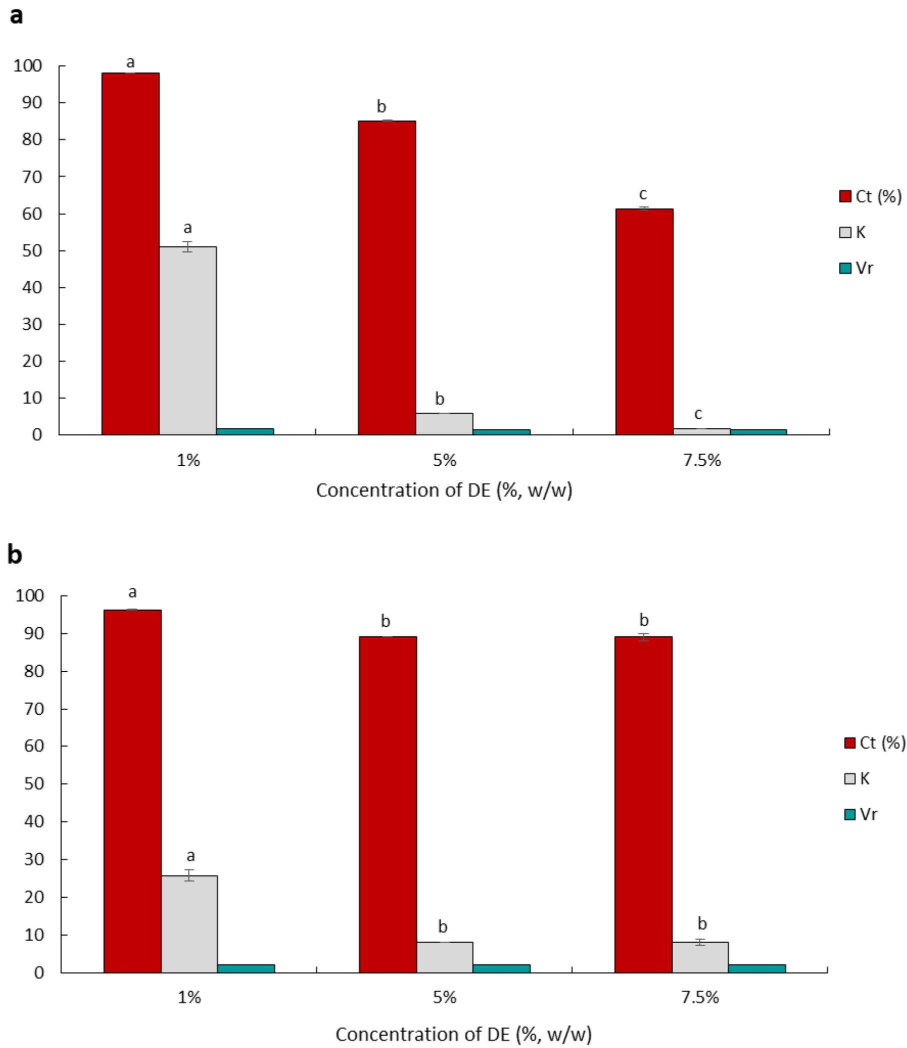


Figure 4-4: Effect of varying concentrations of dried crude extract (DE) (%, w/w) added in ethanol/K_2HPO_4-based LBPS with (a) peel and (b) flesh of red-purple pitaya. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference (*p* < 0.05) using Tukey's test within the same category.

4.4.5 Betacyanins extraction efficiency between LBPS and conventional solvent extractions

The optimised ethanol/K_2HPO_4-based LBPS as well as conventional solvent extractions with 80% ethanol and purified water were conducted to evaluate the betacyanins extraction from peel and flesh of red-purple pitaya (**Figure 4-5**). From our observations, the TBC from peel and flesh of red-purple pitaya extracted with optimised ethanol/K_2HPO_4-based LBPS were much higher compared to that of which were extracted using 80% ethanol and purified water. There was an approximately eight-fold increment in the TBC from the peel extracted with ethanol/K_2HPO_4-based LBPS compared to that of using 80% ethanol extraction method (from 21.961 ± 0.145 to 180.654 ± 2.239 mg BEs/100 g crude extract). Also, an approximately three-fold increment of the TBC from the flesh extracted using ethanol/K_2HPO_4-based LBPS compared to that of with 80% ethanol extraction method was observed (from 44.350 ± 0.068 to 139.645 ± 0.198 mg BEs/100 g crude extract). On the other hand, the TBC from the peel and flesh extracted with purified water extraction method were 100.027 ± 0.028 and 97.363 ± 0.022 mg BEs/100 g crude extract, respectively, in which were significantly lower than that of with the ethanol/K_2HPO_4-based LBPS.

These results reveal that alcohol/salt-based LBPS is more effective in the extraction of phytochemicals and natural products, for example, betacyanins, compared to the use of conventional solvent extractions method. In addition, alcohol/salt-based LBPS only required 0.1 g of crude extract to provide the highest TBC values for both the peel and flesh of red-purple pitaya. In contrast, the conventional solvent extractions method required higher amount of crude extract (i.e. 1 g) and larger amount of solvent, and the results obtained were not satisfactory and promising as compared to the use of LBPS.

Literatures have reported that biphasic system can be a better technology for separation, enrichment and recovery of natural products (Santos et al., 2018; Tan et al., 2017; Zhang et al., 2013).

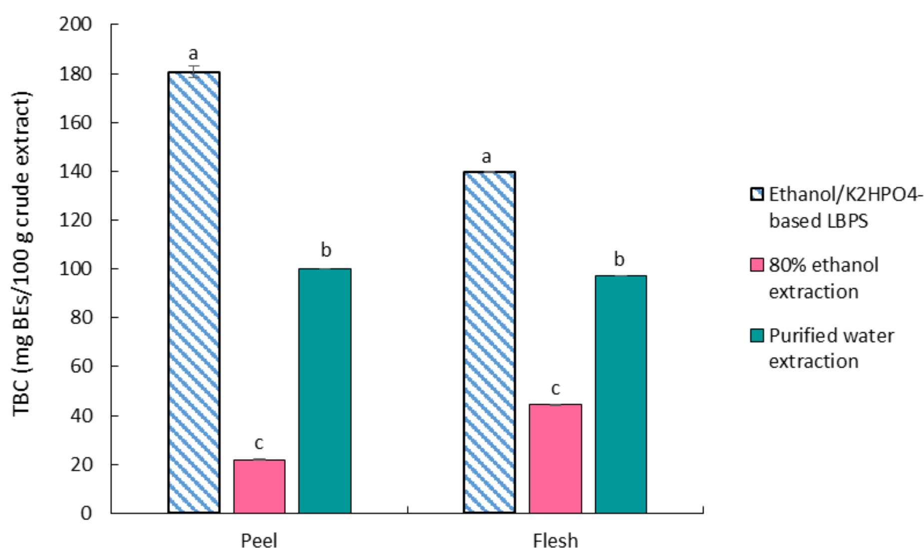


Figure 4-5: Comparison study of betacyanins extractions using the optimised ethanol/K_2HPO_4-based LBPS, 80% ethanol and purified water extraction technique. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within peel and flesh.

4.4.6 Colour characterisation

The colour characterisation was carried out to study the hue, saturation and intensity of colour of the liquid extracts from peel and flesh of red-purple pitaya extracted using the optimised ethanol/K_2HPO_4-based LBPS (alcohol-rich top phase) (**Table 4-1**). The peel extract of red-purple pitaya showed a red-yellowish colour (positive a^* and positive b^*), while the flesh extract revealed in a red-bluish colour (positive a^* and negative b^*). The hue angle (h°) denotes the basic tint of a colour, whereas the chroma (C^*) represents the saturation of colour (Lancaster and Lister, 1997). Overall, both the

peel and flesh liquid extract of red-purple pitaya can be used as natural colourants with different hues and saturations of red colour.

Table 4-1: Colour characterisation for peel and flesh extract of red-purple pitaya extracted using the optimised ethanol/K_2HPO_4-based LBPS.

Colour parameter	Peel extract	Flesh extract
L^*	8.567 ± 0.153	20.300 ± 0.400
a^*	28.467 ± 0.252	49.667 ± 0.115
b^*	8.600 ± 0.265	-21.200 ± 0.100
C^*	29.700 ± 0.300	54.000 ± 0.100
h°	16.800 ± 0.346	336.900 ± 0.173

L^*, a^*, b^*, C^* and h° represent lightness, redness, yellowness, chroma and hue angle, respectively.
Values are mean ± SD of triplicate readings.

4.4.7 Antioxidant properties analysis

The antioxidant properties analysis was conducted to examine the antioxidant capacity and antioxidant compound from peel and flesh of red-purple pitaya. In this study, the peel and flesh extract used were extracted using the optimised ethanol/K_2HPO_4-based LBPS (alcohol-rich top phase), and the results are shown in **Figure 4-6**. FRAP and TEAC are electron transfer-based assays that measure the antioxidant capability of a compound in reducing of an oxidant probe, which is indicated through a colour change of the sample mixture. The oxidant probes used in FRAP and TEAC were TPTZ and ABTS radical cation, respectively. A higher degree of colour change indicates a stronger reducing power of the compound. Both FRAP and TEAC are known as standardised methodologies in antioxidant capability analysis with slightly different working mechanisms due to the use of different oxidant probes (Dai and Mumper, 2010). On the other hand, TPC and TBC assay are used to evaluate antioxidant compound, i.e. polyphenols and betacyanins, respectively.

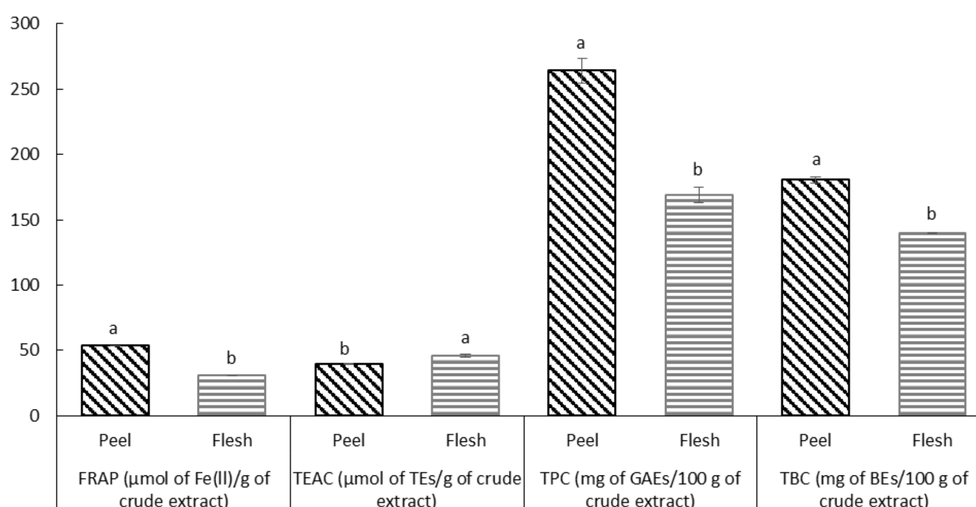


Figure 4-6: Antioxidant properties analysis, including antioxidant capacity assays (i.e. FRAP and TEAC) and antioxidant component assays (i.e. TPC and TBC) for peel and flesh extract of red-purple pitaya extracted using the optimised ethanol/K_2HPO_4-based LBPS. Values are mean ± SD of triplicate readings; different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within FRAP, TEAC, TPC and TBC.

In FRAP assay, the peel and flesh extract of red-purple pitaya showed 53.740 ± 0.109 and 30.770 ± 0.054 µmol of Fe(ll)/g of crude extract, respectively (calibration curve equation: $A_{593} = 0.0007([FeSO_4 \cdot 7H_2O]) + 0.1033$; $R^2 = 0.9977$). On the other hand, the peel and flesh extract showed 39.681 ± 0.664 and 46.078 ± 1.261 µmol of TEs/g of crude extract, respectively (calibration curve equation: Percentage of scavenging (%) = 0.0966([trolox]) + 2.8333; $R^2 = 0.9946$) in TEAC assay. For antioxidant compound analysis, the peel and flesh extract showed 264.125 ± 9.437 and 168.946 ± 5.707 mg of GAEs/100 g of crude extract, respectively (calibration curve equation: $A_{725} = 0.004([\text{gallic acid}]) + 0.0257$; $R^2 = 0.9997$) in determination of polyphenols. For determination of betacyanins, both the peel and flesh extract showed 180.654 ± 2.239 and 139.645 ± 0.198 mg of BEs/100 g of crude extract, respectively.

There is a significant difference ($p < 0.05$) within all the antioxidant properties analysis.

Our findings show that the peel of red-purple pitaya retained more betacyanins and polyphenols compared to the flesh. Similarly, the peel showed a higher FRAP value than that of the flesh. On the other hand, the flesh of red-purple pitaya has a higher TEAC value compared to the peel. This could be possibly explained by the different oxidant probes used in the analysis. Since the peel of red-purple pitaya has significantly high antioxidant activity, it should be fully utilised to avoid food wastage. Moreover, the values of FRAP, TEAC, TPC and TBC of the peel and flesh of red-purple pitaya in the present study were much higher than that of the previously reported studies (Fu et al., 2011; Ramli et al., 2014). The relationships among the antioxidant properties analysis were investigated using Pearson's correlation test. From **Table 4-2**, TEAC showed significant strong negative correlations with FRAP, TPC and TBC. In contrast, FRAP showed significant strong positive correlations with TPC and TBC. Likewise, TPC and TBC also showed a significant strong positive correlation between them. The closer the correlation coefficient (r) value to 1, the stronger the correlation between them.

Table 4-2: Correlation among the antioxidant properties analysis, given correlation coefficient (r).

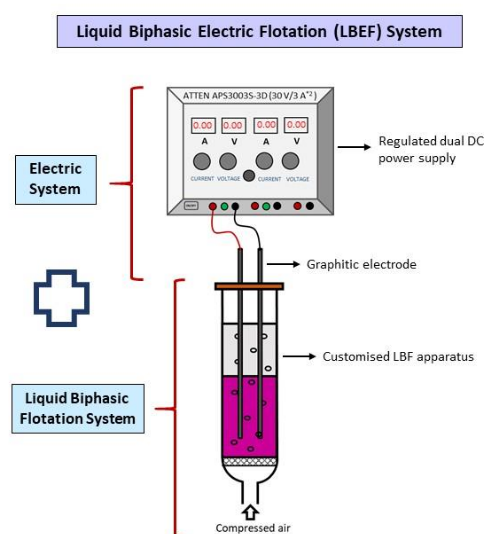
	TEAC	TPC	TBC
FRAP	-0.968*	0.992*	0.998*
TEAC		-0.955*	-0.960*
TPC			0.989*

* Indicated significantly different at $p < 0.01$ (2-tailed).

4.5 Concluding remarks

The extraction process of betacyanins from peel and flesh of red-purple pitaya using alcohol/salt-based LBPS is reported for the first time in this study. The concentrations of the phase-forming components in the 10 g alcohol/salt-based LBPS (%, w/w) for the peel and flesh were as such, 27% of ethanol + 20% of K_2HPO_4 + 50% of purified water + 2% of 0.4 M NaCl + 1% of DE and 33% of ethanol + 20% of K_2HPO_4 + 44% of purified water + 2% of 0.2 M NaCl + 1% of DE, respectively. The LBPS is proved to be a more effective technique for betacyanins extraction as compared to the conventional solvent extractions method. In addition, both the peel and flesh of red-purple pitaya showed different hues and saturations of red colour. They also demonstrated a strong antioxidant activity. Meanwhile, the peel part retained significantly higher antioxidants than that of the flesh part. Thus, it should be fully utilised in food science processing to avoid food wastage. Since alcohol/salt-based LBPS has been proven to be effective in the extraction of natural products like betacyanins, LBPS should be further studied and conducted on a larger scale for potential application in the food industry processes.

CHAPTER 5: Integration Process for Betacyanins Extraction from Peel and Flesh of *Hylocereus polyrhizus* using Liquid Biphasic Electric Flotation System and Antioxidant Activity Evaluation



(A) Betacyanins extraction with LBEF system

1. Optimised LBF system (constant throughout the experiment):
- 1 g of FE (peel or flesh of red-purple pitaya)
- 10 mL of undiluted ethanol
- 20 mL of 200 g/L of K_2HPO_4 solution
- *15 min of flotation time
- 20–30 cc/min of air flow rate

2. Electric system:
- Operation time (5–25 min)
- Voltage apply (1–5 V)
- Position of graphitic electrodes (top, middle, bottom phase)
- Type of crude extract (FE, DE)

*In LBEF system, replace flotation time with operation time

(B) Comparison between LBF and LBEF system
(C) Colour characterisation
(D) Antioxidant activity evaluation

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5.1 Abstract

Recently, betacyanins have added research applications in food science processing industries, owing to their health promoting functional and attractive visual properties. Additionally, red-purple pitaya (*Hylocereus polyrhizus*) is a rich source of betacyanins. Development of a green and effective bioseparation technology is deemed favourably in the downstream processing industries. Hence, this study utilised a liquid biphasic electric flotation (LBEF) system for betacyanins extraction from peel and flesh of red-purple pitaya as well as comparison between LBF and LBEF system, colour characterisation and antioxidant activity evaluation were conducted. In this study, the optimised LBF system (previous experimental results according to Chapter 3 in this thesis) was integrated with electric system. Collectively, the betacyanins extraction using LBEF system showed an optimal betacyanins extraction from the peel and flesh, with the significantly highest values of betacyanins concentration in alcohol-rich top phase (C_t) (99.014% and 96.132%), separation efficiency (E) (98.383% and 96.576%) and partition coefficient (K) (100.814 and 24.883) of betacyanins. Moreover, the peel and flesh extract showed different variations of red colours and antioxidant properties. The present study introduces a new, simple and high efficient bioseparation technology (LBEF system) which is definitely worth to study further.

Keywords: betacyanins; electric system; *Hylocereus polyrhizus*; integration process; liquid biphasic flotation system

5.2 Introduction

Betalains are water-soluble nitrogen-containing natural pigments. These pigments contain betalamic acid as the chromophore in their structure, which either conjugated with *cyclo*-3,4-dihydroxyphenylalanine (*cyclo*-DOPA) to synthesise red-violet betacyanins or with different amino acids or amines to produce yellow-orange betaxanthins. Betalains are restricted to plants of the Caryophylles family including Amaranthaceae and Cactaceae, as well as present in some higher fungi, such as *Amanita muscaria* L. They display not only prominent colouring attributes but also a huge variety of biological properties, for example, anticancer, antimicrobial, antiradical and antioxidant activities (Ciriminna et al., 2018; Khan and Giridhar, 2015; Laqui-Vilca et al., 2018; Moreno et al., 2008). The red-violet betacyanins are presently gaining popularity due to their pH stability ranging from 3 to 7, natural colouring feature (E-162), powerful antioxidant and health promoting functional properties (Leong et al., 2018c). Additionally, they are more stable with respect to betaxanthins in terms of their structural aspects (Azeredo, 2009). The most common betacyanins structure is betanin (betanidin-5-O-β-glucoside). For instance, red-purple pitaya (*Hylocereus polyrhizus*) is a promising source of betacyanins. It is a red-skinned fruit with red-purple flesh and black seeds (Dembitsky et al., 2011; Esatbeyoglu et al., 2015). Hence, betacyanins are known to be valued-added compounds in food science processing industries (Ciriminna et al., 2018; Khan and Giridhar, 2015; Moreno et al., 2008; Stintzing and Carle, 2007).

Conventional solid-liquid extraction procedures (maceration, infusion and Soxhlet extraction) are generally used for the betalains extraction from plants (Castellar et al., 2003; Chong et al., 2014; Gengatharan et al., 2016; Laqui-Vilca et al., 2018; Ramli et

al., 2014; Ravichandran et al., 2013). These methods having several shortcomings, such as time-, energy- and cost-consuming, solvent contamination of the products, not environment-friendly, stability problems as well as relatively low yields (Azmir et al., 2013; Leong et al., 2018a; Wang and Weller, 2006). As a consequence, green and effective separation approaches are deemed necessary to develop as alternative procedures for the betalains extraction. Indeed, aqueous two-phase system (ATPS) (Chandrasekhar et al., 2015; Chethana et al., 2007) and liquid biphasic flotation (LBF) system (Leong et al., 2018a) have been recently applied for the separation, purification and concentration of betalains from plants. Other than that, various extraction techniques like ultrasound (sonication) (Ramli et al., 2014), microwave (dielectric heating) (Bastos and Gonçalves, 2017), high pressure and supercritical fluid processing as well as pulsed electric field (PEF) (Fincan et al., 2004) have been used alongside conventional extraction methods for the betalains extraction. These techniques are proved to minimise the drawbacks of the conventional methods yet they are eco-friendly techniques (Chemat et al., 2017; Ciriminna et al., 2018; Laqui-Vilca et al., 2018; Xu et al., 2017).

In recent times, industrial biotechnology tools including protein engineering and downstream bioprocessing have been employed for the sustainable production of value-added products in food science processing industries (food, pharmaceutical, agricultural etc.). Meanwhile, development of a green, simple, economically viable and effective bioseparation technology is a rapidly growing field in the biotechnology industries (Chemat et al., 2017; Sankaran et al., 2018; Tang and Zhao, 2009; Xu et al., 2017). Results from our previously performed experiment (Leong et al., 2018a) (based on Chapter 3 in this thesis) demonstrated an optimal betacyanins extraction from red-purple pitaya with the utilisation of LBF system. LBF system is a bubbles-assisted

bioseparation method which composed of ATPS and solvent sublation (SS). It is a simple, cost-, energy- and time-effective, high efficient as well as scalable bioseparation technology. The LBF system has been widely applied in the separation of biomolecules, such as lipase, protein, natural products etc. (Bi et al., 2013; Bi et al., 2009; Leong et al., 2018a; Mathiazakan et al., 2016; Sankaran et al., 2018; Show et al., 2013). Therefore, in the present study, we proposed an idea to integrate LBF system with electricity supply in order to further enhance the LBF system for improving biomolecules separation. Electricity treatment not only ameliorates extraction efficiency of biomolecules but also acts as a green extraction technique.

Taking the above into account, this is the first study to investigate the betacyanins extraction from peel and flesh of red-purple pitaya using a new integration process of LBF and electric system (i.e. liquid biphasic electric flotation (LBEF) system). In this study, the betacyanins extraction using the optimised LBF system (Leong et al., 2018a) (previous experimental results according to Chapter 3 in this thesis) with electricity supplied was assessed with various operating parameters. It is worth noting that the LBEF system is a new integration process, and it is also of significance that the biomolecules separation was carried out for the first time with the employment of LBEF system. Moreover, comparison study of betacyanins extraction using the optimised LBF system and LBEF system with initial settings, colour characterisation and antioxidant activity were assessed.

5.3 Materials and methods

5.3.1 Materials

Red-purple pitaya was purchased from a local fruit stall at Semenyih, Selangor, Malaysia. Ultrapure water produced from Milli-Q integral water purification system (Merck, Darmstadt, Germany) was used throughout this experiment. Ethanol, dipotassium hydrogen phosphate (K_2HPO_4), sodium bicarbonate ($NaHCO_3$), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were purchased from R&M Chemicals (Selangor, Malaysia). Acetic acid (CH_3COOH) and sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$) were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was purchased from Fisher Scientific (Selangor, Malaysia). 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), Folin-Ciocalteu (F-C) reagent and gallic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA). All the above mentioned chemicals were of analytical grade (purity $>$ 95%). Graphitic electrodes which were modified from 2B pencil leads (diameter: 2 mm) were purchased from My Family Art & Stationery (Semenyih, Selangor, Malaysia).

5.3.2 Apparatus

The LBF apparatus is a customised glass column with a sintered glass disk (grade 4 (G4) porosity) (height: 20 cm, internal diameter (ID): 2 cm), and a bottom joint connected to the source of compressed air which was acquired from PLT Scientific (Puchong, Selangor, Malaysia). Air bubbles were generated when the gas passed through the sintered glass disk. A flow meter with a range of 0–60 cc/min (Dwyer,

USA) was used to control the air flow rate. A regulated dual direct current (DC) power supply (ATTEN APS3003S-3D, 30 V/3 A[*2]) (Mobicon-Remote Electronic, Petaling Jaya, Selangor, Malaysia) was used to supply electricity, and was kindly provided by the Department of Electrical and Electronic Engineering, University of Nottingham Malaysia Campus (as shown in **Figure 5-1**).

5.3.3 Processing of crude extract

The processing of crude extract was conducted in dim light condition to minimise its pigment losses. The peel and flesh of red-purple pitaya were firstly cut into small cubes, subsequently, they were blended into paste-like substances (i.e. fresh crude extract (FE)) using a blender (Tefal Blendforce, Triple'Ax Technology 400 Watt, Malaysia). To prepare dried crude extract (DE), the FE was freeze dried at -30 °C and 0.37 atm for 48 h using a freeze dryer (CHRIST Alpha 1-2 LDplus, Germany). After that, the freeze-dried crude extract was ground into powder using a grinder (Tefal Blendforce, Triple'Ax Technology 400 Watt, Malaysia). Both FE and DE of the peel and flesh of red-purple pitaya were stored at -20 °C for further use.

5.3.4 Betacyanins extraction with LBEF system

The LBEF system was created by equipping two graphitic electrodes (in which one acted as anode and another one as cathode) into the LBF apparatus; the electrodes were connected to a regulated dual DC power supply in order to supply electricity continuously (**Figure 5-1**). Based on our previously performed experiment, the optimised conditions in the alcohol/salt-based LBF system that achieved an optimal betacyanins extraction from red-purple pitaya was integrated with electric system (LBEF system). The operating parameters of the LBEF system, such as operation time,

voltage apply, position of graphitic electrodes and type of crude extract, were optimised for the betacyanins extraction from peel and flesh of red-purple pitaya (**Table 5-1**). One-factor-at-a-time (OFAT) approach was employed to investigate the effects of the parameters of LBEF system on the betacyanins extraction from aqueous phase (crude extract + salt-rich bottom phase) to alcohol-rich top phase. The initial settings of the LBEF system were 15 min of operation time, 3 V of voltage applied and graphitic electrodes were positioned at bottom phase. The experiment was conducted at room temperature (25 ± 1 °C).

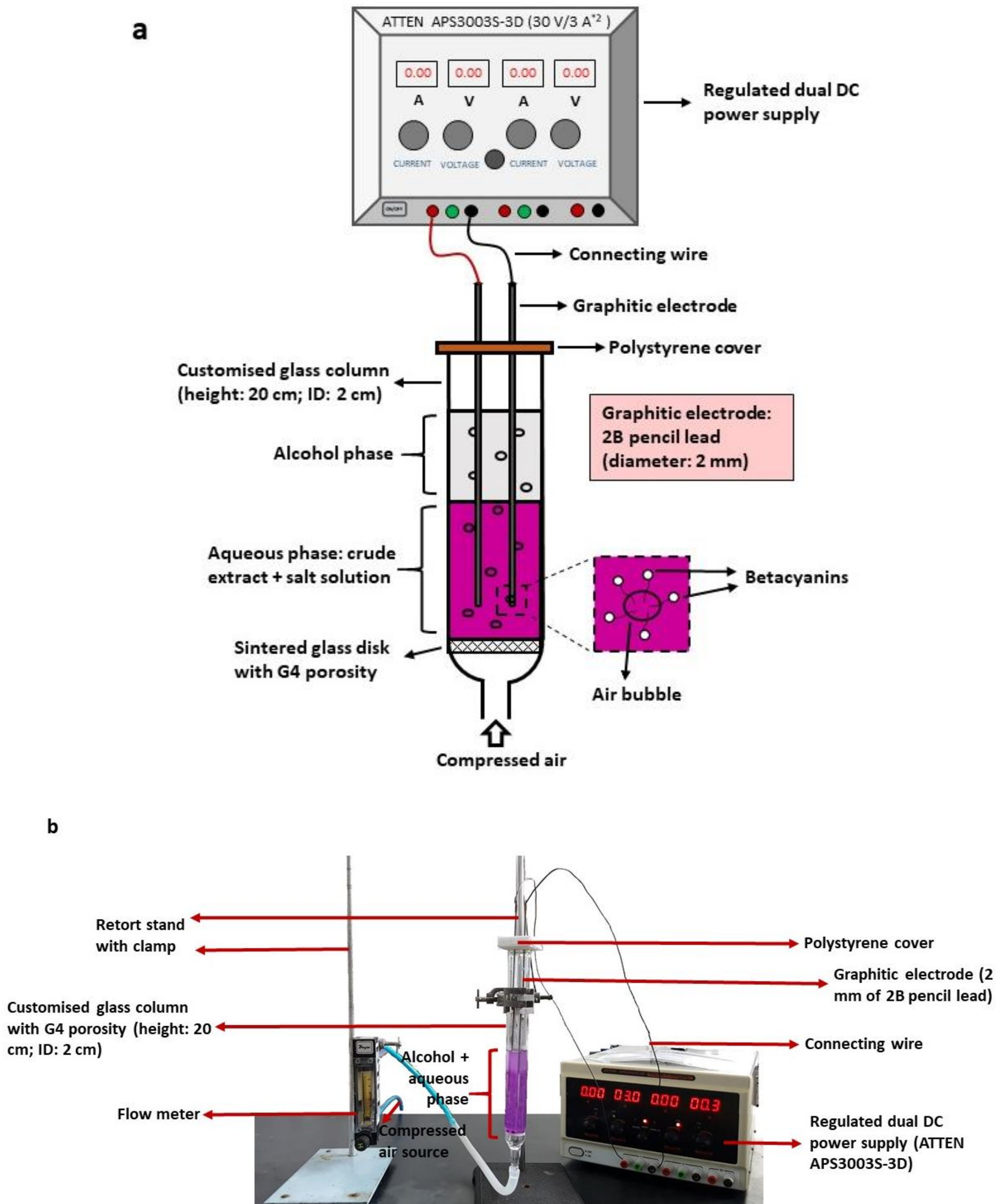


Figure 5-1: (a) Schematic diagram of LBEF system for betacyanins extraction from peel and flesh of red-purple pitaya; the LBF apparatus connected to a regulated dual DC power supply using two graphitic electrodes (anode and cathode; one to detect current flow and another one to control voltage applied) in order to supply electricity continuously. (b) Experimental setup for the betacyanins extraction using LBEF system.

Table 5-1: The operating parameters of LBEF system for betacyanins extraction from peel and flesh of red-purple pitaya.

No.	Condition	Initial setting	Variables	Unit	Justification
1.	Optimised LBF system from our previously performed experiment (constant throughout the experiment): 1 g of FE (peel or flesh of red-purple pitaya) 10 mL of undiluted ethanol (assumed as 100%) 20 mL of 200 g/L of K_2HPO_4 solution 20–30 cc/min of air flow rate				(Leong et al., 2018a)
2.	Operation time	15	5, 10, 20, 25	min	Initial setting: 15 min; as to compare with the optimised LBF system
3.	Voltage apply	3	1, 2, 4, 5	V	(Azmir et al., 2013)
4.	Position of graphitic electrodes	Bottom phase	Top and middle phase	N/A	Middle position: interphase between top and bottom phase
5.	Type of crude extract	1 g of FE	0.1 g of DE	-	(Leong et al., 2018a)

5.3.4.1 Comparison between LBF and LBEF system for betacyanins extraction

The betacyanins extraction from red-purple pitaya using the optimised conditions of LBF system was carried out according to our previously performed experiment. 1 g of FE (peel or flesh) was firstly mixed with 20 mL of 200 g/L of K_2HPO_4 solution, subsequently, they were poured into the LBF apparatus, and 10 mL of ethanol was added slowly. The flotation time and air flow rate were controlled at 15 min and 20–30 cc/min. On the other hand, LBEF system for the betacyanins extraction was conducted using the optimised LBF conditions and continuously supply of electricity, as mentioned in section 5.3.4 (LBEF system with initial settings).

5.3.5 Analytical procedures

5.3.5.1 Colour characterisation

Lightness (L^*), redness (a^*) and yellowness (b^*) of the peel and flesh extract of red-purple pitaya were assessed using a colorimeter (Lovibond LC 100, model RM 200, The Tintometer Ltd, United Kingdom). Additionally, their hue angle (h°) and chroma (C^*) were calculated according to equations (5-1) and (5-2), respectively.

$$h^\circ = \tan^{-1}\frac{b^*}{a^*} \qquad (5\text{-}1)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad (5\text{-}2)$$

5.3.5.2 Determination of total betacyanins content (TBC)

The TBC in the crude extract was analysed using a UV-vis spectrophotometer (UV-1800, Shimadzu Corporation, Japan) at 538 nm. The TBC was expressed as mg of betanin equivalents (BEs) per 100 g of crude extract, and was calculated according to equation (5-3) (Leong et al., 2018a; Ramli et al., 2014):

$$TBC = \frac{A_{538} \times MW \times V \times DF}{\varepsilon \times L \times W} \times 100 \qquad (5\text{-}3)$$

Where A_{538} = absorbance value at 538 nm, MW = molecular weight of betanin (550 $g.mol^{-1}$), V = volume of sample (mL), DF = dilution factor, ε = molar extinction coefficient of betanin (65000 $L.mol^{-1}.cm^{-1}$), L = path length of cuvette (1 cm), W = weight of crude extract (g)

5.3.5.3 Determination of total phenolic content (TPC)

The Folin-Ciocalteu (F-C) method as described in Hajimahmoodi et al. (2013) and Singleton et al. (1999) was employed to evaluate the TPC in the crude extract. A diluted F-C reagent which consists of 10 mL of F-C reagent and 90 mL of purified water was freshly prepared. Next, 100 μL of sample or gallic acid solution (i.e. standard) was mixed with 500 μL of diluted F-C reagent. The mixture was then incubated for 5 min at room temperature under dark condition. Subsequently, 2 mL of 60 g/L of $NaHCO_3$ solution was added to the mixture. The mixture was mixed well and kept for 90 min at room temperature under dark condition. The absorbance value of the mixture was measured at 725 nm using a UV-vis spectrophotometer. The TPC was expressed as mg of gallic acid equivalents (GAEs) per 100 g of crude extract.

5.3.5.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP in the crude extract was evaluated using method that described in literatures (Benzie and Strain, 1996; Leong et al., 2018a). The FRAP reagent was freshly prepared; 10 mL of 10 mmol/L TPTZ solution (0.0031 g of TPTZ in 1 mL of 40 mmol/L HCl) and 10 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution (0.0054 g/mL) for every 100 mL of 300 mmol/L acetate buffer (pH 3.6; mixture of 3.1 g of $C_2H_3NaO_2 \cdot 3H_2O$ with 16 mL of acetic acid per liter of purified water). The FRAP reagent was preheated to 37 °C before use. The FRAP evaluation was carried out by mixing 100 μL of sample or $FeSO_4 \cdot 7H_2O$ solution (i.e. standard), 300 μL of purified water and 3 mL of FRAP reagent. The mixture was subsequently incubated for 4 min at 37 °C. The absorbance value of mixture was measured at 593 nm using a UV-vis spectrophotometer. The result was expressed as μmol of Fe(II) per g of crude extract.

5.3.5.5 Trolox equivalent antioxidant capacity (TEAC) assay

The ABTS radical (ABTS•) method as described in Fu et al. (2011) and Re et al. (1999) was employed to evaluate the TEAC in the crude extract. The ABTS• stock solution (mixture of 7 mmol/L of ABTS solution and 2.45 mmol/L of $K_2O_8S_2$ solution at v:v ratio of 1:1) was first prepared, and was then incubated for 12–16 h at room temperature under dark condition. Next, the ABTS• stock solution was diluted with ethanol to reach an absorbance value of 0.70 ± 0.05 at 734 nm, and was incubated at 30 °C. In order to evaluate the TEAC, 100 µL of sample or trolox solution (i.e. standard) or ethanol (i.e. control) was mixed with 3.8 mL of diluted ABTS• solution. The absorbance value of the mixture was measured at 734 nm using a UV-vis spectrophotometer after 6 min of incubation at 30 °C. The result was expressed as µmol of trolox equivalents (TEs) per g of crude extract. The percentage of scavenging on ABTS• was calculated using equation (5-4) (Leong et al., 2018a):

$$\text{Percentage of scavenging (\%)} = \frac{\text{control} - \text{sample or standard}}{\text{control}} \times 100 \qquad (5\text{-}4)$$

5.3.6 Calculations

Partition coefficient (K) of betacyanins in LBF and LBEF system were calculated according to equation (5-5) (Chandrasekhar et al., 2015):

$$\text{K} = \frac{\text{TBC}_t}{\text{TBC}_b} \qquad (5\text{-}5)$$

Where TBC_t and TBC_b are TBC in the alcohol-rich top phase and salt-rich bottom phase at equilibrium, respectively.

Betacyanins concentrations (%) in alcohol-rich top phase (C_t) and salt-rich bottom phase (C_b) were calculated according to equations (5-6) and (5-7), respectively:

$$C_t(\%) = \frac{\text{TBC in top phase}}{\text{TBC in crude extract}} \times 100 = \frac{TBC_t}{TBC_t + TBC_b} \times 100 \quad (5\text{-}6)$$

$$C_b(\%) = \frac{\text{TBC in bottom phase}}{\text{TBC in crude extract}} \times 100 = \frac{TBC_b}{TBC_t + TBC_b} \times 100 \quad (5\text{-}7)$$

The betacyanins extraction from salt-rich bottom phase to alcohol-rich top phase in LBF and LBEF system were assessed by separation efficiency (E). The E (%) of betacyanins was calculated according to equation (5-8) (Leong et al., 2018a):

$$E\ (\%) = \left(1 - \frac{TBC_b}{TBC_{bi}}\right) \times 100 \quad (5\text{-}8)$$

Where TBC_{bi} and TBC_b are TBC in salt-rich bottom phase collected before and after the LBF and LBEF system, respectively.

5.3.7 Statistical analysis

The statistical analysis was evaluated based on triplicate experimental readings, and the values were expressed as mean ± standard deviation (SD) (n = 3). The experiment was run three times in order to further verify the results. The data were subjected to one-way analysis of variance (ANOVA), and the mean differences were compared using Tukey HSD post-hoc multiple comparisons test. The data were considered for their statistically significant difference where $p < 0.05$. Moreover, the relationship among the antioxidant activities was analysed using Pearson's correlation test. The statistical analysis was performed using IBM SPSS statistics software (SPSS version 23.0 for window, IBM Corporation, Armonk, New York, United States).

5.4 Results and discussion

5.4.1 Comparison study of betacyanins extraction using LBF and LBEF system

The betacyanins extraction from peel and flesh of red-purple pitaya were carried out using the optimised LBF system (Leong et al., 2018a) and an integration process of the optimised LBF and electric system (i.e. LBEF system with initial settings). From our observations, betacyanins extraction from the peel using the LBEF system achieved an optimal betacyanins extraction, as shown by the significantly highest values of C_t (99.014 ± 0.074%), E (98.383 ± 0.215%) and K (100.814 ± 7.324) of betacyanins compared to that of the LBF system (C_t: 95.989 ± 0.479%, E of betacyanins: 88.361 ± 1.708%, K of betacyanins: 24.168 ± 2.949) (**Figure 5-2a**). Likewise, a similar trend was noted for the betacyanins extraction from the flesh, as shown in **Figure 5-2b**. The betacyanins extraction from the flesh using the LBEF system showed the significantly highest values of C_t (96.132 ± 0.154%), E (96.576 ± 0.083%) and K (24.883 ± 1.052) of betacyanins compared to that of the LBF system (C_t: 95.488 ± 0.213%, E of betacyanins: 94.886 ± 0.060%, K of betacyanins: 21.195 ± 1.030). Collectively, application of the LBEF system showed a better betacyanins extraction compared to that of the utilisation of the optimised LBF system.

According to Leong et al. (2018a), in the alcohol/salt-based LBF system, salt solution acted as the first extractive phase and followed by alcohol as the second extractive phase for the betacyanins extraction. Particularly, flotation effect (gas bubbles) promotes the upflow of betacyanins from aqueous phase (salt-rich bottom phase) to alcohol-rich top phase. The betacyanins are adsorbed and adhered on the bubble surfaces of an ascending gas stream, which flowed from the aqueous phase, and subsequently to the alcohol-rich top phase in the column (Mathiazakan et al., 2016;

Pakhale et al., 2013; Sankaran et al., 2018; Show et al., 2013). On the other hand, LBEF system not only possesses the flotation effect but also demonstrates an electrical field influence on the betacyanins extraction. The electricity was passed through the aqueous and alcohol-rich top phase via the two graphitic electrodes located at the bottom phase in the LBEF system (bubbles were observed on the electrodes due to the voltage applied besides the flotation effect). This electrical field is proved to disrupt the cell membrane structure (increased cell permeability in releasing more betacyanins from red-purple pitaya) for increased extraction efficiency. Indeed, in the betacyanins extraction from the peel and flesh of red-purple pitaya using the LBEF system, the C_t, E and K of betacyanins values were greatly ameliorated; due to the flotation and electrical field effect (Azmir et al., 2013; Chemat et al., 2017; Roselló-Soto et al., 2015). Hence, the LBEF system was decided to further investigate for several operating parameters in this study.

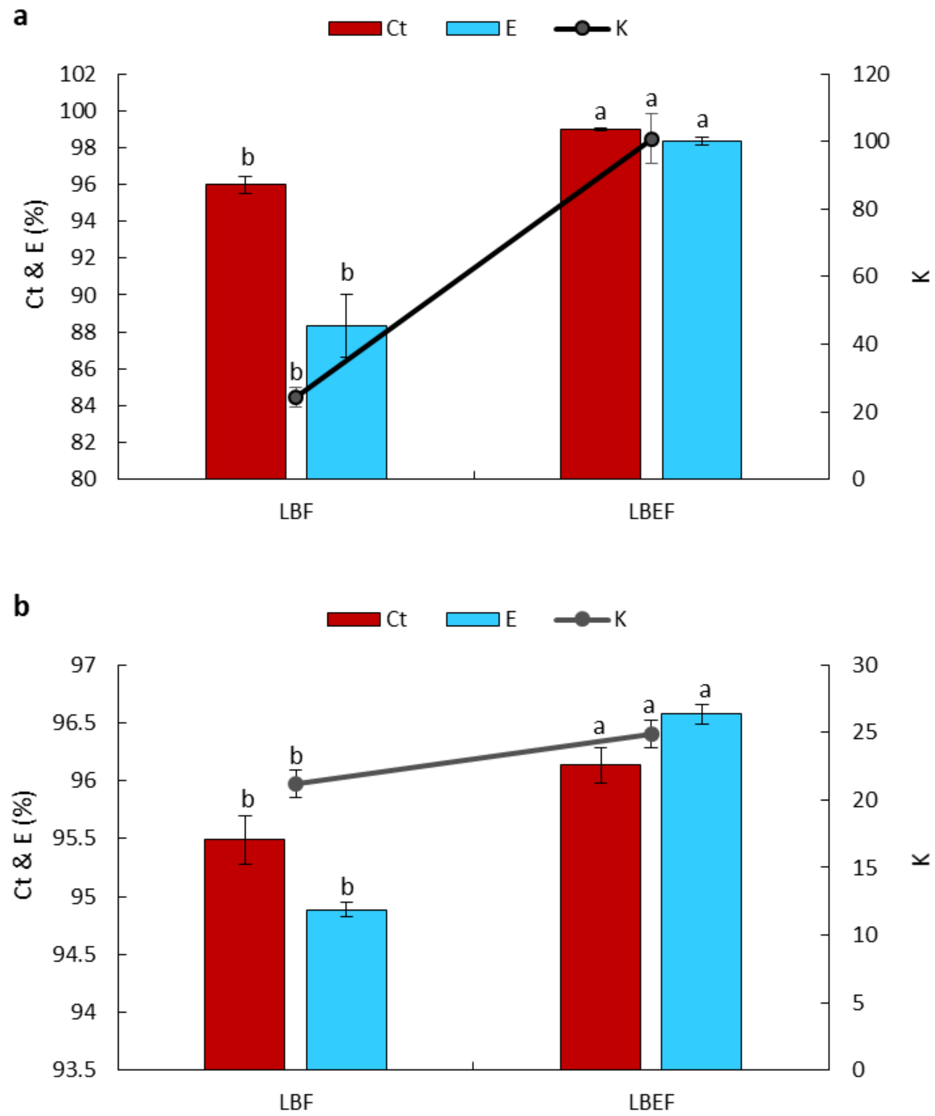


Figure 5-2: Comparison study of betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya using the optimised LBF system and LBEF system with initial settings. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

5.4.2 Effect of the operation time in LBEF system

The betacyanins extraction from peel and flesh of red-purple pitaya using the LBEF system with different operation times from 5 to 25 min were studied; other parameters of the LBEF system included optimised LBF system + 3 V of voltage applied + graphitic electrodes were positioned at bottom phase. The betacyanins extraction from the peel and flesh dramatically increased as the operation time increases from 5 to 15 min, which then slightly decreased for the subsequent operation time in the LBEF system until 25 min. Hence, the betacyanins extraction using the LBEF system with 15 min of operation time showed the significantly highest values of C_t (99.014 ± 0.074%), E (98.383 ± 0.215%) and K (100.814 ± 7.324) of betacyanins from the peel (**Figure 5-3a**), as well as the highest value of C_t (96.132 ± 0.154%), the significantly highest values of E (96.576 ± 0.083%) and K (24.883 ± 1.052) of betacyanins from the flesh (**Figure 5-4a**); E values for the peel between 15 and 20 min, as well as C_t values for the flesh among 15–25 min showed no significantly difference ($p > 0.05$). These highest values inferred an optimal betacyanins extraction, and thus, 15 min of operation time was chosen for the LBEF system.

An increment in the operation time of LBEF system from 5 to 15 min augmented the betacyanins extraction from the peel and flesh of red-purple pitaya. The highest betacyanins extraction was observed on 15 min of operation time in the LBEF system, whereas, further extension in the operation time up to 25 min resulted in slightly lower betacyanins extraction compared to that of with the 15 min of operation time. This might be due to the reason that the maximum betacyanins extraction has reached, and therefore, further increment in the operation time of beyond 15 min is unnecessary for effective betacyanins extraction; the mass transfer in the system has reached

equilibrium. Additionally, longer extraction time might cause the oxidation of betacyanins due to their highly sensitive properties (Khan, 2016; Khan and Giridhar, 2015; Leong et al., 2018a). From our previously performed experiment (Leong et al., 2018a), 15 min of flotation time in the LBF system also showed the maximum betacyanins extraction from peel and flesh of red-purple pitaya.

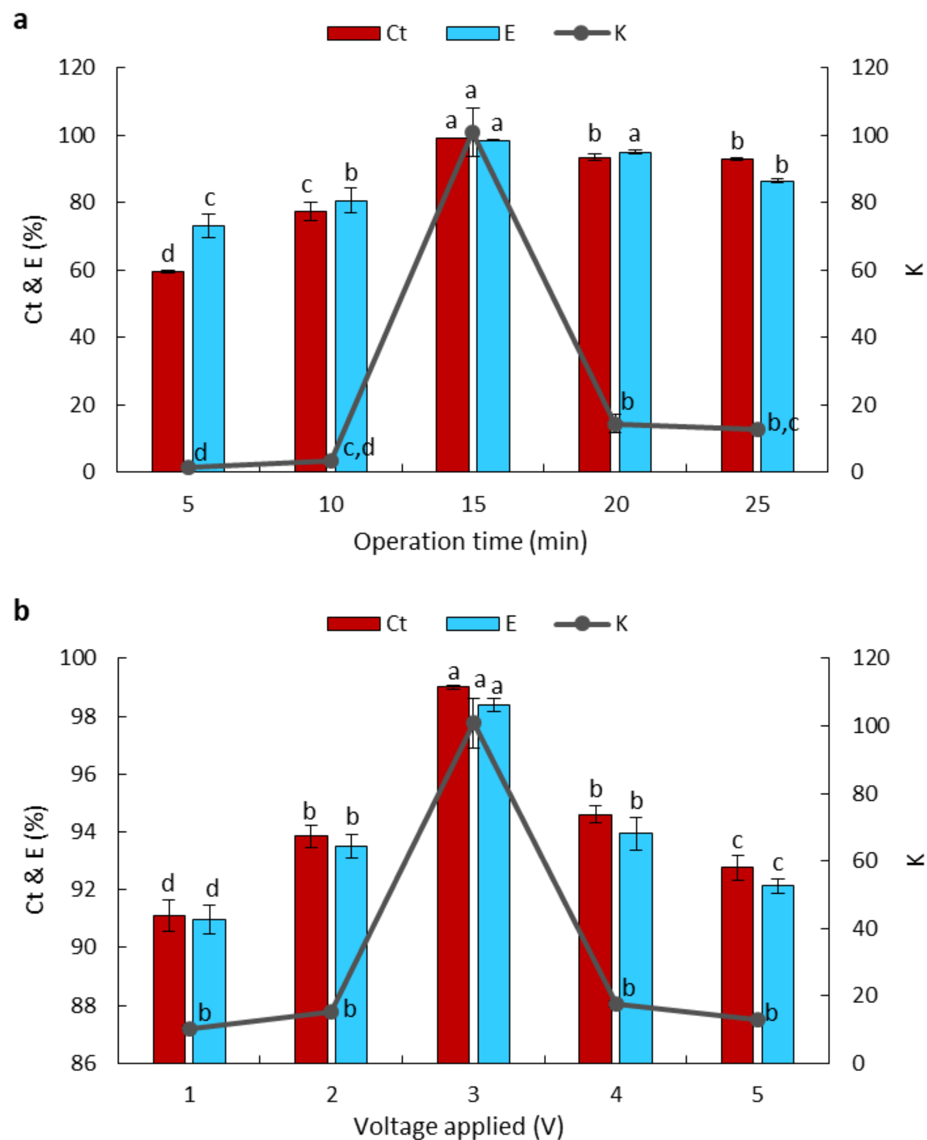
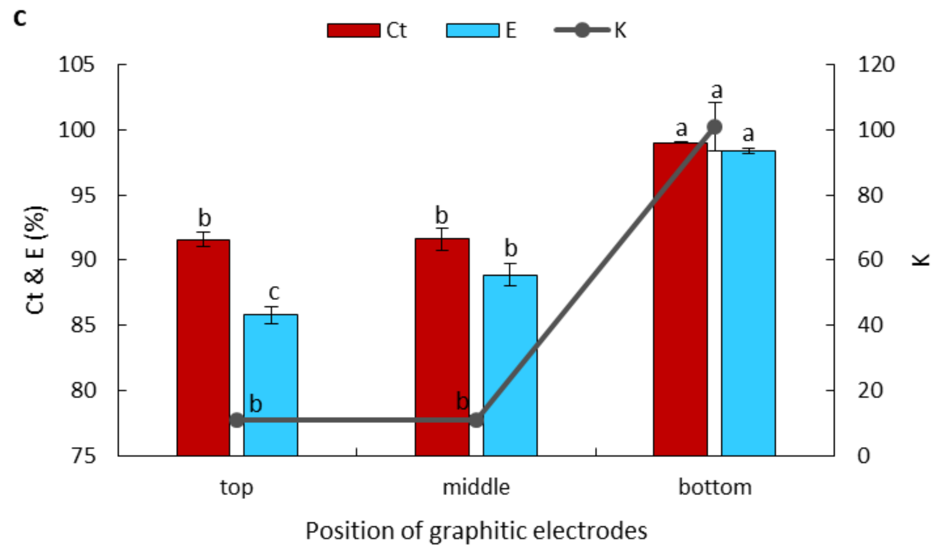


Figure 5-3: Effects of several parameters in LBEF system for betacyanins extraction from peel of red-purple pitaya. OFAT approach was used in the optimisation study: (a) operation time, (b) voltage applied, (c) position of graphitic electrodes and (d) type of crude extract. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.



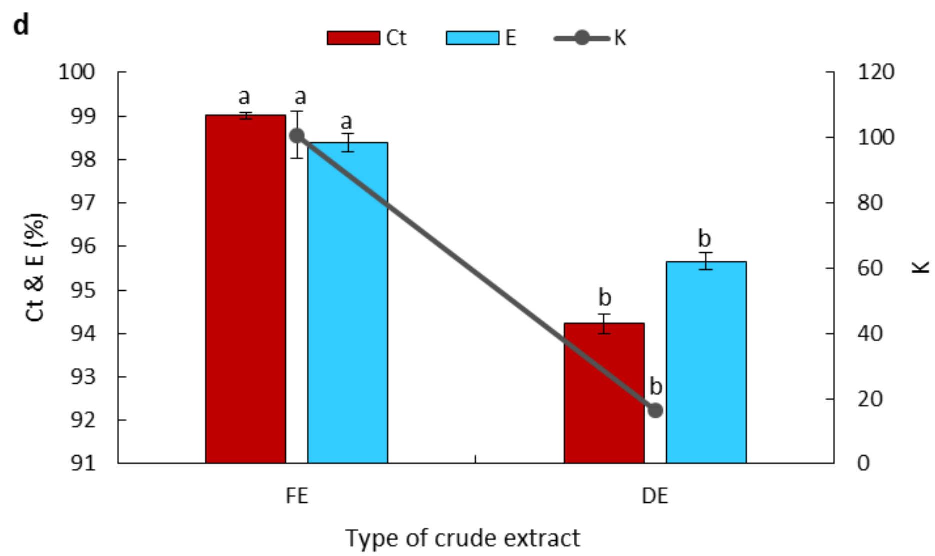


Figure 5-3 continued.

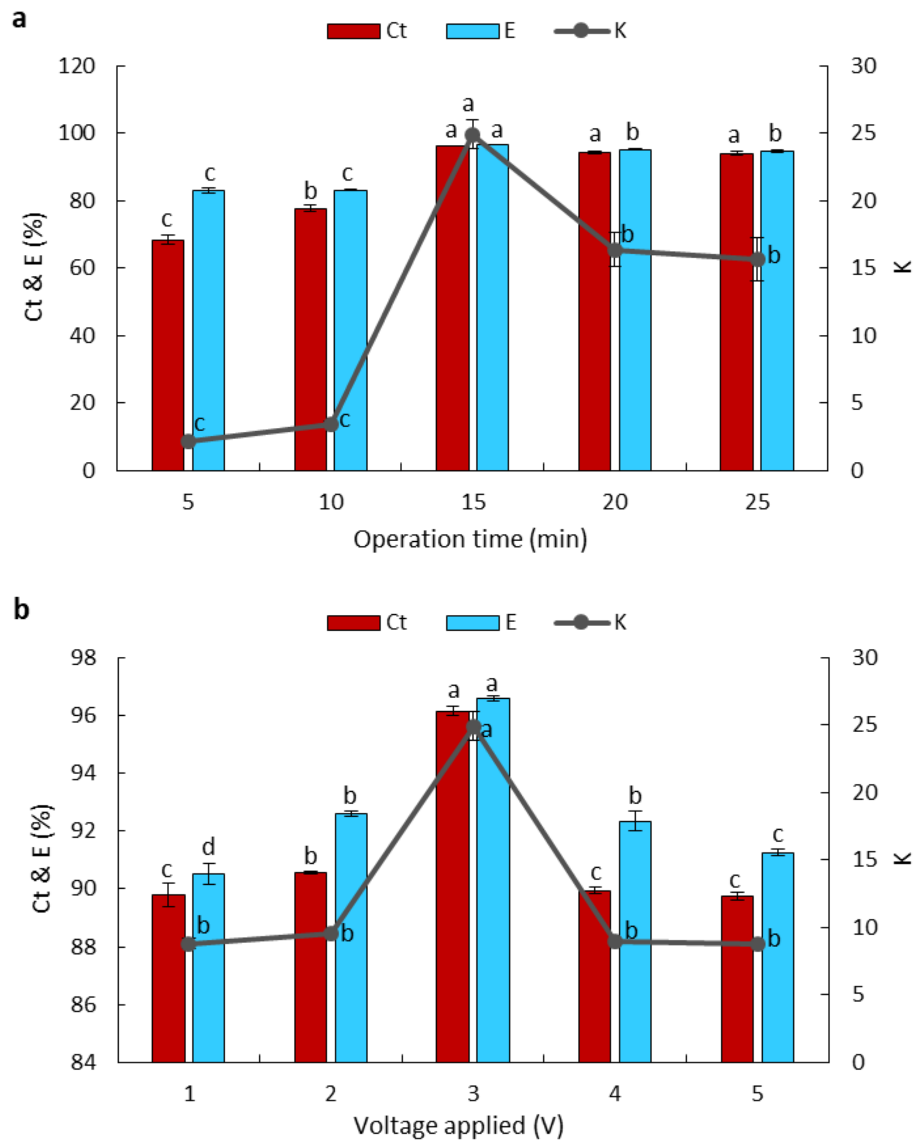


Figure 5-4: Effects of several parameters in LBEF system for betacyanins extraction from flesh of red-purple pitaya. OFAT approach was used in the optimisation study: (a) operation time, (b) voltage applied, (c) position of graphitic electrodes and (d) type of crude extract. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within C_t, E and K.

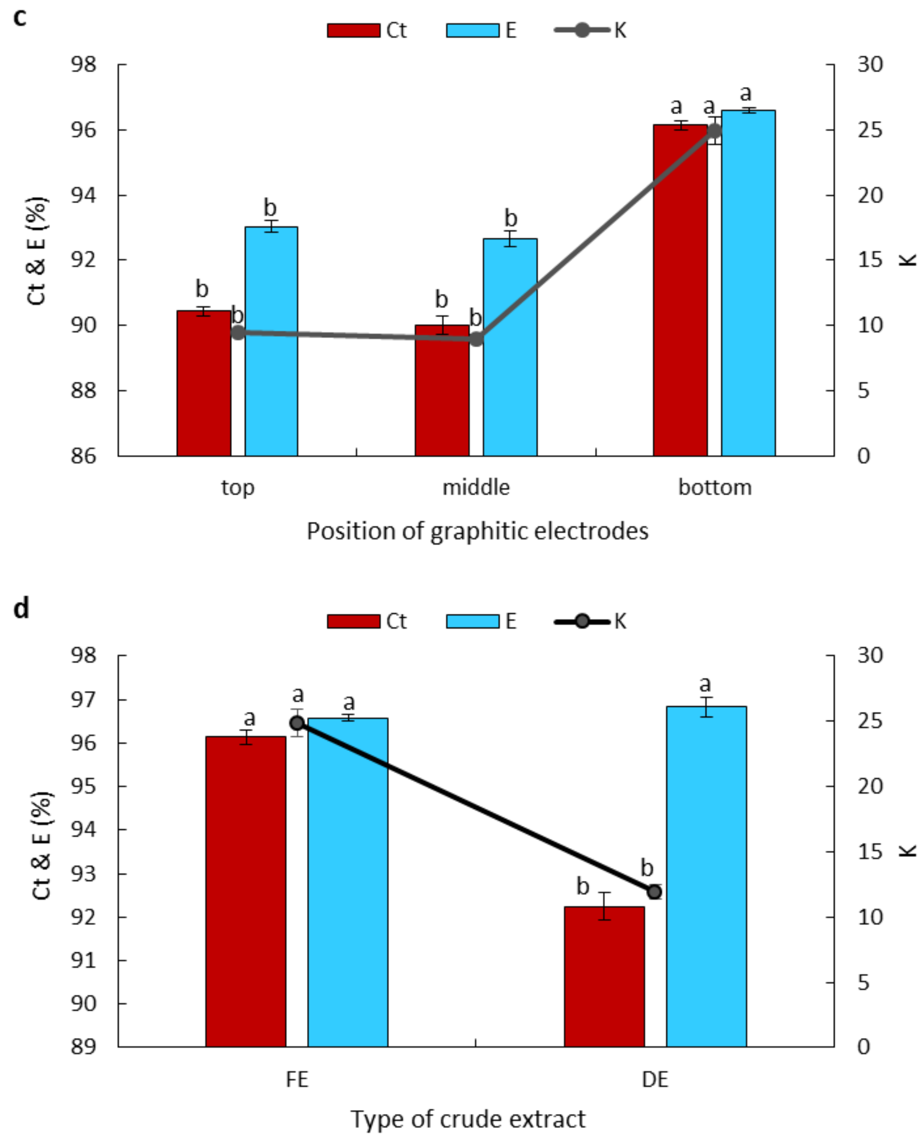


Figure 5-4 continued.

5.4.3 Effect of the voltage applied in LBEF system

The effect of varying voltages applied range from 1 to 5 V in the LBEF system for the betacyanins extraction was investigated after optimising the operation time, and the results are shown in **Figures 5-3b, 5-4b**. An increase in the voltage applied in the LBEF system from 1 to 3 V significantly increased the betacyanins extraction from the peel and flesh, and further increasing in the voltage applied up to 5 V showed a

significant decrement in the betacyanins extraction. A possible explanation for this might be that the mass transfer in the system has achieved equilibrium, and further electricity supplied seem to be unnecessary for this process. Therefore, the LBEF system with 3 V of voltage applied was noted to provide the significantly highest values of C_t, E and K of betacyanins, which revealed in an optimal betacyanins extraction for the peel (99.014 ± 0.074%, 98.383 ± 0.215% and 100.814 ± 7.324, respectively) (**Figure 5-3b**) and flesh (96.132 ± 0.154%, 96.576 ± 0.083% and 24.883 ± 1.052, respectively) (**Figure 5-4b**).

As mentioned earlier in the section 5.4.1, the electricity supplied in the LBEF system caused an electropermeabilisation of red-purple pitaya membrane structure, and hence improved the betacyanins extraction from red-purple pitaya. Increased membrane structure permeability ensures more betacyanins to be released from red-purple pitaya to the surrounding environment. Electrical field treatment is a non-thermal process where an external electric field is applied to a substance. The electricity supplied in this study was determined by the voltage applied (1–5 V). An electrical field passes through the membrane structure, and causes the membrane disruption which due to the dipole nature of the membrane molecules. Electrical field treatment also increases the mass transfer energy of the system. Electropermeabilisation enhances the extraction efficiency of the treated sample because it improves the release of intracellular compounds from the treated sample to the extractive solvent. Moreover, the effectiveness of the electricity treatment strongly depends on the electrical field strength, specific energy input, treatment time, substance to be treated etc. (Azmir et al., 2013; Chemat et al., 2017; Xu et al., 2017).

Roselló-Soto et al. (2015) studied the extraction of pretreated olive kernels with high voltage electrical discharges (HVED), PEF and ultrasound. They reported that HVED treatment as the most effective treatment (highest extraction efficiency) among the others, and it might be possibly due to the propagation of the shock waves and explosion of cavitation bubbles which caused by the application of electrical discharges.

5.4.4 Effect of the position of graphitic electrodes in LBEF system

Subsequently, the LBEF system with 15 min of operation time and 3 V of voltage applied for the betacyanins extraction was further studied using different positions of graphitic electrodes in the system. The electrodes were positioned at top, middle and bottom phase in the LBEF system, and the results are presented in **Figures 5-3c, 5-4c**. According to our findings, the betacyanins extraction using LBEF system with the electrodes positioned at the bottom phase showed a significant highest values of C_t, E and K of betacyanins for peel (99.014 ± 0.074%, 98.383 ± 0.215% and 100.814 ± 7.324, respectively) (**Figure 5-3c**) and flesh (96.132 ± 0.154%, 96.576 ± 0.083% and 24.883 ± 1.052, respectively) (**Figure 5-4c**) of red-purple pitaya. Whereas, the C_t, E and K of betacyanins values for the LBEF system with the electrodes positioned at the top and middle phase showed no significantly difference, except for the E of betacyanins values for the peel.

The possible explanation for these results obtained might be that there is more betacyanins rose through the column in the LBEF system via the flotation effect; the electrodes that were positioned at the bottom phase improved the betacyanins extraction in the aqueous phase through the electrical field influence. On the other hand, the electrodes that were positioned at the top and middle phase restricted the

betacyanins extraction from the aqueous phase, and hence reduced the betacyanins rose through from aqueous phase to alcohol-rich top phase in the column.

5.4.5 Effect of the type of crude extract in LBEF system

Different types of crude extract of peel and flesh of red-purple pitaya, i.e. 1 g of FE and 0.1 g of DE, were assessed for their betacyanins extraction in our optimised LBEF system, which consists of 15 min of operation time, 3 V of voltage applied and the graphitic electrodes were positioned at bottom phase. As comparing between the FE and DE of the peel and flesh of red-purple pitaya, the FE was seen to provide a better betacyanins extraction, owing to their significantly highest values of C_t (99.014 ± 0.074% and 96.132 ± 0.154%), E (98.383 ± 0.215% and 96.576 ± 0.083%) and K (100.814 ± 7.324 and 24.883 ± 1.052) of betacyanins; E of betacyanins values between the FE and DE of the flesh showed no significantly difference (**Figures 5-3d, 5-4d**). This could be explained by both electrical and flotation effect are more favourably exerted in the fresh crude extract compared to the dried crude extract. A higher electropermeabilisation might be achieved in the fresh sample of the red-purple pitaya, which ameliorated the betacyanins extraction. From our previously performed experiment (Leong et al., 2018a), the FE of red-purple pitaya in the LBF system also showed the maximum betacyanins extraction.

5.4.6 Colour characterisation

The peel and flesh extract of red-purple pitaya obtained from the optimised LBEF system (alcohol-rich top phase) were further assessed for their colour characterisation. Their colour are presented in different degrees of lightness (L^*), redness (a^*), yellowness (b^*), chroma (C^*) and hue angle (h°). The flesh extract showed a higher

degree of a^*, b^* and C^*, as well as a smaller degree of L^* and h° compared to that of the peel extract (**Table 5-2**). Nevertheless, both the peel and flesh extract were in the range of red-bluish, owing by positive a^* and negative b^* value. They only presented in different saturations and intensities of the red-bluish colour. As compared with the peel and flesh extract of red-purple pitaya obtained from the optimised LBF system (i.e. our previously performed experiment), all of them are in the same range of colour (red-bluish), and this proves that the electricity treatment did not degrade the colour of the extract. Due to their natural colouring attribute, the peel and flesh extract of red-purple pitaya could be a potential replacement for artificial red colouring.

Table 5-2: Colour characterisation of peel and flesh extract of red-purple pitaya obtained from the optimised LBEF system.

Colour parameter	Peel extract	Flesh extract
L^*	41.433 ± 0.115	32.400 ± 0.100
a^*	10.800 ± 0.000	29.267 ± 0.058
b^*	-4.267 ± 0.058	-26.333 ± 0.252
C^*	11.633 ± 0.058	39.400 ± 0.200
h°	338.367 ± 0.289	318.00 ± 0.265

L^*, a^*, b^*, C^* and h° represent lightness, redness, yellowness, chroma and hue angle, respectively.
Values are mean ± SD of triplicate readings.

5.4.7 Antioxidant properties analysis

Furthermore, antioxidant activity on the peel and flesh extract obtained from the optimised LBEF system (alcohol-rich top phase) were evaluated. They provided different antioxidant values. In the FRAP assessment, the flesh extract (7.202 ± 0.115 μmol of Fe(II)/g of crude extract) showed a significant higher FRAP value compared to that of the peel extract (6.897 ± 0.127 μmol of Fe(II)/g of crude extract) (standard equation: $A_{593} = 0.0006([FeSO_4 \cdot 7H_2O]) + 0.0708$ ($R^2 = 0.9984$); A_{593}: absorbance value at 593 nm & $[FeSO_4 \cdot 7H_2O]$: concentration of $FeSO_4 \cdot 7H_2O$). On the other hand,

the peel and flesh extract showed a non-significant TEAC values of 18.653 ± 0.319 and 18.968 ± 0.403 µmol of TEs/g of crude extract, respectively ($p > 0.05$) (standard equation: Percentage of scavenging (%) = 0.0966([trolox]) + 2.8333 (R^2 = 0.9946)). In addition, the peel and flesh extract showed a non-significant TPC values of 40.787 ± 1.061 and 34.956 ± 4.736 mg of GAEs/100 g of crude extract, respectively, in the TPC evaluation (standard equation: A_{725} = 0.0036([gallic acid]) + 0.0086 (R^2 = 0.9995)). On the other hand, the flesh extract (15.649 ± 0.226 mg of BEs/100 g of crude extract) showed a significant higher TBC value compared to that of the peel extract (8.067 ± 0.420 mg of BEs/100 g of crude extract) (**Figure 5-5**). As compared with our previously performed experiment on the antioxidant activity analysis using the peel and flesh extract of red-purple pitaya obtained from the optimised LBF system (Leong et al., 2018a), all of them are well retained in the antioxidant properties although they showed slightly different in the antioxidant values; the peel and flesh extract in this study showed slightly lower antioxidant values, and this might be due to the batch variation issues.

In the antioxidant activity assessment, the values of FRAP and TEAC are those in measuring the antioxidant capacity of a compound. Their antioxidant capacity evaluation are based on the reducing ability of an oxidant probe (colour change of the mixture being observed); oxidant probes used in the analyses of FRAP and TEAC were TPTZ and ABTS radical cation, respectively. On the other hand, TPC and TBC analysis are used to measure the presence of antioxidant compound; polyphenols and betacyanins, respectively (Dai and Mumper, 2010; Xu et al., 2017). Our results inferred that both the peel and flesh of red-purple pitaya demonstrated antioxidant properties, and therefore, they have health promoting functional feature. Red-purple

pitaya not only can be used as natural colourant but also can be consumed by us in keeping healthy.

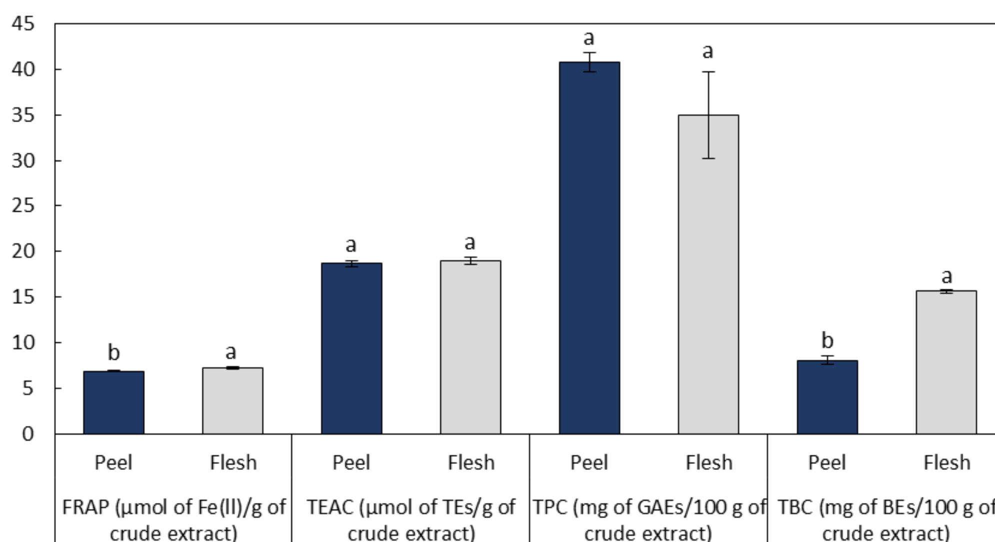


Figure 5-5: Antioxidant activity evaluation of peel and flesh extract of red-purple pitaya obtained from the optimised LBEF system. Values are mean ± SD of triplicate experimental readings. Different letter(s) represent a significant different (*p* < 0.05) using Tukey's test within FRAP, TEAC, TPC and TBC.

Also, the relationship among the antioxidant activities (i.e. FRAP, TEAC, TPC and TBC) was performed using Pearson's correlation test. The closer the correlation coefficient (r) value to 1 indicates a stronger correlation between them. Our results revealed that TEAC and FRAP, TPC and TEAC showed a non-significant weak positive correlation, with r values of 0.207 and 0.208, respectively. In contrast, TPC and FRAP (r = -0.794), TBC and TPC (r = -0.701) showed a non-significant moderate negative correlation, while TBC and TEAC showed a non-significant moderate positive correlation (r = 0.512). A significant strong positive correlation was observed on TBC and FRAP (r = 0.838, $p < 0.05$) (**Table 5-3**).

Table 5-3: Correlation study among antioxidant activities, i.e. FRAP, TEAC, TPC and TBC, given correlation coefficient (r).

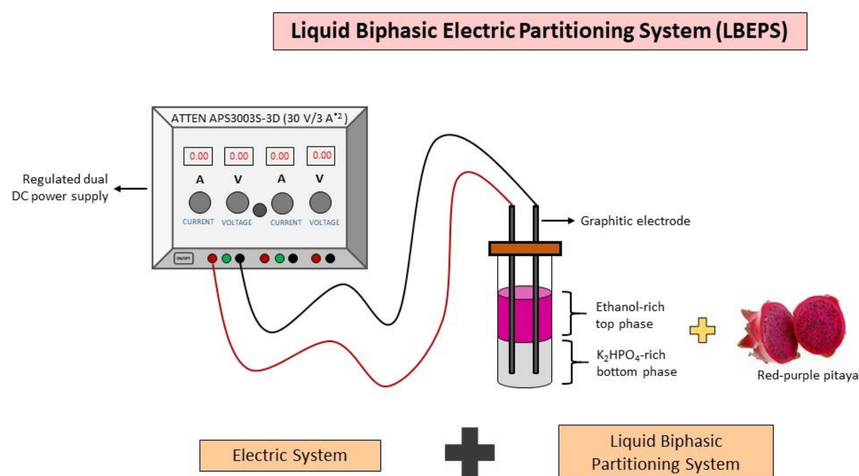
	TEAC	TPC	TBC
FRAP	0.207	-0.794	0.838*
TEAC		0.208	0.512
TPC			-0.701

* Correlation is significant at $p < 0.05$ (2-tailed).

5.5 Concluding remarks

The present study concludes that a satisfactory betacyanins extraction from the peel and flesh of red-purple pitaya were successfully achieved using the LBEF system (i.e. the optimised LBF conditions + 15 min of operation time + 3 V of voltage applied + graphitic electrodes were positioned at bottom phase). Additionally, the peel and flesh extract showed different variations of red colour, and they are well retained in their antioxidant activities. This study offers a new, simple and high efficient green bioseparation technology for the downstream processing industries, which is significantly worth to explore further. A pilot scale LBEF system on the biomolecules separation could also be further studied, owing to its highly potential to serve as an effective bioseparation technology.

CHAPTER 6: Integration Process for Betacyanins Extraction from Peel and Flesh of *Hylocereus polyrhizus* using Liquid Biphasic Electric Partitioning System and Antioxidant Activity Evaluation



This chapter has been submitted to journal of *Frontiers of Chemistry* and being under reviewed:

Hui Yi Leong, Yu-Kaung Chang, Chien Wei Ooi, Chung Lim Law, Advina Lizah Julkifle, Pau Loke Show*. "Liquid biphasic electric partitioning system as a novel integration process for betacyanins extraction from red-purple pitaya and antioxidant properties assessment"

6.1 Abstract

In recent times, downstream processing industries are inclined toward the development of a green and high efficient bioseparation technology. Betacyanins are presently gaining higher interest in the food science processing applications as driven by their high tinctorial strength and health promoting functional properties. In this study, a novel green integration process of liquid biphasic partitioning and electric system, namely liquid biphasic electric partitioning system (LBEPS) was proposed for betacyanins extraction from peel and flesh of red-purple pitaya. Initially, the betacyanins extraction using LBEPS with initial settings was compared with that LBPS (previous experimental results according to Chapter 4 in this thesis), and the results revealed that both systems demonstrated a comparable betacyanins extraction. This was followed by further optimising the LBEPS for better betacyanins extraction. Several operating parameters including operation time, voltage applied and position of graphitic electrodes in the system were investigated in this study. Moreover, comparison between the optimised LBEPS and LBPS with the optimised conditions of electric system (as post-treatment) as well as colour characterisation and antioxidant properties assessment were conducted. Overall, the betacyanins extraction employing the optimised LBEPS showed the significantly highest values of betacyanins concentration in alcohol-rich top phase (C_t) and partition coefficient (K) of betacyanins from peel (99.256% and 133.433) and flesh (97.189% and 34.665) of red-purple pitaya. These results inferred that an optimal betacyanins extraction was successfully achieved by this approach. Also, the LBEPS with the peel and flesh showed phase volume ratio (V_r) values of 1.667 and 2.167, respectively, and this indicated that they have a clear biphasic separation. In addition, the peel and flesh extract obtained from the optimised LBEPS demonstrated different variations of red colour as well as their

antioxidant properties were well retained. This study introduces a new, reliable and effective bioseparation approach, namely LBEPS for the extraction of biomolecules, which is definitely worth to explore further as a bioseparation tool in the downstream processing.

Keywords: antioxidant, betacyanins, electric system, integration process, liquid biphasic partitioning system, red-purple pitaya

6.2 Introduction

Lately, betacyanins are of growing interest in the applications of food science processing, such as foods, nutraceuticals and pharmaceuticals, owing to their versatile properties including attractive visual attributes, pigments stability between pH 3 to 7, natural colouring feature (E-162), powerful antioxidant and health promoting functional properties (Ciriminna et al., 2018; Leong et al., 2018c). The most common and simplest structure of betacyanin found in plants is betanin, also known as betanidin-5-O-β-glucoside. Betacyanins are red-violet pigments which is an important constituent of betalains. Betalains are water-soluble nitrogen-containing natural pigments that have presently received attention as a source of natural colourant (Aberoumand, 2011; Carocho et al., 2015). The pigments contain a chromophore of betalamic acid, in which its conjugation with *cyclo*-3,4-dihydroxyphenylalanine can produce the red-violet betacyanins, whereas yellow-orange betaxanthins can be synthesised through the conjugation of betalamic acid with different amino acids or amines. As compared between betacyanins and betaxanthins, betacyanins are known to be more stable in terms of their structural aspects (Azeredo, 2009). One of the rich sources of betacyanins is red-purple pitaya (*Hylocereus polyrhizus*), in addition to red beetroot and other Caryophyllales. Red-purple pitaya is a type of *Hylocereus* species which belongs to the family of Cactaceae. It is a red-skinned fruit with red-purple flesh and black seeds. Additionally, red-purple pitaya is high in nutritional contents besides as a promising source of betacyanins, and hence, it possess positive effects on health (Ciriminna et al., 2018; Dembitsky et al., 2011; Esatbeyoglu et al., 2015; Khan and Giridhar, 2015; Moreno et al., 2008; Stintzing and Carle, 2007).

Extraction of betalains from various plant sources normally utilise conventional solid-liquid extraction approaches, such as maceration and Soxhlet extraction (Castellar et al., 2003; Celli and Brooks, 2017; Chong et al., 2014; Ramli et al., 2014). These extraction procedures are reported to have limitations, for example, inefficient, time-, energy- and cost-consuming, lower yields production as well as not eco-friendly (Azmir et al., 2013; Ciriminna et al., 2018; Dai and Mumper, 2010; Wang and Weller, 2006). Development of a green, reliable, economically effective and high efficient bioseparation technology is now a rapidly growing field in biotechnology industries including downstream processing industries (Chemat et al., 2017; Sankaran et al., 2018; Tang and Zhao, 2009). To address this need, non-conventional innovative extraction techniques for the betalains extraction have been recently developed. Indeed, several green extraction techniques, such as ultrasound (Laqui-Vilca et al., 2018; Ramli et al., 2014), microwave (Bastos and Gonçalves, 2017), pulsed electric field (Fincan et al., 2004) and high pressure CO_2 (Ciriminna et al., 2018) have been applied along with conventional extraction methods for the betalains extraction, and a better extraction efficiency of betalains yet reduced extraction time were reported (Celli and Brooks, 2017; Xu et al., 2017). Other than that, application of a liquid biphasic system, such as aqueous two-phase system (ATPS) (Chandrasekhar et al., 2015; Chethana et al., 2007; Santos et al., 2018) and liquid biphasic flotation (LBF) system (Leong et al., 2018a) for the separation, purification and concentration of betalains from plants have been studied. The liquid biphasic system is well-known as an easy, scalable, time-, cost- and energy-saving, effective as well as mild and green separation approach for many biotechnological products (Show et al., 2013; Yau et al., 2015; Zimmermann et al., 2017).

Taking the above into account, in the present study, an integration process of liquid biphasic partitioning and electric system, namely liquid biphasic electric partitioning system (LBEPS) was proposed for the betacyanins extraction from red-purple pitaya. In this study, both peel and flesh of red-purple pitaya were fully utilised for their betacyanins extractions. Liquid biphasic partitioning system (LBPS) is a new and green liquid biphasic system for separation of biomolecules as well as possesses the gifted advantages of the current liquid biphasic system. The LBPS was integrated with electricity treatment in this study as to further enhance the LBPS for improving biomolecules separation. The betacyanins extraction using LBEPS was first compared with that of using LBPS (previous experimental results according to Chapter 4 in this thesis), and then followed by optimisation study on the LBEPS for the betacyanins extraction. In addition, comparison between the optimised LBEPS and LBPS with the optimised conditions of electricity treatment (as post-treatment) as well as colour characterisation and antioxidant properties assessment were carried out. To the best of our knowledge, this is the first article in reporting the betacyanins extraction process by employing LBEPS. It is worth noting that the LBEPS is a novel green integration process, and it is also of significance that the separation of biomolecules was performed for the first time using LBEPS.

6.3 Materials and methods

6.3.1 Materials

Red-purple pitaya was purchased from a local fruit stall at Semenyih, Selangor, Malaysia. Ultrapure water produced from Milli-Q integral water purification system (Merck, Darmstadt, Germany) was used throughout this experiment. Graphitic electrodes which were modified from 2B pencil leads (diameter: 2 mm) were acquired

from My Family Art & Stationery (Semenyih, Selangor, Malaysia). Absolute ethanol, dipotassium hydrogen phosphate (K_2HPO_4), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and iron (II) sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) were purchased from R&M Chemicals (Selangor, Malaysia). Acetic acid (CH_3COOH) and sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$) were obtained from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) was purchased from Fisher Scientific (Selangor, Malaysia). 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate ($K_2O_8S_2$), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox), Folin-Ciocalteu (F-C) reagent and gallic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA). All the above mentioned chemicals were of analytical grade (purity > 95%).

6.3.2 Apparatus

A regulated dual direct current (DC) power supply (ATTEN APS3003S-3D, 30 V/3 A^{*2}) (Mobicon-Remote Electronic, Petaling Jaya, Selangor, Malaysia) was used to supply electricity in this experiment, and was kindly provided by the Department of Electrical and Electronic Engineering, University of Nottingham Malaysia Campus.

6.3.3 Processing of crude extract

The processing of crude extract was conducted in dim light condition in order to minimise its pigment losses. The peel and flesh of red-purple pitaya were firstly cut into thin pieces after washed and dried with tissue towel, subsequently, they were stored at -80 °C for 48 h. To prepare dried crude extract (DE), the sample was freeze dried at -30 °C and 0.37 atm for 48 h using a freeze dryer (CHRIST Alpha 1-2 LDplus,

Germany). After that, the freeze-dried crude extract was ground into powder using a grinder (Tefal Blendforce, Triple'Ax Technology 400 Watt, Malaysia). The DE of the peel and flesh of red-purple pitaya were stored at -20 °C for further use.

6.3.4 Betacyanins extraction with LBEPS

The LBEPS was created by equipping two graphitic electrodes into the LBPS. The electrodes (anode and cathode; former to apply voltage and latter to detect current flow) were connected to a regulated dual DC power supply in order to supply electricity continuously (**Figure 6-1**). A 10 g LBPS with optimised conditions was further incorporated with electric system (i.e. LBEPS). The operating parameters of the 10 g LBEPS including operation time, voltage apply and position of graphitic electrodes were optimised using one-factor-at-a-time (OFAT) approach for the betacyanins extraction from peel and flesh of red-purple pitaya. The initial settings of the LBEPS were 15 min of operation time, 3 V of voltage applied and graphitic electrodes were positioned at bottom phase (**Table 6-1**). The experiment was conducted at room temperature (25 ± 1 °C).

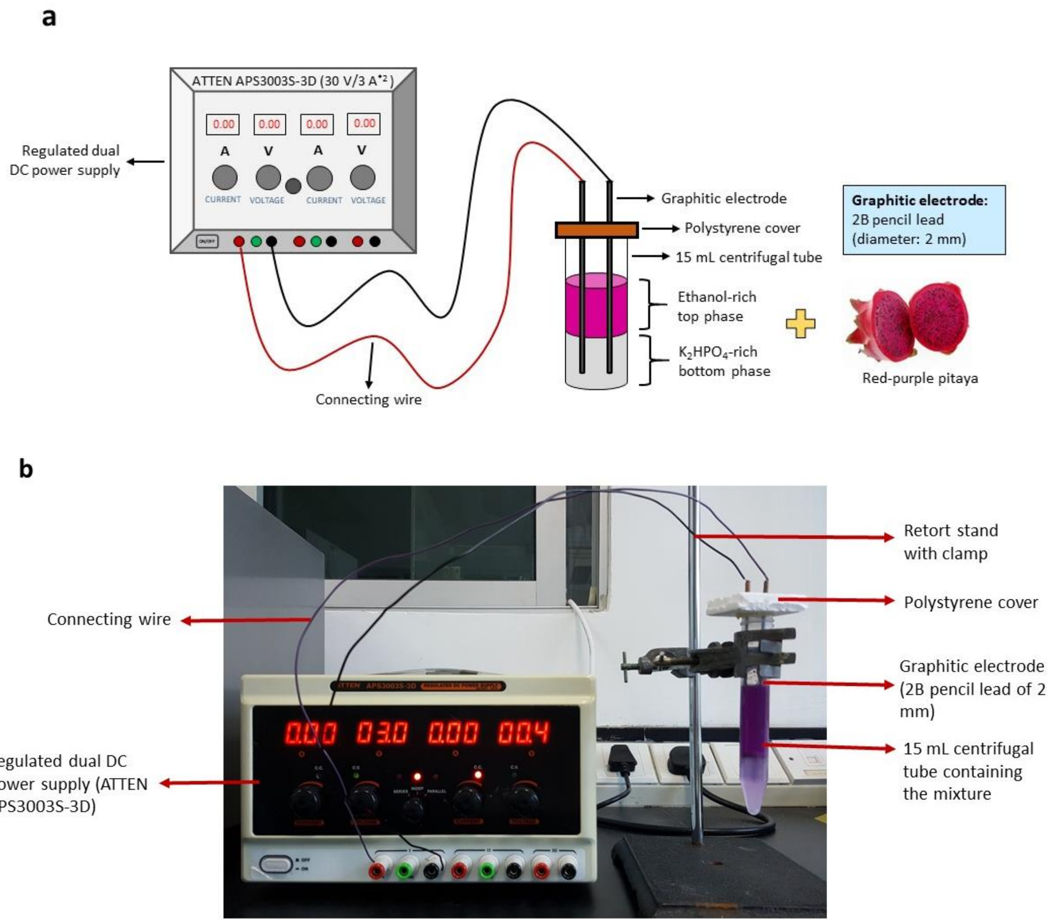


Figure 6-1: (a) Schematic diagram of LBEPS for betacyanins extraction from peel and flesh of red-purple pitaya. (b) Experimental setup for the betacyanins extraction using LBEPS.

Table 6-1: The operating parameters of LBEPS for betacyanins extraction from peel and flesh of red-purple pitaya.

No.	Condition	Initial setting	Variables	Unit
1.	10 g LBPS with optimised conditions (constant throughout the experiment) (%, w/w)			
	Peel		Flesh	
	1% of DE 27% of undiluted ethanol 20% of K_2HPO_4 solution 50% of purified water 2% of 0.4M NaCl		1% of DE 33% of undiluted ethanol 20% of K_2HPO_4 solution 44% of purified water 2% of 0.2M NaCl	
	Electric system			
2.	Operation time	20	10, 15, 25, 30	min
3.	Voltage apply	3	1, 2, 4, 5	V
4.	Position of graphitic electrodes	Bottom phase	Top and middle phase; middle position refers to the interphase between top and bottom phase	N/A

6.3.4.1 Comparison between LBPS and LBEPS

A 10 g LBPS was prepared in a 15 mL graduated centrifugal tube by mixing phase-forming components and DE in accordance with their respective compositions (%, w/w), as shown in **Table 6-1**. After thorough mixing of all the components by gentle agitation, the mixture was centrifuged at 3000 rpm for 20 min to induce a phase separation. On the other hand, in the 10 g LBEPS, a biphasic system was firstly formed; the phase-forming components were mixed well and centrifuged at 3000 rpm for 5 min, subsequently, 1% of DE was added to the biphasic system and gently mixed. After that, the mixture was supplied with electricity, as mentioned in section 6.3.4 (i.e. LBEPS with initial settings). The volume of the top and bottom phase in LBPS and LBEPS were then measured, followed by the collection of sample from both phases for analysis of total betacyanins content (TBC).

6.3.5 Comparison between optimised LBEPS and LBPS with optimised conditions of electricity treatment as post-treatment

A 10 g LBPS was firstly prepared, as mentioned in section 6.3.4.1, subsequently, the optimised conditions of the electricity treatment (operation time, voltage applied and position of graphitic electrodes in the LBEPS) were used in further treatment of the mixture (i.e. post-treatment). The results obtained were compared with the optimised LBEPS.

6.3.6 Analytical procedures

6.3.6.1 Colour characterisation

Lightness (L^*), redness (a^*) and yellowness (b^*) of the peel and flesh extract of red-purple pitaya were analysed using a colorimeter (Lovibond LC 100, model RM 200, The Tintometer Ltd, United Kingdom). Additionally, their hue angle (h°) and chroma (C^*) were calculated according to equations (6-1) and (6-2), respectively.

$$h^\circ = \tan^{-1}\frac{b^*}{a^*} \quad (6\text{-}1)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (6\text{-}2)$$

6.3.6.2 Determination of total betacyanins content (TBC)

The TBC in the crude extract was analysed using a UV-vis spectrophotometer (UV-1800, Shimadzu Corporation, Japan) at 538 nm. The TBC was expressed as mg of betanin equivalents (BEs) per 100 g of crude extract, and was calculated according to equation (6-3) (Leong et al., 2018a; Ramli et al., 2014):

$$\mathrm{TBC} = \frac{A_{538} \times \mathrm{MW} \times \mathrm{V} \times \mathrm{DF}}{\varepsilon \times \mathrm{L} \times \mathrm{W}} \times 100 \qquad (6\text{-}3)$$

Where A_{538} = absorbance value at 538 nm, MW = molecular weight of betanin (550 $g.mol^{-1}$), V = volume of sample (mL), DF = dilution factor, ε = molar extinction coefficient of betanin (65000 $L.mol^{-1}.cm^{-1}$), L = path length of cuvette (1 cm), W = weight of crude extract (g)

6.3.6.3 Determination of total phenolic content (TPC)

The Folin-Ciocalteu (F-C) method as described in Fu et al. (2011), Hajimahmoodi et al. (2013) and Singleton et al. (1999) was employed to analyse the TPC in the crude extract. A diluted F-C reagent which consists of 10 mL of F-C reagent and 90 mL of purified water was freshly prepared. Next, 100 µL of sample or gallic acid solution (i.e. standard) was mixed with 500 µL of diluted F-C reagent. The mixture was then incubated for 5 min at room temperature under dark condition. Subsequently, 2 mL of 60 g/L of $NaHCO_3$ solution was added to the mixture. The mixture was mixed well and kept for 90 min at room temperature under dark condition. The absorbance value of the mixture was measured at 725 nm using a UV-vis spectrophotometer. The TPC was expressed as mg of gallic acid equivalents (GAEs) per 100 g of crude extract.

6.3.6.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP in the crude extract was analysed using method as described in literatures (Benzie and Strain, 1996; Fu et al., 2011). The FRAP reagent was freshly prepared; 10 mL of 10 mmol/L TPTZ solution (0.0031 g of TPTZ in 1 mL of 40 mmol/L HCl) and 10 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution (0.0054 g/mL) for every 100 mL of 300 mmol/L acetate buffer (pH 3.6; mixture of 3.1 g of $C_2H_3NaO_2 \cdot 3H_2O$ with 16 mL of acetic acid per liter of purified water). The FRAP reagent was preheated

to 37 °C before use. The FRAP assessment was carried out by mixing 100 μL of sample or $FeSO_4 \cdot 7H_2O$ solution (i.e. standard), 300 μL of purified water and 3 mL of FRAP reagent. The mixture was subsequently incubated for 4 min at 37 °C. The absorbance value of mixture was measured at 593 nm using a UV-vis spectrophotometer. The result was expressed as μmol of Fe(II) per g of crude extract.

6.3.6.5 Trolox equivalent antioxidant capacity (TEAC) assay

The ABTS radical (ABTS•) method as described in Fu et al. (2011) and Re et al. (1999) was employed to analyse the TEAC in the crude extract. The ABTS• stock solution (mixture of 7 mmol/L of ABTS solution and 2.45 mmol/L of $K_2O_8S_2$ solution at v:v ratio of 1:1) was first prepared, and was then incubated for 12–16 h at room temperature under dark condition. Next, the ABTS• stock solution was diluted with ethanol to reach an absorbance value of 0.70 ± 0.05 at 734 nm, and was incubated at 30 °C. As to analyse the TEAC, 100 μL of sample or trolox solution (i.e. standard) or ethanol (i.e. control) was mixed with 3.8 mL of diluted ABTS• solution. The absorbance value of the mixture was measured at 734 nm using a UV-vis spectrophotometer after 6 min of incubation at 30 °C. The result was expressed as μmol of trolox equivalents (TEs) per g of crude extract. The percentage of scavenging on ABTS• was calculated using equation (6-4) (Leong et al., 2018a):

$$\text{Percentage of scavenging (\%)} = \frac{\text{control} - \text{sample or standard}}{\text{control}} \times 100 \qquad (6\text{-}4)$$

6.3.7 Calculations

Partition coefficient (K) of betacyanins in LBPS and LBEPS were calculated according to equation (6-5) (Leong et al., 2018a):

$$K = \frac{TBC_t}{TBC_b} \qquad (6\text{-}5)$$

Where TBC_t and TBC_b are TBC in the alcohol-rich top phase and salt-rich bottom phase at equilibrium, respectively.

Betacyanins concentrations (%) in alcohol-rich top phase (C_t) and salt-rich bottom phase (C_b) were calculated according to equations (6-6) and (6-7), respectively:

$$C_t(\%) = \frac{\text{TBC in top phase}}{\text{TBC in crude extract}} \times 100 = \frac{TBC_t}{TBC_t + TBC_b} \times 100 \qquad (6\text{-}6)$$

$$C_b(\%) = \frac{\text{TBC in bottom phase}}{\text{TBC in crude extract}} \times 100 = \frac{TBC_b}{TBC_t + TBC_b} \times 100 \qquad (6\text{-}7)$$

Phase volume ratio (V_r) is defined as ratio of the volume of alcohol-rich top phase to the volume of salt-rich bottom phase at equilibrium, and was calculated according to equation (6-8).

$$V_r = \frac{V_t}{V_b} \qquad (6\text{-}8)$$

Where V_t and V_b are volume of the alcohol-rich top phase and salt-rich bottom phase at equilibrium, respectively.

6.3.8 Statistical analysis

The statistical analysis was performed by IBM SPSS statistics software (SPSS version 23.0 for window, IBM Corporation, Armonk, New York, United States). Triplicate experimental readings were recorded and were used in the analysis, and the values were expressed as mean ± standard deviation (SD) (n = 3). The experiment was run three times in order to further verify the results. The data were subjected to one-way analysis of variance (ANOVA), and the mean differences were compared using

Tukey HSD post-hoc multiple comparisons test. The data were considered for their statistically significant difference where $p < 0.05$. Moreover, the relationship among the antioxidant properties was analysed using Pearson's correlation test.

6.4 Results and discussion

6.4.1 Comparison study of betacyanins extraction using LBPS and LBEPS

An initial study was conducted by comparing the betacyanins extraction from peel and flesh of red-purple pitaya using LBPS with optimised conditions and LBEPS (i.e. an integration process of LBPS and electricity treatment) with initial settings. Our findings showed that the betacyanins extraction from the peel using LBPS was slightly better than that of using LBEPS. The application of LBPS showed the significantly highest values of C_t (98.080 ± 0.051%) and K of betacyanins (51.097 ± 1.354) compared to that of the values obtained from LBEPS (C_t: 96.820 ± 0.046% and K of betacyanins: 30.450 ± 0.459). Both systems showed a similar value of V_r (1.667) (**Figure 6-2a**). Likewise, a similar trend was observed for the betacyanins extraction from the flesh, as shown in **Figure 6-2b**. However, the betacyanins extraction from the flesh using LBPS showed the non-significantly highest values of C_t (96.256 ± 0.207%) and K of betacyanins (25.764 ± 1.525) as compared to that of the LBEPS (C_t: 96.010 ± 0.144% and K of betacyanins: 24.086 ± 0.911) ($p > 0.05$). Both systems also showed a similar value of V_r (2.167). The similar value of V_r revealed that the biphasic separation was not affected by the difference in these two extraction methods. In LBPS, centrifugation process alongside biphasic system was utilised in the betacyanins extraction, whereas electrical field effect alongside biphasic system was applied to the betacyanins extraction in LBEPS. Both approaches are different in terms of their working mechanisms. Although LBPS resulted in the slightly higher

betacyanins extraction, we decided to further optimise our LBEPS for the betacyanins extraction in this study. The reasons are that both systems showed a comparable betacyanins extraction from red-purple pitaya and LBEPS seems to be promising in achieving a more efficient betacyanins extraction than LBPS due to the fact that its conditions used in this study could still be optimised.

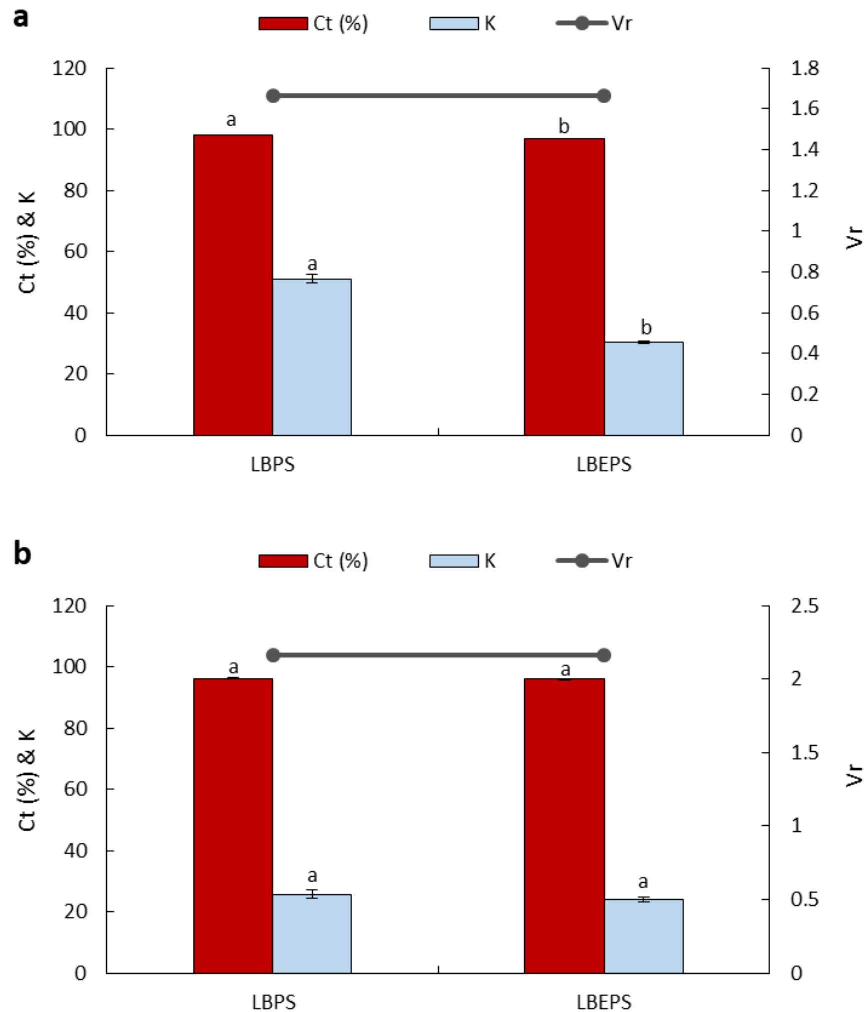


Figure 6-2: Comparison study of betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya using LBPS and LBEPS with initial settings. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within C_t and K.

6.4.2 Effect of the operation time in LBEPS

The betacyanins extraction from the peel and flesh using LBEPS were further studied by optimising the operation time (10–30 min) in the system. Other parameters of the LBEPS included 3 V of voltage applied and graphitic electrodes located at bottom phase in the system. As depicted in **Figure 6-3a**, an increase in the operation time from 10 to 15 min in the LBEPS greatly improved the betacyanins extraction

from the peel, and further extending the operation time up to 30 min resulted in a slightly lower betacyanins extraction compared to that of with 15 min of operation time. Meanwhile, LBEPS with 15 min of operation time showed a better betacyanins extraction compared to LBPS with 20 min of operation time (control). The significantly highest values of C_t and K of betacyanins obtained from the LBEPS with 15 min of operation time were 99.256 ± 0.014% and 133.433 ± 2.566, respectively, among the others. The control and LBEPS with different operation times showed a similar V_r value of 1.667, and this inferred that their biphasic separation was not affected by the different extraction methods (as mentioned earlier in section 6.4.1). Also, a similar trend was noted for the betacyanins extraction from the flesh, as shown in **Figure 6-4a**. The significantly highest values of C_t and K of betacyanins obtained from the LBEPS with 15 min of operation time were 97.189 ± 0.172% and 34.665 ± 2.253, respectively, among the others. The control and LBEPS with different operation times also showed a similar V_r value (2.167).

Collectively, a higher betacyanins extraction from the peel and flesh were achieved through the use of LBEPS with 15 min of operation time compared to that of with LBPS with 20 min of operation time as well as to that of with LBEPS with other operation times. Application of LBEPS can shorten the extraction time from 20 to 15 min; compared to control (i.e. LBPS). The possible reason might be due to the different working extraction mechanisms between LBPS and LBEPS, as mentioned earlier in section 6.4.1. The LBEPS uses electrical field effect alongside biphasic system for the betacyanins extraction, while LBPS employs centrifugation process alongside biphasic system for the betacyanins extraction. The electrical field treatment occurred between the two graphitic electrodes is suggested to enhance the betacyanins extraction and cause a higher extraction efficiency compared to the centrifugation process. Other than

that, as compared among the 10 to 30 min of operation time of LBEPS, 15 min of the operation time was noted to be the most effective extraction time. This could be explained by the mass transfer energy of the system has reached equilibrium and maximum at 15 min. Moreover, longer extraction time can cause oxidation of betacyanins due to their highly sensitive features (Celli and Brooks, 2017; Ciriminna et al., 2018; Esatbeyoglu et al., 2015; Khan, 2016; Leong et al., 2018a). Hence, 15 min of operation time was chosen for the betacyanins extraction using LBEPS.

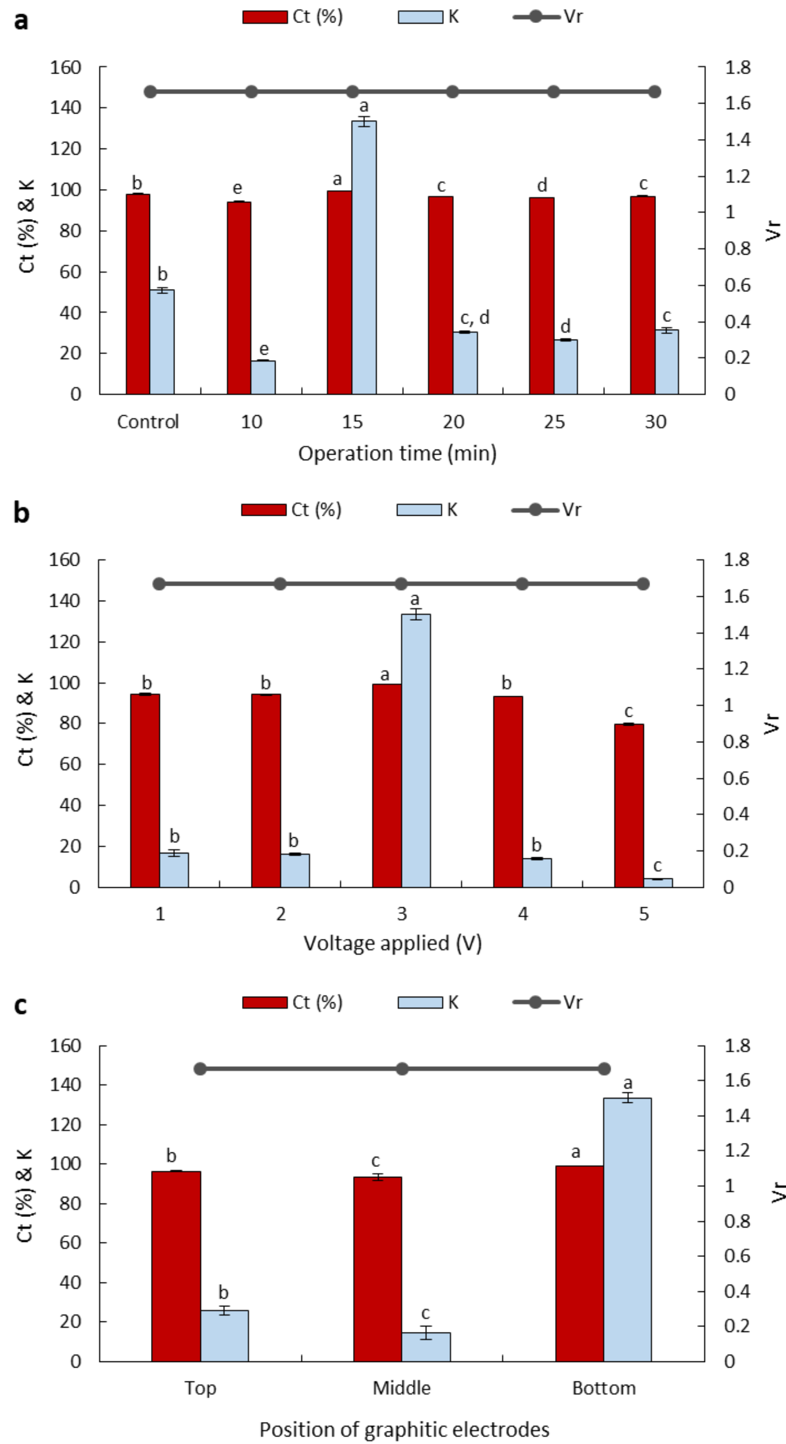


Figure 6-3: Effects of various parameters in LBEPS for betacyanins extraction from peel of red-purple pitaya. OFAT approach was applied in the optimisation study: (a) operation time (control: LBPS), (b) voltage applied and (c) position of graphitic electrodes. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference (p < 0.05) using Tukey's test within C_t and K.

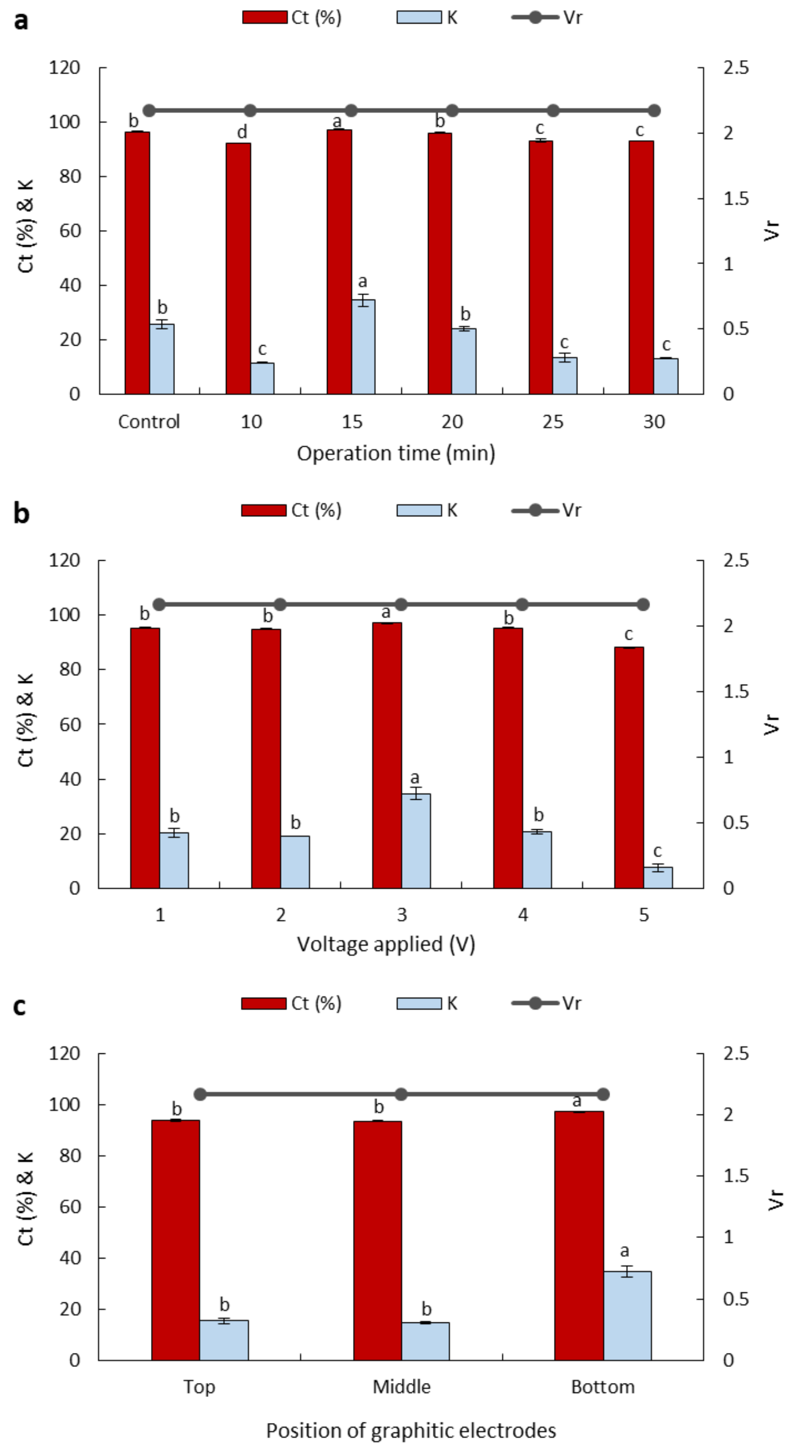


Figure 6-4: Effects of various parameters in LBEPS for betacyanins extraction from flesh of red-purple pitaya. OFAT approach was applied in the optimization study: (a) operation time (control: LBPS), (b) voltage applied and (c) position of graphitic electrodes. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within C_t and K.

6.4.3 Effect of the voltage applied in LBEPS

The effect of different voltages applied range from 1 to 5 V in the LBEPS for betacyanins extraction from peel and flesh of red-purple pitaya was investigated, and the results are presented in **Figures 6-3b,6-4b**, respectively. The betacyanins extraction using LBEPS with 3 V of voltage applied showed the significantly highest values of C_t and K of betacyanins from the peel (99.256 ± 0.014% and 133.433 ± 2.566, respectively) and flesh (97.189 ± 0.172% and 34.665 ± 2.253, respectively). Meanwhile, the values of C_t and K of betacyanins from the peel and flesh obtained from the betacyanins extraction using LBEPS with 1, 2 and 4 V of voltage applied were noted to be no significant difference among them ($p > 0.05$). However, the betacyanins extraction using LBEPS with 5 V of voltage applied showed the significantly lowest values of C_t and K of betacyanins from the peel (79.737 ± 0.419% and 3.937 ± 0.101, respectively) and flesh (88.231 ± 0.162% and 7.498 ± 0.116, respectively). Additionally, with the 5 V of voltage applied, the graphitic electrodes were oxidised and graphite residue was observed in the salt-rich bottom phase, as shown in **Figure 6-5**. Furthermore, V_r values in the LBEPS with different voltages applied for the peel and flesh showed that different voltages do not influence the biphasic separation much, due to their similar values among the others; LBEPS with peel: 1.667 and LBEPS with flesh: 2.167.

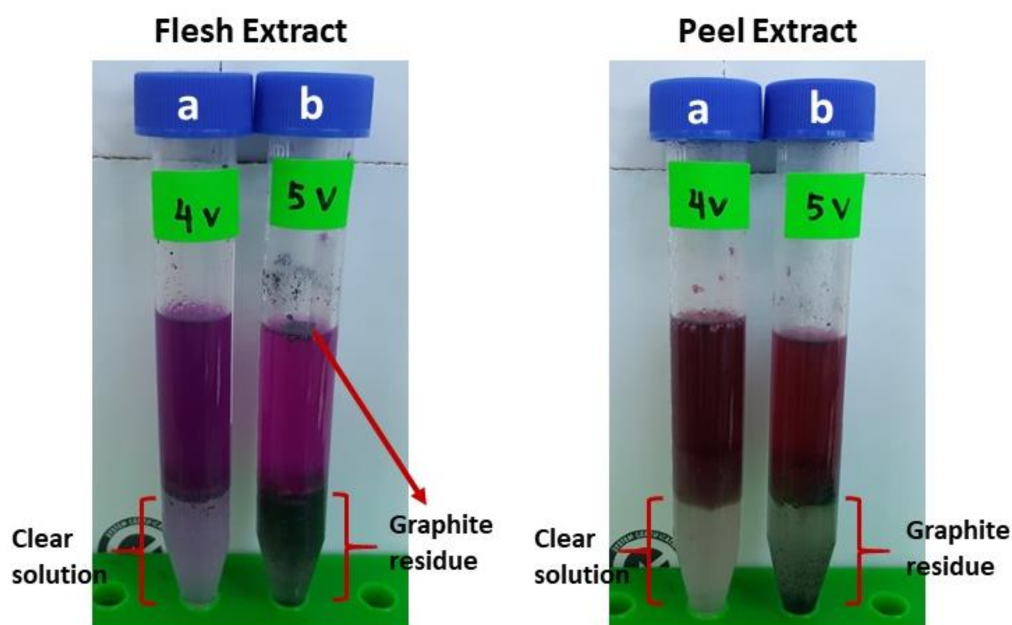


Figure 6-5: Effect of different voltages applied in LBEPS; comparison between (a) 4 V and (b) 5 V of voltage applied for the flesh and peel extract.

Our results inferred that LBEPS with 3 V of voltage applied achieved an optimal betacyanins extraction from the peel and flesh as shown by their significantly highest values of C_t and K of betacyanins. The electrical field effect supplied by the 3 V of voltage applied in the system is proved to have the highest electropermeabilization of red-purple pitaya membrane structure, in which the cell membrane structure was disrupted by short and intense electric pulses, and the increased cell membrane permeability led to release of more betacyanins from red-purple pitaya to the extractive solvent in the system. This eventually increase the extraction efficiency of betacyanins. The betacyanins adhered to the electrodes and moved along from bottom to top phase of the system via electrical field effect. This electrical field treatment is known as a non-thermal process where an external electricity is supplied to a substrate. Additionally, the effectiveness of the electrical field treatment strongly depends on the electrical field strength, specific energy input, treatment duration, substance to be

treated etc. (Azmir et al., 2013; Boussetta and Vorobiev, 2014; Celli and Brooks, 2017; Chemat et al., 2017; Xu et al., 2017). A study conducted by Roselló-Soto et al. (2015) reported that high voltage electrical discharges was the most effective pre-treatment for the extraction of olive kernels among the other pre-treatments of pulsed electric field and ultrasound, due to its highest extraction efficiency. This result was explained by the occurrence of propagation of the shock waves and explosion of cavitation bubbles during the pre-treatment of olive kernel that induced by the application of electrical discharges.

Moreover, in the present study, oxidation of the graphitic electrodes was observed when 5 V of voltage was applied in the LBEPS. In the same instance, this system showed the lowest betacyanins extraction from the peel and flesh. This could be explained by the 10 g LBEPS cannot withstand the strong electrical field effect which induced by the 5 V of voltage applied in the system. Also, the lowest betacyanins extraction might be caused by the presence of graphite residue due to oxidation of the graphitic electrodes, in which they degrade the betacyanins since betacyanins are highly sensitive pigments.

6.4.4 Effect of the position of graphitic electrodes in LBEPS

Subsequently, the LBEPS with 15 min of operation time and 3 V of voltage applied for the betacyanins extraction was further assessed using different positions of graphitic electrodes in the system; the electrodes were positioned at top, middle and bottom phase in the LBEPS. Our results revealed that the electrodes located at the bottom phase in LBEPS augmented the betacyanins extraction, with the significantly highest values of C_t and K of betacyanins from the peel (99.256 ± 0.014% and 133.433 ± 2.566, respectively (**Figure 6-3c**) and flesh (97.189 ± 0.172% and 34.665 ± 2.253,

respectively (**Figure 6-4c**). The top and middle position of the graphitic electrodes seemed to provide a slightly lower betacyanins extraction, owing to their smaller values of C_t and K of betacyanins. The possible explanation could be, in the bottom position, the electrodes have the maximum electrical field influence that allows betacyanins to move along from bottom to top phase. V_r values for the LBEPS in this case with the peel and flesh inferred that different positions of electrodes in the system do not have a significant impact on the biphasic separation, due to their similar values among the others (LBEPS with peel: 1.667 and LBEPS with flesh: 2.167).

6.4.5 Comparison study of betacyanins extraction using optimised LBEPS and LBPS with optimised conditions of electricity treatment as post-treatment

The optimised conditions of electricity treatment in the LBEPS included 15 min of operation time, 3 V of voltage applied and the electrodes located at bottom phase. The optimised LBEPS augmented the betacyanins extraction from peel and flesh of red-purple pitaya. As a result, these optimised conditions were incorporated to the LBPS as post-treatment for the betacyanins extraction, and the results obtained were compared with that of the optimised LBEPS (**Figure 6-6**). Our findings showed that the optimised LBEPS owned a better betacyanins extraction from the peel and flesh compared to that of using LBPS with the electricity treatment as post-treatment (C_t and K of betacyanins from the peel were 95.296 ± 0.309% and 20.323 ± 1.431, respectively, as well as from the flesh were 93.825 ± 0.012% and 15.195 ± 0.032, respectively). The reason for the results obtained could be that the LBPS with electricity treatment as post-treatment requires longer extraction time (i.e. 35 min) that might cause oxidation of betacyanins to occur. This eventually might reduce the betacyanins extraction. On the other hand, the V_r values are similar for both

approaches. Therefore, an optimised LBEPS with 15 min of operation time, 3 V of voltage applied and the electrodes located at bottom phase was chosen for the betacyanins extraction from the peel and flesh of red-purple pitaya.

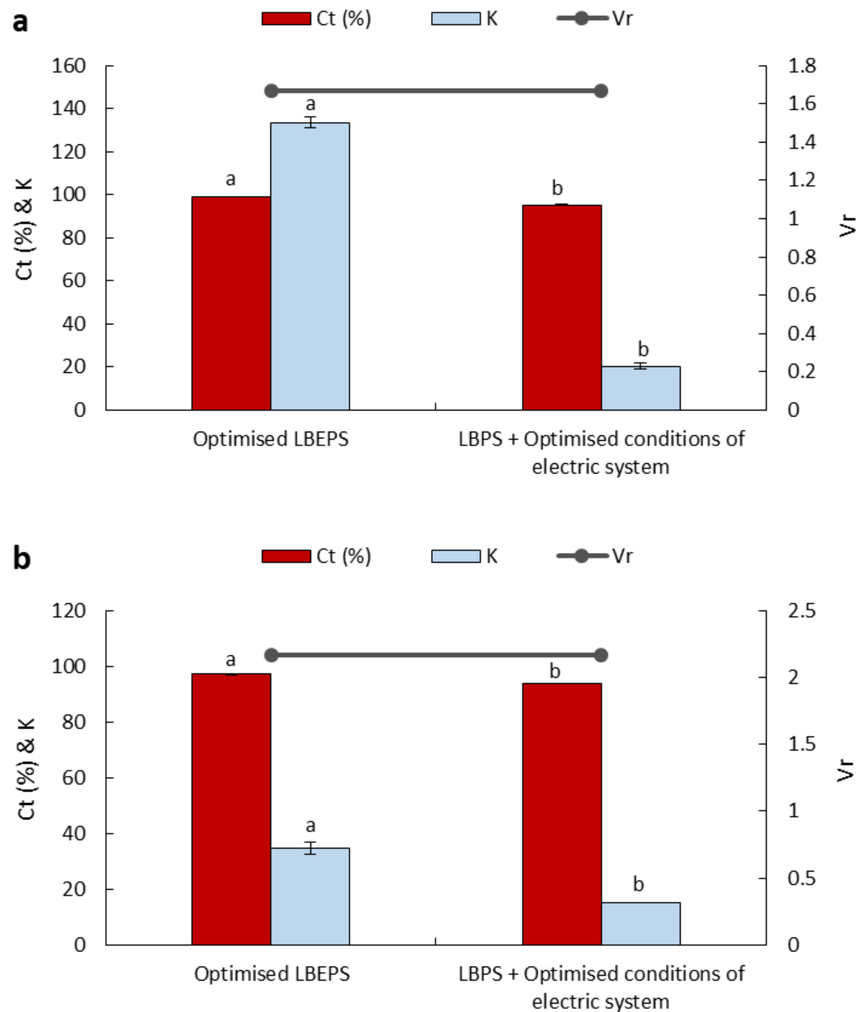


Figure 6-6: Comparison study of betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya using the optimised LBEPS and LBPS with the optimised conditions of electric system as post-treatment. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference (p < 0.05) using Tukey's test within C_t and K.

6.4.6 Colour characterisation

After the most effective extraction approach for the betacyanins extraction was determined, the colour characterisation of the extract was analysed further. In this case, peel and flesh extract of red-purple pitaya used in the analysis were obtained from the optimised LBEPS (alcohol-rich top phase). Both extracts are presented in different hues, lightness, saturations and intensities of colour. They showed different degrees of lightness (L^*), redness (a^*), yellowness (b^*), chroma (C^*) and hue angle (h°), as presented in **Table 6-2**. The peel extract showed a higher degree of a^* and b^* as well as a smaller degree of L^*, C^* and h° compared to that of the flesh extract. Our results revealed that the peel and flesh extract showed different ranges of red colour. The peel extract showed a red-yellowish colour (positive a^* and b^*), whereas the flesh extract showed a red-bluish/purplish colour (positive a^* and negative b^*). The C^* and h° measure the basic tint and saturation of a colour, respectively (Lancaster and Lister, 1997). Owing to their natural colouring attribute, the peel and flesh extract can be applied as natural colourants with different variations of red colour, and therefore replace the artificial dyes.

Table 6-2: Colour characterisation of peel and flesh extract of red-purple pitaya obtained from the optimised LBEPS.

Colour parameter	Peel extract	Flesh extract
L^*	7.933 ± 0.306	11.033 ± 0.208
a^*	23.633 ± 0.651	36.100 ± 0.529
b^*	4.567 ± 0.306	-17.167 ± 0.153
C^*	24.067 ± 0.651	39.967 ± 0.569
h°	10.933 ± 0.666	334.533 ± 0.153

L^*, a^*, b^*, C^* and h° represent lightness, redness, yellowness, chroma and hue angle, respectively.
Values are mean ± SD of triplicate readings.

6.4.7 Antioxidant properties analysis

Lastly, the antioxidant properties on peel and flesh extract of red-purple pitaya were assessed in order to examine their antioxidant capability and the presence of antioxidant compound. These extracts were obtained from the optimised LBEPS (alcohol-rich top phase). Particularly, FRAP and TEAC are used to measure the antioxidant capability, while TPC and TBC are determined the quantity of antioxidant compound (Dai and Mumper, 2010). In the FRAP assessment, the peel extract (61.767 ± 1.460 µmol of Fe(II)/g of crude extract) showed a significant higher FRAP value compared to that of the flesh extract (35.916 ± 0.489 µmol of Fe(II)/g of crude extract) (standard equation: A_{593} = 0.0006([$FeSO_4 \cdot 7H_2O$]) + 0.0708 (R^2 = 0.9984); A_{593}: absorbance value at 593 nm & [$FeSO_4 \cdot 7H_2O$]: concentration of $FeSO_4 \cdot 7H_2O$). On the other hand, the peel extract (35.460 ± 0.443 µmol of TEs/g of crude extract) showed a significant lower TEAC value compared to that of the flesh extract (59.606 ± 0.857 µmol of TEs/g of crude extract) (standard equation: Percentage of scavenging (%) = 0.0966([trolox]) + 2.8333 (R^2 = 0.9946)). Other than that, in the TPC and TBC assessment, the peel extract showed a significant higher values of TPC (310.741 ± 8.486 mg of GAEs/100 g of crude extract) and TBC (156.877 ± 2.655 mg of BEs/100 g of crude extract) compared to that of the flesh extract (TPC: 292.019 ± 7.517 mg of GAEs/100 g of crude extract, TBC: 147.840 ± 3.038 mg of BEs/100 g of crude extract); standard equation for TPC: A_{725} = 0.0036([gallic acid]) + 0.0086 (R^2 = 0.9995) (**Figure 6-7**).

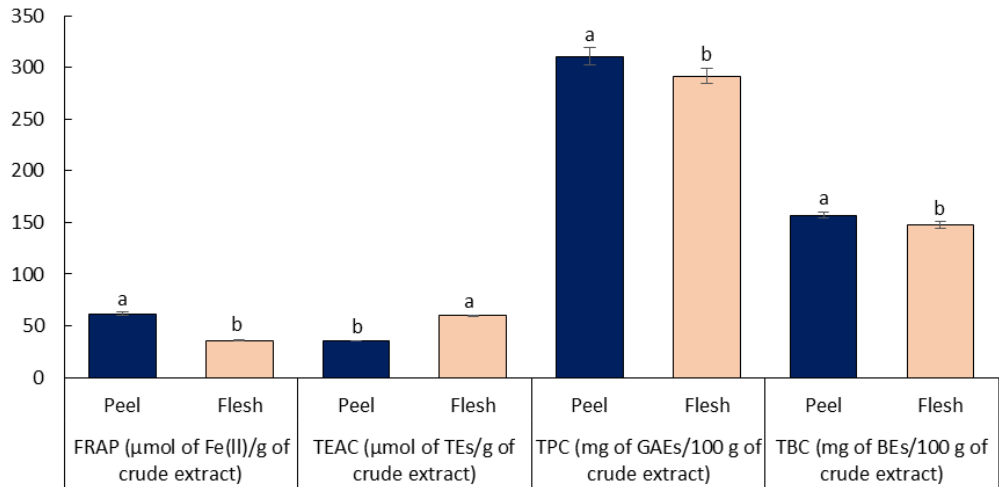


Figure 6-7: Antioxidant properties assessment of peel and flesh extract of red-purple pitaya obtained from the optimised LBEPS. Values are mean ± SD of triplicate readings. Different letter(s) represent a significant difference ($p < 0.05$) using Tukey's test within FRAP, TEAC, TPC and TBC.

Our results concluded that electricity treatment did not reduce the antioxidant properties from the red-purple pitaya, but in fact it enhanced them as the antioxidant values obtained in this study are much higher compared to the previously reported studies (Fu et al., 2011; Ramli et al., 2014). Moreover, our Pearson's correlation study conveyed that the FRAP with TEAC, TPC and TBC were correlated among them with a significant strong positive relationship. TEAC showed a non-significant strong negative with TPC ($p > 0.05$), whereas a significant strong negative relationship between TEAC and TBC was noted. TPC and TBC demonstrated a non-significant strong positive relationship (**Table 6-3**).

Table 6-3: Correlation study among the antioxidant properties assays, given correlation coefficient (r).

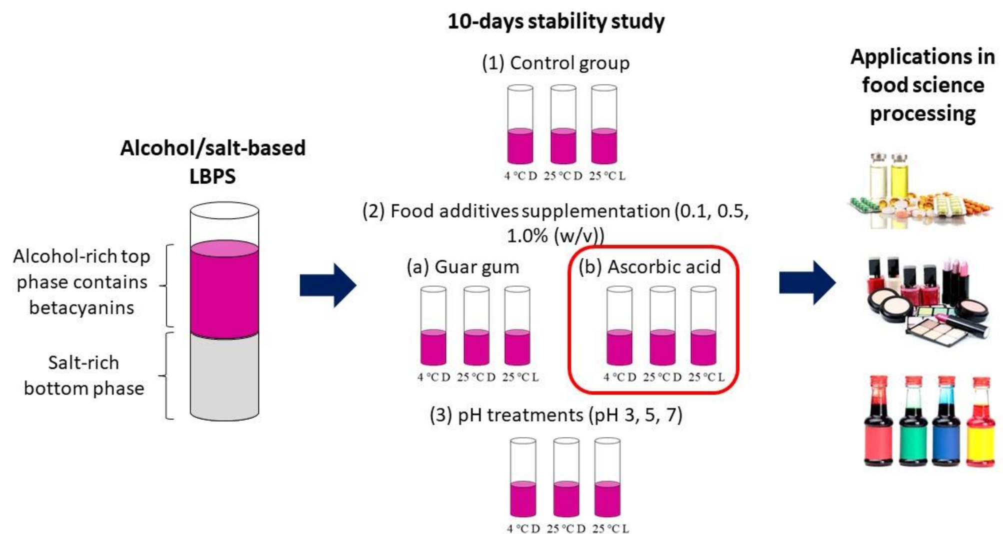
	TEAC	TPC	TBC
FRAP	-0.997**	0.853*	0.899*
TEAC		-0.811	-0.902*
TPC			0.788

** Correlation is significant at $p < 0.01$ (2-tailed).
* Correlation is significant at $p < 0.05$ (2-tailed).

6.5 Concluding remarks

This study concludes that a satisfactory betacyanins extraction from the peel and flesh of red-purple pitaya was successfully achieved with the utilisation of the optimised LBEPS. In the system, electricity treatment greatly improves extraction of biomolecules like betacyanins. In addition, the peel and flesh extract showed different variations of red colour, and they are proved to demonstrate appreciable antioxidant properties. Overall, the present study introduces a new, easy and effective green bioseparation technology for the biomolecules separation which could be applied in the downstream processing industries. LBEPS is significantly worth to explore further. For instance, a pilot scale LBEPS on the biomolecules separation could be investigated, owing to its high potential to serve as an effective bioseparation technology.

CHAPTER 7: Investigation of Betacyanins Stability from Peel and Flesh of *Hylocereus polyrhizus* with Food Additives Supplementation and pH Treatments



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7.1 Abstract

Betacyanins are potential source of natural red dyes. Generally, natural plant pigments, such as betacyanins, are highly unstable and prone to degradation due to internal and external factors. Hence, this study investigated the stability of betacyanins extracted from peel and flesh of red-purple pitaya using alcohol/salt-based liquid biphasic partitioning system (LBPS) over a 10-days storage period. Various stability treatments, including addition of food additives, pH treatments and different storage conditions were conducted. Overall, the results reveal that the peel and flesh extract added with 0.5% (w/v) of ascorbic acid and without any pH adjustments showed an optimum betacyanins stability under all storage conditions. The betacyanins retentions of the peel and flesh extract at 4 °C dark storage (4 °C D) were the highest, as indicated at 114.976% and 105.903%, respectively. In addition, kinetic analysis of the degradation of betacyanins was studied, and the extracts at 4 °C D showed no degradation sign of betacyanins. Stabilised betacyanins with low degradation rate would be beneficial to be used in food science processing.

Keywords: betacyanins; food additives; pH treatments; red-purple pitaya; stability

7.2 Introduction

Food products with appealing appearance are attractive and appetising, and they are always related to colouring matters as colourants play a significant role in the development of food science. In recent years, arising of the potential deleterious health problems with the use of artificial food dyes, such as occurrence of intolerance and allergenic responses, have elevated manufactures and consumers' consciousness toward the importance on the usage of natural ingredients as food dyes. Natural colourants are food-safe and environmentally benign compounds, besides offer health-promoting benefits due to the presence of antioxidant contents (Carocho et al., 2015; Gengatharan et al., 2016; Martins et al., 2016). At present, betacyanins, which belong to the class of betalains, have received attention as substitute for artificial red dyes in food science industries. This is because betacyanins are believed to have huge potentials in the food science processing, mainly due to their powerful antioxidant capability and pH stability ranging from 3–7 (Carocho et al., 2015; Gengatharan et al., 2017; Leong et al., 2018c; Moreno et al., 2008; Shaaruddin et al., 2017; Wrolstad and Culver, 2012).

Betacyanins (i.e. red-violet pigments) are synthesised through condensation of betalamic acid with *cyclo*-3,4-dihydroxyphenylalanine (*cyclo*-DOPA). They are water soluble ammonium conjugates, and also known to be chemically more stable compared to betaxanthins (Azeredo, 2009; Moreno et al., 2008). Betanin is the major constituent of betacyanins (Castellar et al., 2003). Betacyanins demonstrated protective roles against chronic diseases, such as cancers and oxidative-stress related disorders, and thus beneficial to humans' health. For example, red-purple pitaya and red beetroot are

two promising sources of betacyanins (Esatbeyoglu et al., 2015; Gengatharan et al., 2015; Khan and Giridhar, 2015; Leong et al., 2018c).

Red beetroot has been commercially used as a natural red colourant (i.e. E-162) in the food industry because it is the only permitted source of betalains and exempted from batch certification (Castellar et al., 2003). Nevertheless, the use of natural red food dye extracted from red beetroot has been restricted in recent years, due to its major drawbacks, such as formation of carcinogenic nitrosamines, and presence of unfavourable earthy odour mainly caused by geosmin and pyrazine derivatives (Carocho et al., 2015). In this case, red-purple pitaya represents a potential replacement for red beetroot. This is because both red-purple pitaya and red beetroot are promising sources of betacyanins, besides red-purple pitaya has a pleasant smell and does not produce carcinogenic compounds (Moreno et al., 2008; Pavokovic and Krsnik-Rasol, 2011; Woo et al., 2011). Red-purple pitaya or commonly known as red dragon fruit or red-purple pitahaya (*Hylocereus polyrhizus*) is an exotic and underutilised berry fruit, which is native to Mexico, Central and South America. It is also widely cultivated in the Southeast Asian countries, such as Malaysia. Red-purple pitaya is belongs to the family of Cactaceae, and it is rich in nutritional values (Dembitsky et al., 2011).

Conventional solvent extraction technique which normally used in the extraction of phytochemicals has the limitations of being time-consuming, costly and not sufficiently environmental-friendly, which mainly due to the large amount of volatile and toxic solvent consumption (Dai and Mumper, 2010; Tan et al., 2017). Thus, in this study, we utilised a green bioseparation technique, i.e. liquid biphasic partitioning system (LBPS) as our extraction approach. The peel and flesh extract used in the stability study were obtained using LBPS composed of alcohol and salt (in line with

Chapter 4 in this thesis). The LBPS having benefits of being simple to perform, cost-effective, environmental-friendly and ensure an efficient extraction of biotechnological products.

Natural pigments that extracted from biological sources are highly unstable and prone to degradation by both internal (i.e. pigment concentration) and external (i.e. pH, temperature etc.) factors (Herbach et al., 2006b). They are only stable when their pigment concentration are high, at pH ranging from 3–7, presence of antioxidants and chelating agents as stabilisers, in dark and at low temperature environments, as well as contain high degree of glucosylation and acylation (Stintzing and Carle, 2007). Therefore, this study investigated the betacyanins stability from peel and flesh of red-purple pitaya with several stability treatments over a 10-days storage period. The stability study was first conducted using addition of two types of food additives (i.e. guar gum and ascorbic acid) as to compare their stabilising effects on betacyanins, in which due to their well-known food stabiliser feature (Herbach et al., 2006a; Herbach et al., 2006b; Khan and Giridhar, 2014; Mudgil et al., 2014). After that, pH treatments were conducted for the chosen food additive. In addition, the extracts were kept at different storage conditions. Heat treatment was not carried out in this study because betacyanins are reported to be thermo-sensitive (Fernández-López et al., 2013) with their degradation rate increased starting from 30–100 °C (Gengatharan et al., 2016). Last but not least, kinetic analysis of the degradation of betacyanins was conducted.

7.3 Materials and methods

7.3.1 Materials

Ethanol, dipotassium hydrogen phosphate (K_2HPO_4), sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from R&M Chemicals (Selangor, Malaysia). Hydrochloric acid (HCl) was obtained from Fisher Scientific (Selangor, Malaysia). Guar gum and ascorbic acid were acquired from Sigma-Aldrich (St. Louis, MO, USA). All of the above mentioned chemicals were of analytical grade (purity > 95%). Ultrapure water produced from Milli-Q integral water purification system (Merck, Darmstadt, Germany) was used throughout this study. Red-purple pitaya was purchased from a local fruit stall at Semenyih, Selangor, Malaysia.

7.3.2 Processing of crude extract

The processing of red-purple pitaya was carried out in a dim light condition for the purpose of minimising its pigments loss. Peel of red-purple pitaya was first removed manually from its flesh using a knife. Subsequently, the peel and flesh were cut into thin pieces, and were stored at -80 °C for 24 h. As for the preparation of dried crude extract (DE), the wet biomass was freeze-dried at -30 °C, 0.37 atm for 48 h using a freeze dryer (CHRIST Alpha 1-2 LDplus, Germany). The freeze-dried biomass was ground into powder using a grinder (Tefal Blendforce, Triple'Ax Technology 400 Watt, Malaysia), and was kept at -20 °C for further use.

7.3.3 Betacyanins extraction with alcohol/salt-based LBPS

A 10 g biphasic system was prepared by mixing phase-forming components and DE in accordance with their respective compositions (%, w/w), as shown in **Table 7-**

1. After thorough mixing of all the components by gentle agitation, the mixture was centrifuged at 3000 rpm for 20 min to induce a phase separation. Betacyanins were mostly remained in alcohol-rich top phase. The top phase (red-purple pigments extract) was then carefully collected and used for stability study while the bottom phase was removed.

Table 7-1: Phase-forming components in alcohol/salt-based LBPS for peel and flesh of red-purple pitaya.

Crude extract	Concentration of phase-forming component (%, w/w)				
	Ethanol	K_2HPO_4	Purified water	DE	NaCl
Peel	27	20	50	1	2% of 0.4M NaCl
Flesh	33	20	44	1	2% of 0.2M NaCl

7.3.4 Stability study

The red-purple pigments extracts of peel and flesh of red-purple pitaya (alcohol-rich top phase) were subjected to different stability treatments, which include addition of food additives and pH treatments using one-factor-at-a-time (OFAT) approach. Food additives supplementation were first conducted, and then followed by pH treatments. Also, a control group was carried out. The stability study was run twice and three experimental replicates were analysed for each study. The extracts (i.e. 7 mL each) were kept at different storage conditions: 4 °C dark storage (4 °C D) (i.e. centrifugal tube was covered with aluminium foil to create the dark condition and kept at 4 °C refrigerator), 25 °C dark storage (25 °C D) (i.e. centrifugal tube was covered with aluminium foil and kept at free space in the laboratory; room temperature of the laboratory is 25 ± 1 °C) and 25 °C daylight storage (25 °C L) (i.e. centrifugal tube was kept at free space in the laboratory). Total betacyanins content (TBC) of the extracts was monitored for 10 days.

7.3.4.1 Addition of food additives

Two types of food additive, namely guar gum and ascorbic acid (well-known food stabiliser), were added to the extract for the assessment of their stabilising effects on betacyanins. Concentration of food additives at 0.1, 0.5 and 1.0% (w/v) was added to the extract at a v:v ratio of 1:3.5.

7.3.4.2 pH treatments

Once the food additive with the best stabilising effect on betacyanins was chosen, the extract was further subjected to pH-stability studies covering from pH 3, 5 and 7 (pH stability range for betacyanins). The pH of the extract was adjusted with 1M of NaOH and 1M of HCl.

7.3.5 Calculations

The TBC was analysed with a UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) at 538 nm. As shown in equation (7-1), TBC was expressed as mg of betanin equivalents (BEs) per 100 g of DE (Ramli et al., 2014).

$$\mathrm{TBC} = \frac{A_{538} \times \mathrm{MW} \times \mathrm{V} \times \mathrm{DF}}{\varepsilon \times \mathrm{L} \times \mathrm{W}} \times 100 \qquad (7\text{-}1)$$

Where A_{538} = absorbance value at 538 nm, MW = molecular weight of betanin (550 $g.mol^{-1}$), V = volume of extract (mL), DF = dilution factor, ε = molar extinction coefficient of betanin (65000 $L.mol^{-1}.cm^{-1}$), L = path length of cuvette (1 cm), W = weight of DE (g)

The betacyanins stability was determined based on betacyanins retention of the extract over a 10-days storage period. The betacyanins retention was calculated according to equation (7-2).

$$\text{Betacyanins retention } (\%) = \left(\frac{\text{TBC}_f}{\text{TBC}_i}\right) \times 100 \qquad (7\text{-}2)$$

Where TBC_i and TBC_f are TBC of the extract at day 0 and the following day, respectively.

7.3.6 Kinetic analysis of betacyanins degradation rate

The kinetic analysis of betacyanins degradation rate was calculated by the reaction rate which reported in literatures (Fernández-López et al., 2013; Van den Broeck et al., 1998). The degradation rate of betacyanins was assumed to follow a first-order kinetic model, indicating a logarithmic order of degradation, which is mathematically expressed as equation (7-3).

$$\ln\left(\frac{N_t}{N_0}\right) = -kt \qquad (7\text{-}3)$$

Where N_0 and N_t are TBC before and after the stability treatment at day t, respectively. t and k are time (day) and degradation rate constant (day^{-1}), respectively, in the stability treatment.

Half-life ($t_{1/2}$) is defined as the period of time required by a given quantity of substance to degrade half of its initial value, and was calculated according to equation (7-4).

$$t_{1/2} = \frac{t\ \ln(2)}{\ln(N_0) - \ln(N_t)} \qquad (7\text{-}4)$$

7.3.7 Statistical analysis

The statistical analysis was performed using IBM SPSS statistics program (SPSS version 23.0 for window, IBM Corporation, Armonk, New York, United States). Triplicate experimental readings were recorded and were used in the statistical analysis, and the values were expressed as mean ± standard deviation (SD) ($n = 3$). The data were subjected to one-way ANOVA (analysis of variance), and the mean differences were compared using Tukey HSD post-hoc multiple comparisons test. The data were considered statistically significant difference where $p < 0.05$.

7.4 Results and discussion

7.4.1 Effects of addition of food additives

The control groups (i.e. devoid of food additives supplementation and pH treatments) for peel and flesh extract of red-purple pitaya were first examined under different storage conditions over 10 days, and the results are shown in **Figure 7-1**. The betacyanins retention of control group for the peel extract at 4 °C dark storage was 42.763 ± 1.143% after 10 days. On the other hand, the betacyanins retention of the peel extract at 25 °C daylight storage (50.782 ± 1.099%) was higher compared to that of at 25 °C dark storage (29.470 ± 0.368%) after 1-day incubation, and this could be due to the daylight storage has both light and dark condition during storage period. The purpose of using daylight storage condition because we would like to mimic the normal storage situation. However, both extracts showed a rapid betacyanins degradation from day 1 to 10, and were retained in a betacyanins concentration at 3.724 ± 0.100% and 3.642 ± 0.057%, respectively, after 10 days (**Figure 7-1a**).

A similar trend was noted on the betacyanins stability of control group for the flesh extract, as shown in **Figure 7-1b**. The betacyanins retention of the flesh extract at 4 °C dark storage was 51.722 ± 1.180% after 10 days. On the other hand, the betacyanins retentions of the flesh extracts at 25 °C dark and daylight storage were 3.006 ± 0.140% and 3.645 ± 0.147%, respectively, after 10 days. In addition, the peel and flesh extract changed from red-purple to yellowish-brown during the betacyanins degradation process. This colour change is believed to be caused by the formation of neobetanin, which is resulted from the dehydrogenation of betanin (i.e. the major constituent of betacyanins) (Azeredo, 2009). Our results imply that the storage conditions at 4 °C and in dark environment could retain a higher amount of betacyanins because betacyanins are relatively stable at low temperature and without light intensity. Higher light intensity and temperature increase the degradation reactivity of natural pigments, such as betacyanins (Azeredo, 2009; Esatbeyoglu et al., 2015; Woo et al., 2011).

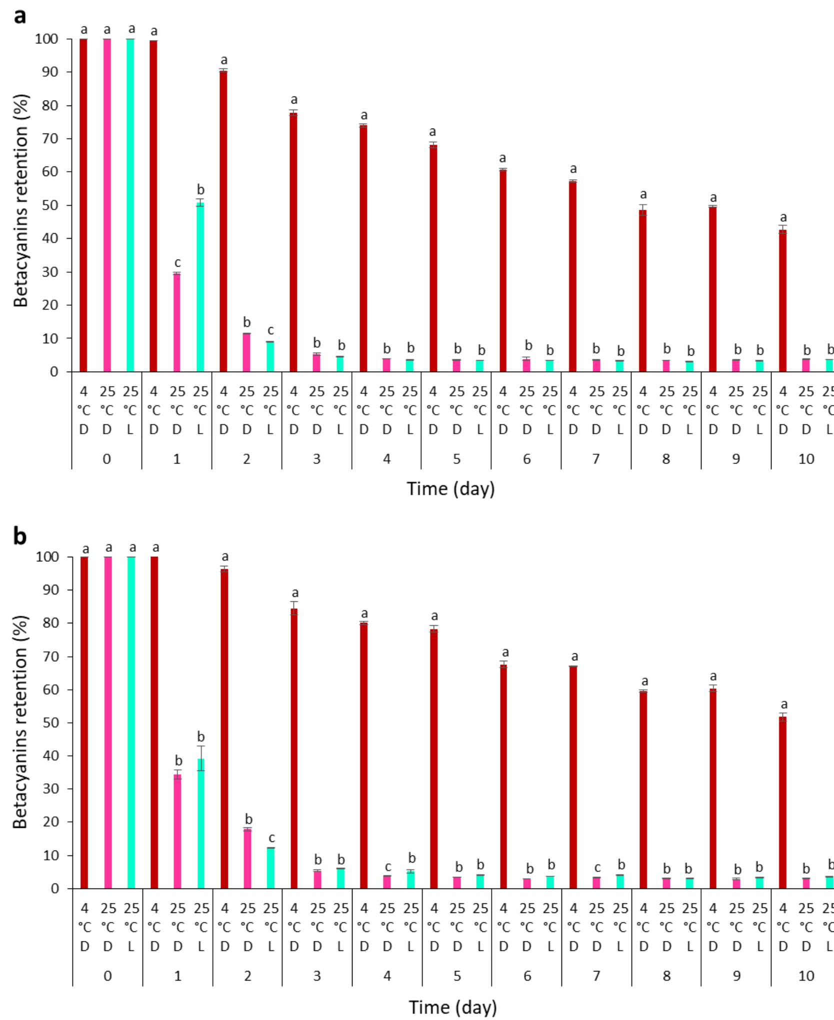


Figure 7-1: Betacyanins retentions (%) for control groups of (a) peel and (b) flesh extract of red-purple pitaya over a 10-days storage period. Values are mean ± SD of triplicate readings. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different (*p* < 0.05) using Tukey's test within day.

7.4.1.1 Addition of guar gum

Guar gum is a type of plant-based natural additive, and it is a well-known food stabiliser used in the food science processing. For instance, guar gum has been applied as a stabiliser in the production of dairy products (Garcia-Ochoa and Casas, 1992). Additionally, it has been affirmed as a "generally recognised as safe" (GRAS) compound, and proved to have health-promoting benefits upon a lower concentration of consumption at around 0.5–1.0% of weight basic (Mudgil et al., 2014). Hence, in the present study, guar gum was added to the red-purple pigments extract at varying concentrations (0.1–1.0% (w/v)) to evaluate its stabilising effect on betacyanins, and the results are depicted in **Figure 7-2**.

As depicted in **Figure 7-2a-I to a-III**, the betacyanins retentions of the peel extracts supplemented with 0.1, 0.5 and 1.0% of guar gum at 4 °C dark storage were 47.842 ± 0.760%, 63.700 ± 1.384% and 59.134 ± 2.165%, respectively, after 10 days. In contrast, the peel extracts added with 0.1, 0.5 and 1.0% of guar gum at both 25 °C dark and daylight storage suffered from the loss of almost 50% of betacyanins after 1-day incubation, and approximately 96% of the pigments losses after 10 days. Likewise, a similar trend was observed on the betacyanins stability of the flesh extracts supplemented with 0.1–1.0% of guar gum and kept at different storage conditions (**Figure 7-2b-I to b-III**). The betacyanins retentions of the flesh extracts supplemented with 0.1, 0.5 and 1.0% of guar gum at 4 °C dark storage were 65.883 ± 2.539%, 64.506 ± 1.499% and 55.512 ± 0.865%, respectively, after 10 days. On the other hand, the flesh extracts added with 0.1, 0.5 and 1.0% of guar gum at both 25 °C dark and daylight storage retained in a betacyanins concentration at approximately 4.500%.

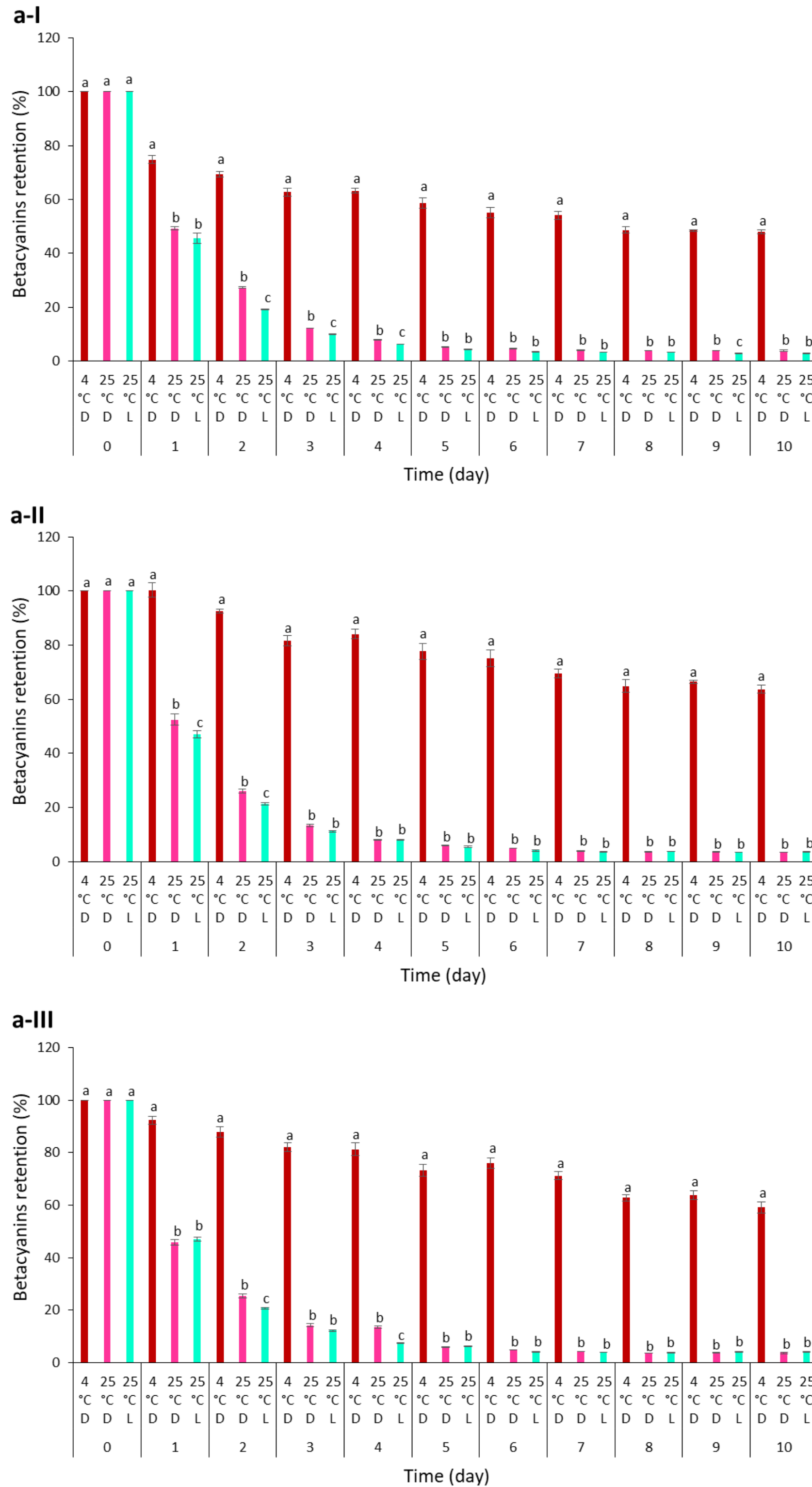


Figure 7-2: Betacyanins retentions (%) of (a) peel and (b) flesh extract of red-purple pitaya added with different concentrations of guar gum over a 10-days storage period. Values are mean ± SD of triplicate readings. I, II and III represent 0.1, 0.5 and 1.0% (w/v) of guar gum, respectively. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different (*p* < 0.05) using Tukey's test within day.

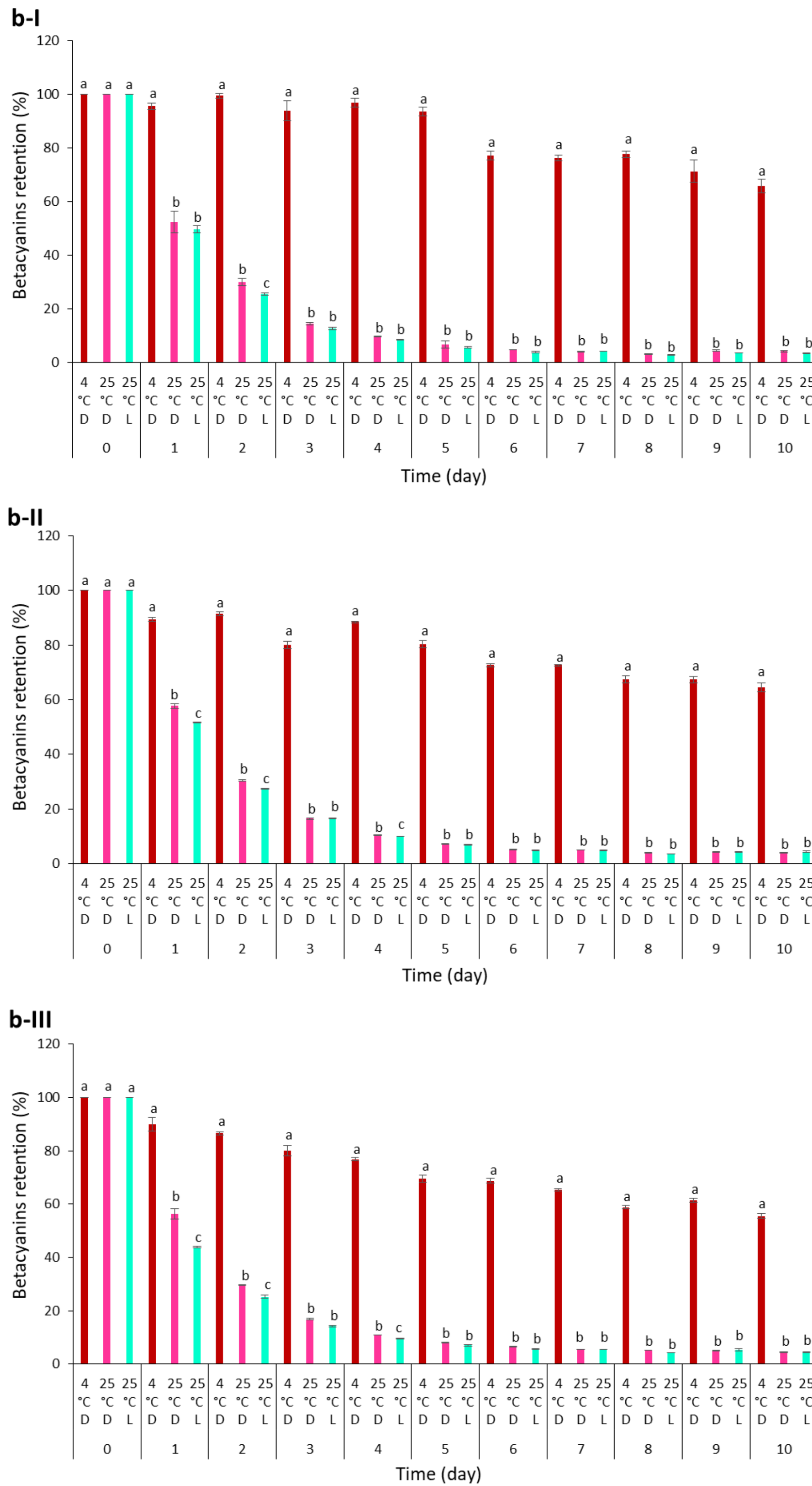


Figure 7-2 continued.

Our findings conclude that the peel and flesh extract supplemented with different concentrations of guar gum (0.1–1.0% (w/v)) at 4 °C dark storage showed a higher

betacyanins retention (47–66%) compared to that of the control groups (42–52%), as well as those extracts at 25 °C dark and daylight storage (3–4.5%) after 10-days stability study. Collectively, 0.5% of guar gum supplementation and 4 °C dark storage are recommended because the peel and flesh extract in these conditions showed a higher betacyanins retention among the others. This could be due to 0.5% of guar gum exhibited the best protective role against the betacyanins degradation at 4 °C dark storage; neither too weak (0.1%) or too strong (1.0%) the protective role could cause degradation of betacyanins. In this case, although the flesh extract added with 0.1% of guar gum amounted a slightly higher betacyanins content (65.883 ± 2.539%) compared to that of added with 0.5% of guar gum (64.506 ± 1.499%), but there was insignificance difference between them (1.377%). As compared between the peel and flesh extract at 25 °C dark and daylight storage, both control group and supplementation of different concentrations of guar gum showed no difference in the betacyanins retention (3–4.5%) after 10-days stability study.

According to Mudgil et al. (2014), guar gum has been largely used as a stabiliser and thickener in various applications, such as food processing, paper making, pharmaceuticals and cosmetics production, oil well drilling etc., because of its ability to form hydrogen bond with water molecule. We hypothesised that the formation of hydrogen bond might aid in a much slower degradation rate of betacyanins. However, its stabilising effect on betacyanins is not very promising. This could be explained that the degradation of betacyanins mostly caused by oxidation, and the formation of hydrogen bond could not prevent betacyanins from being oxidised and degraded. It is worth noting that this study is the first ever on the usage of guar gum in the stability study of natural plant-based bioactive compounds, such as betacyanins.

7.4.1.2 Addition of ascorbic acid

Ascorbic acid, a well-known food stabiliser (Herbach et al., 2006a; Herbach et al., 2006b), was evaluated for its stabilising effect on betacyanins at concentration range of 0.1–1.0% (w/v) (**Figure 7-3**). The betacyanins retentions of the peel extracts added with 0.1, 0.5 and 1.0% of ascorbic acid at 4 °C dark storage were 93.310 ± 2.844%, 114.976 ± 1.113% and 116.420 ± 1.914%, respectively, after 10 days. On the other hand, the peel extracts added with 0.1, 0.5 and 1.0% of ascorbic acid at 25 °C dark storage (20.543 ± 0.309%, 75.092 ± 0.728% and 62.359 ± 0.573%, respectively) retained a slightly higher amount of betacyanins compared to its respective group at 25 °C daylight storage (20.132 ± 0.383%, 74.363 ± 1.198% and 52.490 ± 1.115%, respectively) after 10 days (**Figure 7-3a-I to a-III**). Furthermore, the betacyanins stability of the flesh extracts supplemented with different concentrations of ascorbic acid ranging from 0.1–1.0% and kept at different storage conditions are shown in **Figure 7-3b-I to b-III**. The betacyanins retentions of the flesh extracts supplemented with 0.1, 0.5 and 1.0% of ascorbic acid at 4 °C dark storage were 106.391 ± 0.867%, 105.903 ± 1.589% and 111.311 ± 1.576%, respectively, after 10 days. A notable reduction in the betacyanins retentions was observed as the storing temperature increases. The flesh extracts added with 0.1, 0.5 and 1.0% of ascorbic acid at 25 °C dark storage (25.694 ± 0.127%, 49.166 ± 0.612% and 27.249 ± 3.105%, respectively) retained a higher amount of betacyanins compared to its respective group at 25 °C daylight storage (17.099 ± 0.320%, 41.368 ± 0.243% and 15.571 ± 0.669%, respectively) after 10 days.

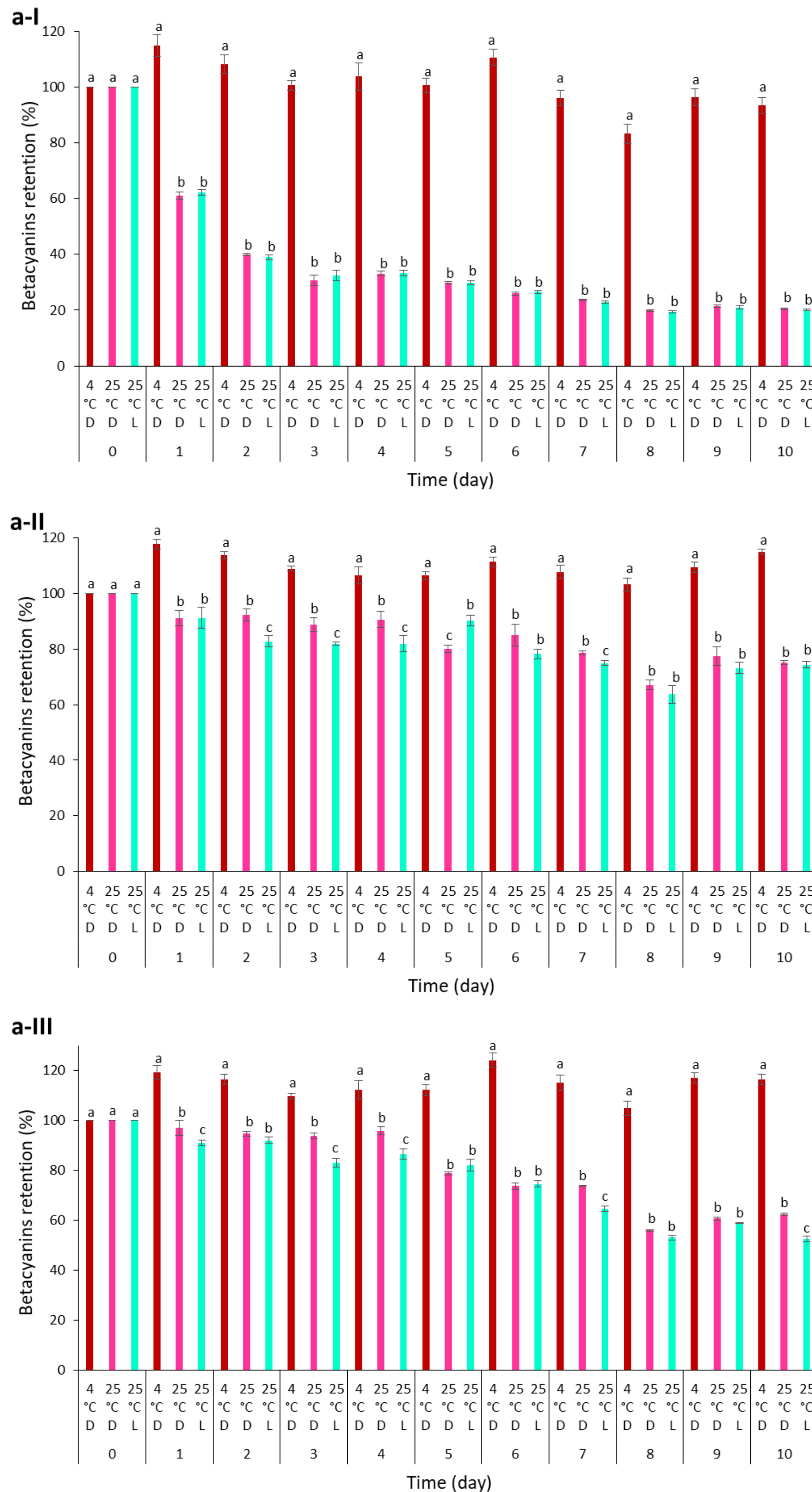
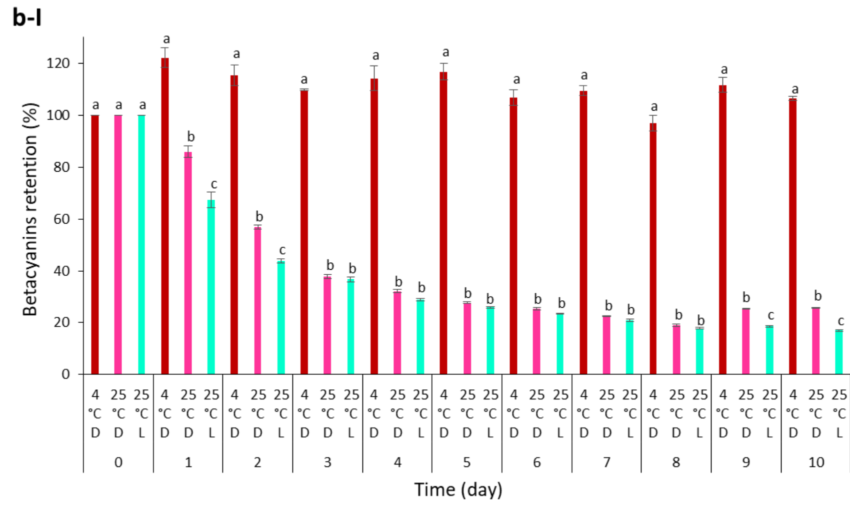
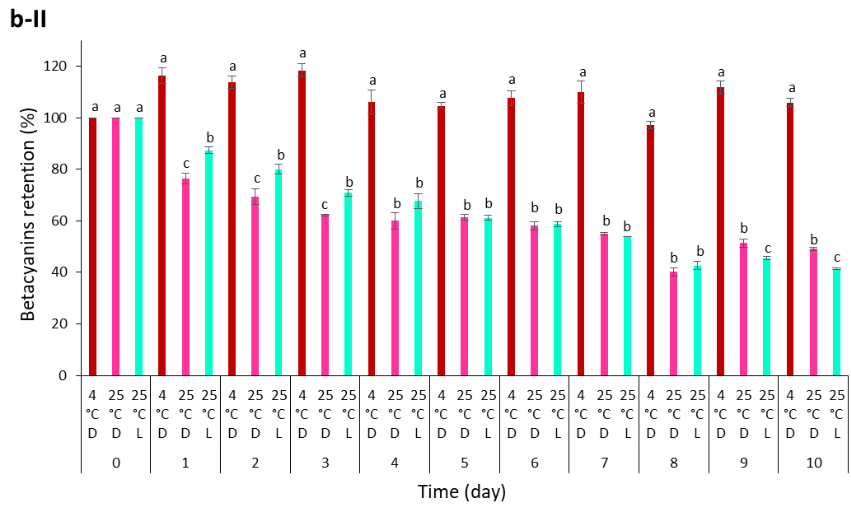


Figure 7-3: Betacyanins retentions (%) of (a) peel and (b) flesh extract of red-purple pitaya added with different concentrations of ascorbic acid over a 10-days storage period. Values are mean ± SD of triplicate readings. I, II and III represent 0.1, 0.5 and 1.0% (w/v) of ascorbic acid, respectively. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different (p < 0.05) using Tukey's test within day.





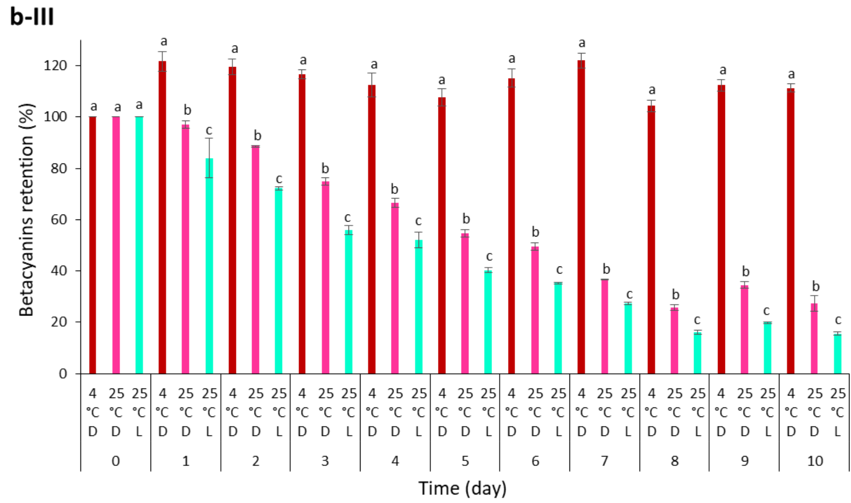


Figure 7-3 continued.

Our observations showed that those extracts exhibited a betacyanins retention of more than 100% infer that the degradation of betacyanins did not occur, while there was a formation of more betacyanins in these extracts. It is suggested that there could be regeneration of betacyanins due to the existence of building blocks of betacyanins, i.e. betalamic acid and *cyclo*-DOPA in these extracts (Gengatharan et al., 2016). They might undergo condensation reaction to regenerate betacyanins. The regeneration of betacyanins might occur in the extracts added with ascorbic acid and stored at low temperature of 4 °C and in dark environment. Czapski (1985) reported that storage at low temperature (5 °C) can induce betacyanins regeneration on heat-treated samples. Also, Herbach et al. (2006a) reported that cold storage condition can regenerate betacyanins on thermal-treated samples. In addition, ascorbic acid has higher oxidation potential, and thus it will be first oxidised as to protect betacyanins from being oxidised and degraded. This could be contributed by the bleaching effects of hydrogen peroxide during the oxidation of ascorbic acid (Azeredo, 2009; Carocho et al., 2015; Herbach et al., 2006a; Herbach et al., 2006b). It is worth noting that the supplementation of ascorbic acid as well as storage conditions at low temperature and in dark environment are used to prevent the degradation of betacyanins.

Overall, our results reveal that the peel and flesh extract supplemented with different concentrations of ascorbic acid ranging from 0.1–1.0% (w/v) at 4 °C dark storage showed a higher betacyanins retention (93–117%) compared to that of the control groups (42–52%) and the extracts added with different concentrations of guar gum (0.1–1.0% (w/v)) (47–66%), as well as those extracts at 25 °C dark and daylight storage (15–75%) after 10-days stability study. In addition, the peel and flesh extract added with varying concentrations of ascorbic acid greatly enhanced the betacyanins retention at both 25 °C dark and daylight storage compared to that of the control

groups, and those added with varying concentrations of guar gum. Collectively, 0.5% (w/v) of ascorbic acid supplementation was chosen for further studies in pH treatments because of its relatively higher betacyanins retention at 25 °C dark and daylight storage. At the 25 °C storages, the lower oxidation potential of 0.1% of ascorbic acid showed weak protection against betacyanins degradation, while 1.0% of ascorbic acid might has too strong oxidation potential, and thus it caused the betacyanins degradation. The storage condition at room temperature (25 °C) would be convenient for maintaining the betacyanins in the extracts, and hence could be beneficial to food science processing.

7.4.2 Effect of pH treatments

From our previous results, 0.5% (w/v) of ascorbic acid supplementation was recommended for further studies in the pH treatments (pH 3, 5 and 7). The initial pH values of the peel and flesh extract added with 0.5% of ascorbic acid were 7.44 and 6.25 (i.e. without pH adjustment), respectively. The betacyanins contents of the peel extracts added with 0.5% of ascorbic acid and adjusted to pH 3, 5 and 7 at 4 °C dark storage degraded slightly by 15–30% after 10 days (**Figure 7-4a-I to a-III**). On the other hand, the peel extracts added with 0.5% of ascorbic acid and adjusted to pH 3, 5 and 7 at 25 °C dark and daylight storage suffered from the loss of approximately 35–60% of betacyanins after 10 days. Similarly, the flesh extracts added with 0.5% of ascorbic acid and adjusted to pH 3, 5 and 7 at different storage conditions demonstrated a similar trend (**Figure 7-4b-I to b-III**). The betacyanins contents of the flesh extracts adjusted to different pH values and kept at different storage conditions degraded slowly over the 10-days incubation. The flesh extracts at 4 °C dark storage managed

to retain 77–95% of betacyanins content, whereas the flesh extracts at 25 °C dark and daylight storage suffered from the loss of approximately 20–50% of betacyanins.

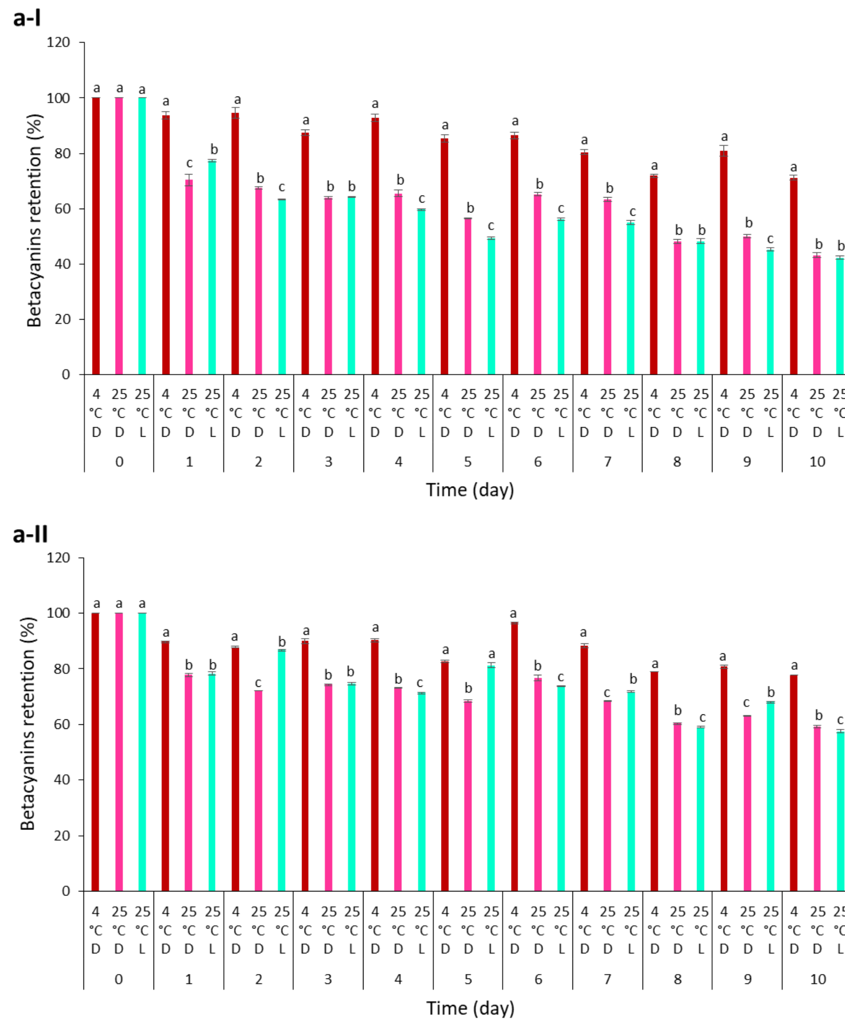
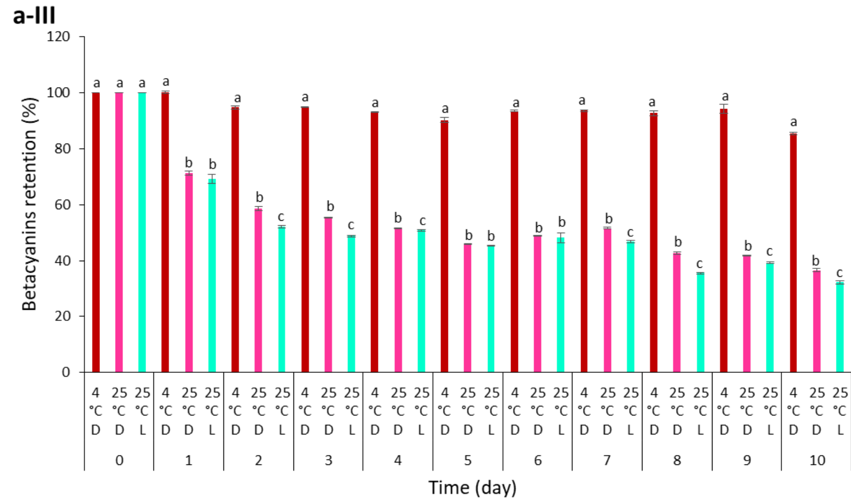
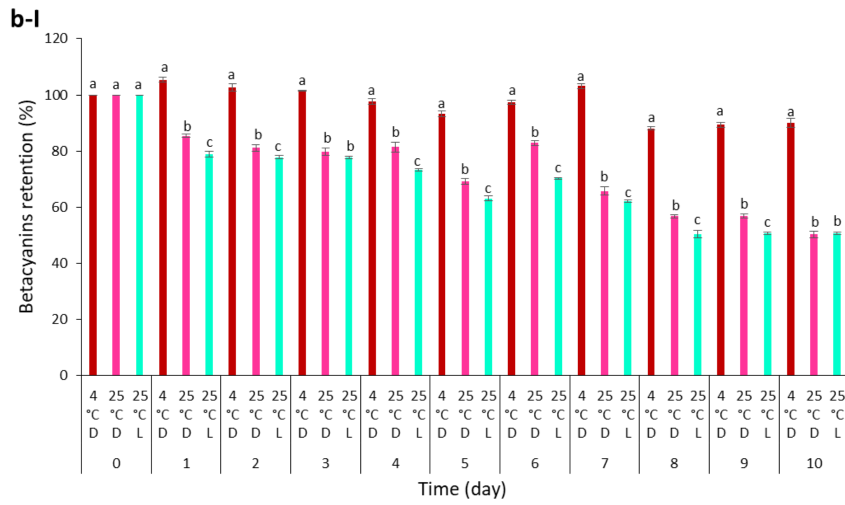


Figure 7-4: Betacyanins retentions (%) of (a) peel and (b) flesh extract of red-purple pitaya added with 0.5% (w/v) of ascorbic acid and with different pH treatments over a 10-days storage period. Values are mean ± SD of triplicate readings. I, II and III represent pH 3, 5 and 7, respectively. 4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively. Different letter(s) represent a significant different ($p < 0.05$) using Tukey's test within day.





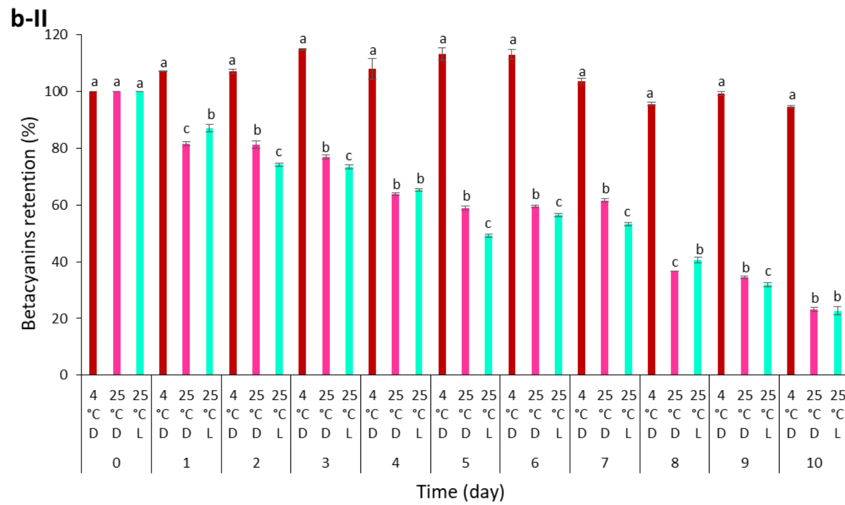


Figure 7-4 continued (1).

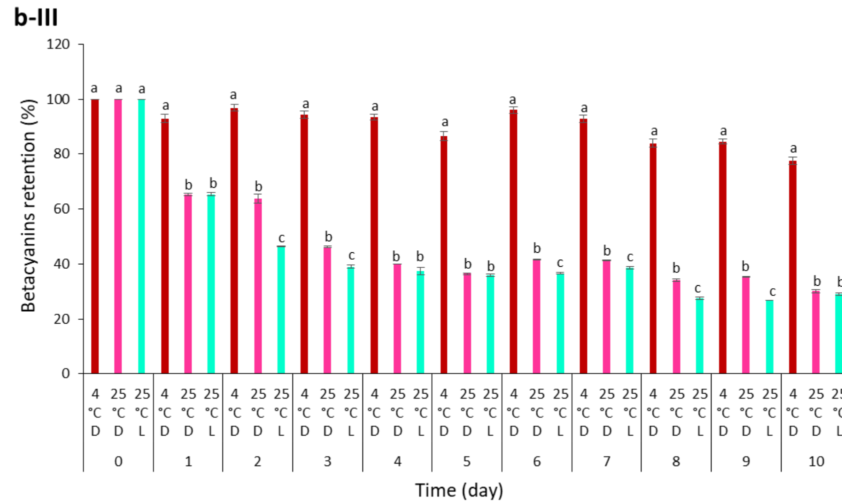


Figure 7-4 continued (2).

Our results infer that the betacyanins retention of the peel extract added with 0.5% of ascorbic acid at 4 °C dark storage increased with increasing pH from 3 to 7.44, and the highest betacyanins retention (114.976 ± 1.113%) was achieved at pH 7.44 (i.e. initial pH; **Figure 7-3a-II**). On the other hand, the betacyanins retention of the flesh extract added with 0.5% of ascorbic acid at 4 °C dark storage increased with increasing pH from 3 to 6.25, and the highest betacyanins retention (105.903 ± 1.589%) was achieved at pH 6.25 (i.e. initial pH; **Figure 7-3b-II**), which was then followed by a drop in the betacyanins retention at pH 7. For the peel and flesh extract at 25 °C dark and daylight storage, their betacyanins retentions were lower than that of at 4 °C dark storage. Dehydrogenation is observed at acidic environment (pH < 3), while alkaline environment caused hydrolytic cleavage of aldimine bond. Both mechanisms caused degradation of betacyanins, and resulted in the fading of red-purple colour (Azeredo, 2009; Woo et al., 2011).

Overall, the peel and flesh extract added with 0.5% (w/v) of ascorbic acid and without any pH adjustments (i.e. initial pH; 7.44 and 6.25, respectively) at all storage

conditions showed the highest betacyanins retention among the others, except for the flesh extracts at 25 °C dark and daylight storage. The flesh extracts added with 0.5% of ascorbic acid and without any pH adjustments (pH 6.25) at 25 °C dark and daylight storage (49.166 ± 0.612% and 41.368 ± 0.243%, respectively) showed a slightly lower betacyanins retention compared to that of those adjusted to pH 3 (50.301 ± 1.267% and 50.742 ± 0.506%, respectively) after 10 days. This could be due to the flesh extracts adjusted to pH 3 have higher starting betacyanins concentration, and thus they are relatively stable.

7.4.3 Kinetic analysis of degradation of betacyanins

The kinetic analysis of betacyanins degradation in the extracts for all the stability treatments was evaluated, and the results are presented in **Table 7-2**. In this analysis, the degradation rate constant (k) and half-life ($t_{1/2}$) were calculated. Smaller k value indicates a lower degradation rate, whereas bigger $t_{1/2}$ value means a given quantity of substance required longer time to degrade half of its initial quantity. In other words, a longer shelf life of a compound can be indicated by smaller k and bigger $t_{1/2}$ value. From **Table 7-2**, the peel and flesh extract supplemented with 0.5% (w/v) of ascorbic acid and without any pH adjustments demonstrated the most stable condition for the betacyanins stability, because it showed no degradation rate at 4 °C dark storage (negative values for both k and $t_{1/2}$), as well as a longer shelf life at 25 °C dark and daylight storage.

Table 7-2: Kinetic analysis of degradation of betacyanins in the extracts for all the stability treatments.

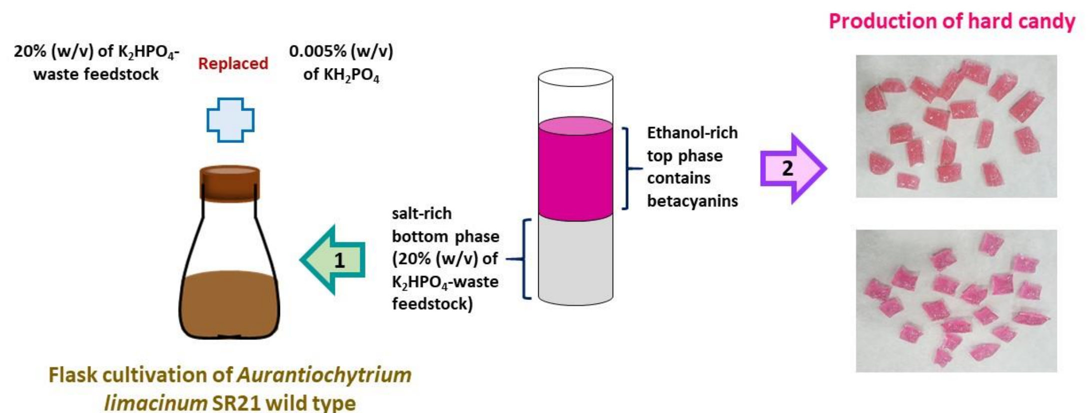
Parameter	Storage condition	Peel extract		Flesh extract	
		k (day^{-1})	$t_{1/2}$ (day)	k (day^{-1})	$t_{1/2}$ (day)
Control	4 °C D	0.085 ± 0.003^{j}	8.164 ± 0.259^{d}	0.066 ± 0.002^{f,g}	10.519 ± 0.368^{e,f}
	25 °C D	0.329 ± 0.003^{n,o,p}	2.106 ± 0.017^{d}	0.351 ± 0.005^{q}	1.978 ± 0.026^{f}
	25 °C L	0.331 ± 0.002^{n,o,p}	2.092 ± 0.010^{d}	0.331 ± 0.004^{o,p}	2.093 ± 0.025^{f}
0.1% of guar gum	4 °C D	0.074 ± 0.002^{i}	9.404 ± 0.204^{d}	0.042 ± 0.004^{e}	16.684 ± 1.506^{e,f}
	25 °C D	0.326 ± 0.005^{n,o}	2.128 ± 0.030^{d}	0.315 ± 0.007^{n}	2.204 ± 0.052^{f}
	25 °C L	0.353 ± 0.004^{q}	1.965 ± 0.025^{d}	0.336 ± 0.005^{p}	2.065 ± 0.029^{f}
0.5% of guar gum	4 °C D	0.045 ± 0.002^{f}	15.388 ± 0.735^{c,d}	0.044 ± 0.002^{e}	15.834 ± 0.859^{e,f}
	25 °C D	0.336 ± 0.002^{p}	2.064 ± 0.012^{d}	0.322 ± 0.002^{n,o}	2.150 ± 0.012^{f}
	25 °C L	0.329 ± 0.003^{n,o,p}	2.106 ± 0.019^{d}	0.312 ± 0.004^{n}	2.225 ± 0.028^{f}
1.0% of guar gum	4 °C D	0.053 ± 0.004^{f,g}	13.226 ± 0.949^{c,d}	0.059 ± 0.002^{f}	11.781 ± 0.315^{e,f}
	25 °C D	0.332 ± 0.006^{o,p}	2.087 ± 0.040^{d}	0.311 ± 0.003^{n}	2.227 ± 0.021^{f}
	25 °C L	0.324 ± 0.004^{n}	2.139 ± 0.026^{d}	0.313 ± 0.005^{n}	2.214 ± 0.036^{f}
0.1% of ascorbic acid	4 °C D	0.007 ± 0.003^{b}	114.643 ± 52.913^{b}	-0.006 ± 0.001^{a,b}	-113.128 ± 13.814^{a}
	25 °C D	0.158 ± 0.002^{m}	4.380 ± 0.042^{d}	0.136 ± 0.001^{j,k}	5.101 ± 0.019^{f}
	25 °C L	0.160 ± 0.002^{m}	4.324 ± 0.051^{d}	0.177 ± 0.002^{m}	3.925 ± 0.042^{f}
0.5% of ascorbic acid*	**4 °C D**	**-0.014 ± 0.001^{a}**	**-49.845 ± 3.560^{a}**	**-0.006 ± 0.001^{a,b}**	**-126.116 ± 29.482^{a}**
	25 °C D	**0.029 ± 0.001^{d,e}**	**24.214 ± 0.822^{c,d}**	**0.071 ± 0.001^{g}**	**9.764 ± 0.172^{e,f}**
	25 °C L	**0.030 ± 0.002^{d,e}**	**23.440 ± 1.302^{c,d}**	**0.088 ± 0.001^{h}**	**7.853 ± 0.052^{e,f}**
1.0% of ascorbic acid	4 °C D	-0.015 ± 0.002^{a}	-45.955 ± 4.703^{a}	-0.011 ± 0.001^{a}	-65.430 ± 8.006^{b}
	25 °C D	0.047 ± 0.001^{f,g}	14.680 ± 0.285^{c,d}	0.130 ± 0.011^{i,j}	5.341 ± 0.477^{f}
	25 °C L	0.064 ± 0.002^{h}	10.760 ± 0.359^{d}	0.186 ± 0.004^{m}	3.727 ± 0.086^{f}
0.5% of ascorbic acid + pH 3	4 °C D	0.034 ± 0.001^{e}	20.405 ± 0.768^{c,d}	0.011 ± 0.001^{c}	66.874 ± 11.461^{d}
	25 °C D	0.084 ± 0.002^{j}	8.249 ± 0.199^{d}	0.069 ± 0.003^{f,g}	10.093 ± 0.366^{e,f}
	25 °C L	0.086 ± 0.001^{j}	8.045 ± 0.138^{d}	0.068 ± 0.001^{f,g}	10.218 ± 0.150^{e,f}
0.5% of ascorbic acid + pH 5	4 °C D	0.025 ± 0.000^{d}	27.437 ± 0.042^{c,d}	0.005 ± 0.000^{b,c}	129.941 ± 11.210^{c}
	25 °C D	0.052 ± 0.001^{f,g}	13.260 ± 0.210^{c,d}	0.146 ± 0.003^{k,l}	4.743 ± 0.105^{f}
	25 °C L	0.055 ± 0.001^{g}	12.549 ± 0.221^{d}	0.148 ± 0.006^{l}	4.683 ± 0.206^{f}
0.5% of ascorbic acid + pH 7	4 °C D	0.016 ± 0.001^{c}	44.430 ± 1.554^{c}	0.025 ± 0.002^{d}	27.403 ± 1.976^{e}
	25 °C D	0.101 ± 0.001^{k}	6.890 ± 0.103^{d}	0.120 ± 0.002^{i}	5.774 ± 0.097^{e,f}
	25 °C L	0.113 ± 0.002^{l}	6.118 ± 0.113^{d}	0.124 ± 0.002^{i,j}	5.593 ± 0.083^{e,f}

Values are mean ± SD of triplicate readings.
Values in a column followed by different letter(s) are significantly different ($p < 0.05$) using Tukey's test.
* pH values for peel and flesh extract added with 0.5% of ascorbic acid are 7.44 and 6.25, respectively.
4 °C D, 25 °C D and 25 °C L represent 4 °C dark storage, 25 °C dark storage and 25 °C daylight storage, respectively.

7.5 Concluding remarks

This study concludes that the peel and flesh extract of red-purple pitaya supplemented with 0.5% (w/v) of ascorbic acid and without any pH adjustments exhibited higher retention of betacyanins with low degradation rate under all storage conditions. In addition, low temperature of 4 °C can promote a betacyanins regeneration. In other words, this study unravels some important aspects that are vital for the stabilisation of betacyanins which can be used as natural dyes in the food science processing. Furthermore, the peel of red-purple pitaya was used in this study as to fully utilise fruit by-product.

CHAPTER 8: Development of *Aurantiochytrium limacinum* SR21 Wild Type Cultivation using Salt-Rich Waste Feedstock for Docosahexaenoic Acid Production and Application of Natural Colourant in Food Product



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8.1 Abstract

Microalgae biorefinery is presently receiving a lot of attention as driven by its production of high value-added products. In this study, an oleaginous microalga *Aurantiochytrium limacinum* SR21 wild type was cultured for docosahexaenoic acid (DHA) production using 20% (w/v) of K_2HPO_4-waste feedstock to replace 0.005% (w/v) of KH_2PO_4 in the flask culture. DHA is an essential nutrient for human's brain functionalities. The effects of different parameters on the microalgal biomass concentration and lipid production were investigated, including number of moles of phosphate ions (PO_4^{3-}) and working concentration of the phosphate salts. Collectively, the K_2HPO_4-waste feedstock with working concentration of 0.005% (w/v) in the cultivation prompted a higher lipid content (8.29%) and DHA production (128.81 $mg.L^{-1}$), whereas there was no significantly change in biomass concentration after 6 days of cultivation when compared with others. Moreover, natural plant pigment products containing stabilised betacyanins were utilised as natural red colourants for hard candy production. This study develops microalgal cultivation using salt-rich waste feedstock for a higher lipid and DHA content as well as application of natural colouring agents in food products.

Keywords: *Aurantiochytrium limacinum* SR21 wild type; microalgae biorefinery; docosahexaenoic acid; natural colourant; salt-rich waste feedstock

8.2 Introduction

Microalgal biotechnology applications are presently a rapidly growing field. In particular, microalgae biorefinery is gaining global interest as an emerging biomass transformation approach. Generation of high value-added products in addition to biofuel through microalgae biorefinery have been developed. For instance, microalgae biomass can be transformed into pigments, proteins, lipids, polyunsaturated fatty acids (PUFAs), carbohydrates, vitamins and antioxidants (Yen et al., 2013). These high value-added materials are subsequently applied in various commercial and industrial applications, such as foods, cosmetics, pharmaceuticals and nutraceuticals (Chew et al., 2017; Pulz and Gross, 2004; Spolaore et al., 2006; Wang et al., 2017). One of the major benefits of culturing microalgae is that they can accumulate high percentages of lipid in their bodies (approximately 20–50% of their total weight) (Brennan and Owende, 2010). Among the microalgal lipids is a long chain PUFA called docosahexaenoic acid (DHA) which play a vital role as health food supplements (Borowitzka, 2013; Tan et al., 2016).

DHA (22:6n-3) is classified as an important lipid in the omega-3 family because it is an essential nutrient for neurological and cognitive functions in humans (Bradbury, 2011; Calderon and Kim, 2004; Kawakita et al., 2006; Kim, 2007). A DHA-rich diet formulation is required for infants as DHA improves the growth and functional development of the brain in infants. DHA consumption is also important in adults as it aids in maintaining normal brain functionalities. Moreover, sufficient DHA intake showed a preventive role in non-communicable diseases, such as diabetes, cardiovascular diseases, neurodegenerative diseases, heart diseases and cancers

(Bazan et al., 2011; Hashimoto, 2018; Horrocks and Yeo, 1999; Huang et al., 2012; Stillwell and Wassall, 2003).

Aurantiochytrium limacinum SR21, or previously known as *Schizochytrium limacinum* SR21 (Yokoyama and Honda, 2007), is a highly oleaginous microalga strain and reported as an excellent DHA producer. In addition, *Aurantiochytrium* species is easy to cultivate with short cultivation time. Previous studies have reported that cultivation of *A. limacinum* SR21 under optimised culture conditions produced high concentrations of lipid, DHA and biomass. Usage of different carbon sources, such as glycerol, crude glycerol and glucose, have prompted for higher lipid accumulation and microalgal growth in the cultivation of *A. limacinum* SR21 (Chi et al., 2007; Ethier et al., 2011; Gao et al., 2013; Huang et al., 2012; Li et al., 2015; Lung et al., 2016).

In recent years, natural colourants are receiving a lot of attention both from the consumers and food industries, as driven by the arising of negative health impacts on the utilisation of artificial colourants for food applications. Consumers prefer more safer, nutritious and healthier food products in addition to their appealing and delightful appearance. Natural colouring agents not only safe to consume but also offering some healthy functional. They can be obtained from natural pigments, such as betalains, anthocyanins and carotenoids. Betalains, especially betacyanins, are of growing interest due to their pH stability ranging from 3 to 7 which enable wide applications in colouring low acid to neutral foodstuffs (Carocho et al., 2015; Delgado-Vargas et al., 2000; Leong et al., 2018c; Martins et al., 2016; Moreno et al., 2008).

Taking the above into account, this study aimed to culture *A. limacinum* SR21 wild type (WT) using salt-rich waste feedstock, in addition to the use of glycerol as carbon

source for DHA production. *A. limacinum* SR21 is known to be a microalga species with high lipid and DHA content. 20% (w/v) of K_2HPO_4-waste feedstock was used as a replacement ingredient for 0.005% (w/v) of KH_2PO_4 in the flask cultivation. The KH_2PO_4 and K_2HPO_4-waste feedstock with different number of moles of phosphate ions (PO_4^{3-}) were first evaluated, followed by working concentration of the salts. Analyses of microalgal growth and lipid production, in particular DHA, were assessed. Subsequently, natural plant pigment products containing stabilised betacyanins (Leong et al., 2018b) were utilised as natural red colourants for hard candy production. This study renews attention towards the potentiality of microalgae biorefinery in respect to economic and environment evaluations as well as sustainable management on the salt-rich waste feedstock. Also, natural colouring agents were applied in the food products.

8.3 Materials and methods

8.3.1 Materials

Microalga strain *A. limacinum* SR21 WT was kindly provided by the Biorefinery and Bioprocess Engineering Laboratory, Yuan Ze University, Taoyuan, Taiwan. 20% (w/v) of K_2HPO_4-waste feedstock was obtained from our previously conducted experiment using liquid biphasic systems. Natural colouring agents (peel and flesh extract of red-purple pitaya) were obtained from our previously performed experiment (Leong et al., 2018b) (in line with Chapter 7 in this thesis). Food grade ethanol (99.8% (v/v)) was purchased from R&M Chemicals (Selangor, Malaysia). Other chemicals used in this experiment were of analytical grade (AG) (purity > 95%).

8.3.2 Flask cultivation of *A. limacinum* SR21 WT

The microalga was first pre-cultured in a shaking incubator (TLT-806080, Cherny Huei Co. Ltd., Taiwan) for 3 days. This was then followed by transference of 10% (v/v) of inoculum from the pre-cultured stock into a 500 mL conical flask containing 90 mL of artificial seawater medium supplemented with 3% (w/v) of glycerol (total working volume was 100 mL). The culture medium was adjusted to pH 7.5. Subsequently, the microalga was incubated at 22 °C for 6 days at a shaking speed of 150 rpm with 12 h of illumination. The *A. limacinum* SR21 flourish under conditions of low temperature and mild alkaline due to better photosynthesis process (Lung et al., 2016).

The culture medium was prepared according to UTEX culture collection of algae with slight modifications (UTEX, 2018). It is an artificial seawater medium and composed of NaCl (18 $g.L^{-1}$), $MgSO_4 \cdot 7H_2O$ (2.6 $g.L^{-1}$), KCl (0.6 $g.L^{-1}$), $NaNO_3$ (1 $g.L^{-1}$), $CaCl_2 \cdot 2H_2O$ (0.3 $g.L^{-1}$), KH_2PO_4 (0.05 $g.L^{-1}$), tricine (4.48 $g.L^{-1}$; stock solution (224 $g.L^{-1}$) was adjusted to pH 8), NH_4Cl (0.027 $g.L^{-1}$), 10 $mL.L^{-1}$ of P-II metal solution and 1 $mL.L^{-1}$ of chelated iron solution. The P-II metal solution was composed of $Na_2EDTA \cdot 2H_20$ (1 $g.L^{-1}$), H_3BO_3 (1.14 $g.L^{-1}$), $FeCl_3 \cdot 6H_2O$ (49 $mg.L^{-1}$), $MnSO_4 \cdot H_2O$ (164 $mg.L^{-1}$), $ZnSO_4 \cdot 7H_2O$ (22 $mg.L^{-1}$) and $CoCl_2 \cdot 6H_2O$ (4.8 $mg.L^{-1}$). The chelated iron solution was prepared using 10 g of $Na_2EDTA \cdot 2H_2O$ per 500 mL of distilled water and 0.81 g of $FeCl_3 \cdot 6H_2O$ per 450 mL of 0.1 M HCl; the total volume was brought up to 500 mL.

8.3.2.1 Replacement of KH_2PO_4 by K_2HPO_4-waste feedstock

The KH_2PO_4 in the culture medium (0.005% (w/v)) for *A. limacinum* SR21 WT cultivation was replaced by 20% (w/v) of K_2HPO_4-waste feedstock. The experiment was conducted using (1) different no. of moles of PO_4^{3-} (mol/L) and (2) similar working concentration (%, w/v) of the salts; the subsequent study with the chosen salt was further optimised using different working concentrations, in order to obtain an optimal result. In addition, 20% (w/v) of K_2HPO_4 salt (AG) was used to further confirm our experimental results obtained from the K_2HPO_4-waste feedstock.

8.3.3 Hard candy production

The peel and flesh extract of red-purple pitaya which play a role as natural red colourant were obtained from our previously performed experiment. To produce hard candy, 100 g of sugar, 33 g of glucose syrup and 20 g of water were first mixed and boiled to 155 °C, followed by addition of some natural colouring and flavouring to the candy.

8.3.4 Analytical procedures

8.3.4.1 Analysis of microalga cell concentration

The optical density (OD) of the microalga wet biomass concentration was analysed every 24 h using a UV-vis spectrophotometer (Ultrospec 2100 Pro, Amersham Biosciences Corporation, Little Chalfont, United Kingdom) at 680 nm for 6 days. On day 6, the microalga wet biomass was collected and centrifuged at 10000 rpm, 4 °C for 15 min to obtain a supernatant (centrifuge: Universal 320R, Hettich Zentrifugen, Germany). After that, the collected supernatant was washed twice using distilled water and was stored overnight at -20 °C before freeze dried at -40 °C and 0.13 atm for 6 h

using a freeze dryer (Labconco Corporation, Kansas City, MO). It was subsequently stored at -20 °C for further analysis.

8.3.4.2 Pre-treatment for analysis of lipid content

The pre-treatment for analysis of lipid content was divided into two parts: extraction and transesterification of lipid. Lipid transesterification procedure was carried out on the extracted lipid from freeze-dried microalga biomass in order to analyse the lipid content in respect to fatty acid methyl esters (FAMEs). A direct conversion of triglycerides into FAMEs occurred in the lipid transesterification.

(a) ***Extraction of lipid***

The extraction of lipid was conducted according to Folch et al. (1957) with slight modifications. An approximately 0.05 g of freeze-dried microalga biomass was used in the lipid extraction procedure. Chloroform solution (1 mL) was firstly added to the sample as solvent and subsequently put in a sonicator (E120 H Elmasonic, Sunway Scientific Corporation, Taipei) for 30 min in order to breakdown the cell wall. This was then followed by centrifugation (centrifuge: FORCE MINI, SN 0703 0026, Korea) at 5000 rpm for 5 min to obtain a supernatant. These procedures were repeated several times and suspended when the added solvent became colourless. Chloroform in the sample was removed by putting the sample in the oven at 100 °C for 12 h. The total lipid content in the sample was calculated using equation (8-1).

$$\text{Total lipid content (\%)} = \frac{\text{weight of lipid crude}}{\text{weight of dried algae}} \times 100\% \qquad (8\text{-}1)$$

(b) ***Transesterification of lipid***

The transesterification of lipid was conducted according to Cheah et al. (2018) with slight modifications. The sample was added with 3 mL of 0.5 N KOH/methanol solution and was mixed well before subjected to saponification at 100 °C for 10 min. The sample was then allowed to cool at room temperature before proceeded to esterification. The cooled sample was added with 3 mL of 0.7 N HCl/methanol solution and 1 mL of 14% (v/v) of BF_3/methanol solution, and was mixed well before brought to 100 °C for 10 min. Subsequently, 5 mL of saturated NaCl solution was added into the cooled FAMEs to prevent emulsification. Hexane was then added into the FAMEs. The layer containing hexane and FAMEs was used in the analysis of lipid content by gas chromatography (GC).

8.3.4.3 Analysis of lipid content

The lipid content in the microalga sample was analysed using GC equipped with a flame ionisation detector (FID) (Agilent 6890 GC, Agilent Technologies, USA). A mixed standard composed of 37 types of FAME was used to quantify the lipid content. The capillary column used was 0.25 µm DB-Wax (length: 30 m, internal diameter: 0.25 mm). Other operating conditions included: temperatures of injector and detector were set at 250 and 280 °C, respectively, injection volume of 3 µL (split ratio of 1/50), hydrogen as carrier gas with flow rate of 40 $mL.min^{-1}$ and oven temperature was set at 50 °C for 1 min, increased from 50–200 °C at a rate of 25 $°C.min^{-1}$, 200–230 °C at a rate of 3 $°C.min^{-1}$ and held at 230 °C for 18 min.

8.3.4.4 Colour characterisation

The colour parameters of lightness (L^*), redness (a^*) and yellowness (b^*) of the hard candy were analysed using a colorimeter (Lovibond LC 100, model RM 200, The Tintometer Ltd, United Kingdom). Other parameters, such as hue angle (h°) and chroma (C^*) were calculated according to equations (8-2) and (8-3), respectively.

$$h^\circ = \tan^{-1}\frac{b^*}{a^*} \qquad (8\text{-}2)$$

$$C^* = \sqrt{a^{*2} + b^{*2}} \qquad (8\text{-}3)$$

8.3.5 Statistical analysis

The statistical analysis was performed using IBM SPSS statistics software (SPSS version 23.0 for window, IBM Corporation, Armonk, New York, United States). The data were expressed as mean ± standard deviation (SD) (n = 3 or n = 6). The data were subjected to one-way analysis of variance (ANOVA), and the mean differences were compared using Tukey HSD post-hoc multiple comparisons test. The statistically difference was considered at $p < 0.05$.

8.4 Results and discussion

8.4.1 Effects of number of moles of PO_4^{3-} on microalgal growth and lipid production

In this study, we aimed to utilise 20% (w/v) of K_2HPO_4-waste feedstock as a replacement for 0.005% (w/v) of KH_2PO_4 (working concentration in the 100 mL flask culture) in the *A. limacinum* SR21 WT cultivation. Therefore, the effects of no. of moles of PO_4^{3-} in KH_2PO_4 and K_2HPO_4-waste feedstock on microalgal growth and

lipid production were investigated, followed by an optimisation study using cultivation with different no. of moles of PO_4^{3-} in the salts. The working concentration of 0.005% of KH_2PO_4 comprises 2.564×10^{-4} mol/L of PO_4^{3-}.

According to our findings, the stationary growth phase of *A. limacinum* SR21 WT in all instances occurred on day 6 (harvesting period), as shown in **Figure 8-1**. As shown in **Figure 8-1a**, the cultivation using KH_2PO_4 with 2.564×10^{-4} mol/L of PO_4^{3-} exhibited a slightly higher biomass concentration (12.93 ± 0.78 $g.L^{-1}$) compared to that of 1.282×10^{-4} (10.35 ± 0.49 $g.L^{-1}$) and 5.128×10^{-4} mol/L of PO_4^{3-} (10.15 ± 0.41 $g.L^{-1}$) on day 6. On the other hand, the biomass concentration on day 6 in the cultivation using replacement salt of K_2HPO_4-waste feedstock with 1.282×10^{-4} mol/L of PO_4^{3-} was slightly higher (13.11 ± 0.65 $g.L^{-1}$) compared to that of 2.564×10^{-4} and 5.128×10^{-4} mol/L of PO_4^{3-} (11.86 ± 0.76 and 12.43 ± 0.54 $g.L^{-1}$) (**Figure 8-1b**). Collectively, the microalgal cultivation using different phosphate salts with different no. of moles of PO_4^{3-} showed a biomass concentration ranging from 10.15–13.11 $g.L^{-1}$ with similar growth curves.

Moreover, the experimental data obtained from K_2HPO_4-waste feedstock was repeated using K_2HPO_4 salt (**Figure 8-1c**). Our results reveal that both K_2HPO_4 cultivations showed a similar trend on the microalgal growth curve and biomass concentration after 6 days of cultivation. The biomass concentrations using K_2HPO_4 salt with 1.282×10^{-4}, 2.564×10^{-4} and 5.128×10^{-4} mol/L of PO_4^{3-} were 12.04 ± 0.37, 11.77 ± 0.41 and 12.02 ± 0.51 $g.L^{-1}$, respectively. Overall, we can conclude that replacement of KH_2PO_4 by K_2HPO_4-waste feedstock in the culture comprises 2.564×10^{-4} mol/L of PO_4^{3-} , even with different no. of moles of PO_4^{3-} did not significantly influence the biomass concentration after 6 days of cultivation, as

indicated by the insignificant change on the biomass concentration. However, K_2HPO_4-waste feedstock can be utilised as a potential replacement for KH_2PO_4 in the microalgal cultivation in order to reduce the cultivation cost.

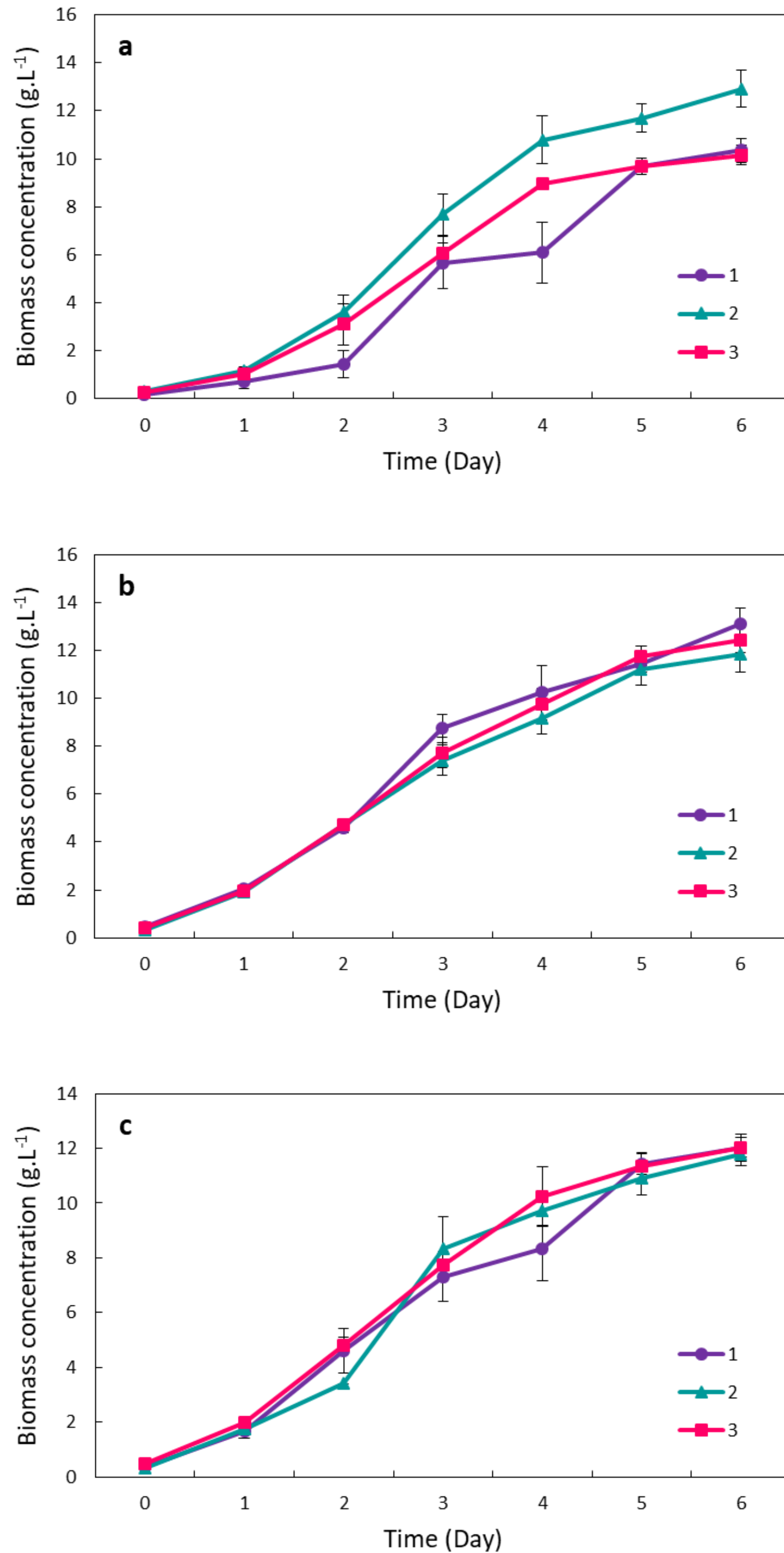


Figure 8-1: Effect of different phosphate salts with varying number of moles of PO_4^{3-} on biomass concentration of *A. limacinum* SR21 WT cultivation; (a) 0.005% (w/v) of KH_2PO_4, (b) 20% (w/v) of K_2HPO_4-waste feedstock, (c) 20% (w/v) of K_2HPO_4 (AG). 1, 2 and 3 represent 1.282×10^{-4}, 2.564×10^{-4} and 5.128×10^{-4} mol/L of PO_4^{3-}, respectively. Values are mean ± SD (n = 3).

As shown in **Table 8-1**, total lipid content in the microalgal cultivations using K_2HPO_4-waste feedstock with 1.282×10^{-4}, 2.564×10^{-4} and 5.128×10^{-4} mol/L of PO_4^{3-} were 7.92 ± 0.08%, 7.51 ± 0.06% and 7.02 ± 0.02%, respectively, in which they were significantly higher compared to that of the cultivations using KH_2PO_4 with 1.282×10^{-4}, 2.564×10^{-4} and 5.128×10^{-4} mol/L of PO_4^{3-} (0.62 ± 0.01%, 0.54 ± 0.03% and 0.68 ± 0.01%, respectively). Also, the total lipid content obtained in the cultivation with K_2HPO_4-waste feedstock were further affirmed by the cultivation using K_2HPO_4 salt, and both showed almost similar results. Brennan and Owende (2010), Chew et al. (2017) reported that microalgal cultivation under stress conditions, for example high salinity environment, could accelerate lipid accumulation. However, from **Table 8-1**, different no. of moles of PO_4^{3-} for each phosphate salt showed no observable difference on lipid accumulation. This could be explained by the similar salt concentration exhibited in the cultivation environment due to the low molar concentration of PO_4^{3-} (of the order of 10^{-4} mol/L). Interestingly, with the replacement of KH_2PO_4 by K_2HPO_4-waste feedstock in the culture medium, a 10 to 14-folds higher lipid content was achieved.

Similarly, the use of K_2HPO_4-waste feedstock in the cultivation resulted in a significant higher DHA content compared to that of KH_2PO_4, and our results were further confirmed by cultivation with K_2HPO_4 salt. The use of K_2HPO_4-waste feedstock with 1.282×10^{-4}, 2.564×10^{-4} and 5.128×10^{-4} mol/L of PO_4^{3-} produced 107.71 ± 4.88, 87.69 ± 3.67 and 56.31 ± 3.00 mg.L^{-1} DHA, respectively, and 1.282×10^{-4} mol/L of PO_4^{3-} produced the highest DHA content. Moreover, **Table 8-1** also shows a FAMEs profile obtained from the microalgal cultivation using different phosphate salts comprise different no. of moles of PO_4^{3-}. These results indicated that there is other

fatty acids being produced, for example C4, C14, C16 etc. besides DHA in the cultivation.

Based on our results, the use of KH_2PO_4 and K_2HPO_4-waste feedstock significantly influenced the lipid and DHA production but not the biomass concentration after 6 days of microalgal cultivation. In addition, the effect of different no. of moles of PO_4^{3-} in the salts only seem to affect the DHA production. These results infer that there is a potentiality of utilisation of K_2HPO_4-waste feedstock in the *A. limacinum* SR21 WT cultivation in order to produce high value-added products like DHA. This study achieves a good management of waste feedstock by microalgal cultivation. In addition, microalgae are known to be effective in biological treatment by removing nutrients (such as nitrogen, phosphorus etc.) and toxic metals, and as a result, they showed potentiality in wastewater treatment (Cai et al., 2013). Also, Xia and Murphy (2016) reported that the benefits of microalga-based liquid digestate treatment that not only can greatly reduce the microalgal cultivation cost but also involve in the management of biogas by-products.

Table 8-1: Lipid analysis of *A. limacinum* SR21 WT cultivation using different phosphate salts with varying number of moles of PO_4^{3-}.

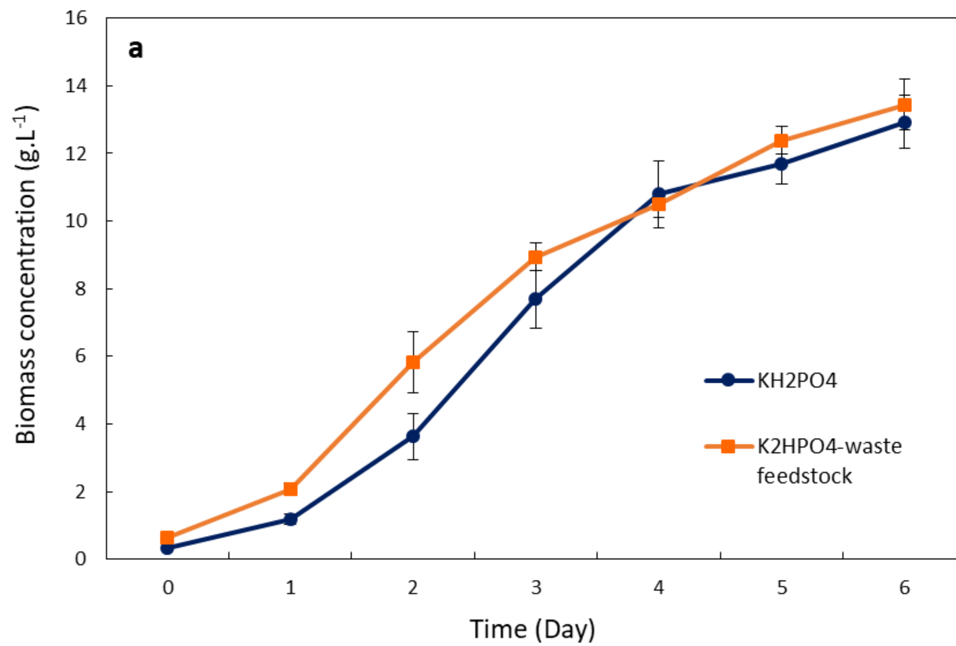
	KH_2PO_4		
	No. of moles of PO_4^{3-} (mol/L)		
FAMEs profile (mg.L^{-1})	1.282×10^{-4}	2.564×10^{-4}	5.128×10^{-4}
C4	15.00 ± 0.74	32.00 ± 2.00	22.95 ± 1.01
C6	8.70 ± 0.34	5.26 ± 0.33	8.88 ± 0.39
C14	2.45 ± 0.15		2.55 ± 0.11
C15	1.09 ± 0.07		1.13 ± 0.05
C16	15.13 ± 0.69	19.87 ± 1.24	16.95 ± 0.74
C17	0.82 ± 0.05	0.34 ± 0.59	0.85 ± 0.04
C18:1	4.84 ± 0.26	1.13 ± 1.96	2.84 ± 0.12
C21	1.36 ± 0.08		
C22	2.72 ± 0.17	1.13 ± 1.96	4.29 ± 0.19
C22:1	1.36 ± 0.08		5.62 ± 0.45
*C22:6 (DHA)	5.26 ± 0.39^{c}	10.27 ± 0.50^{a}	5.62 ± 0.45^{b}
C24	5.16 ± 0.27		
*Total lipid content (%)	0.62 ± 0.01^{b}	0.54 ± 0.03^{c}	0.68 ± 0.01^{a}
	K_2HPO4-waste feedstock		
	No. of moles of PO_4^{3-} (mol/L)		
FAMEs profile (mg.L^{-1})	1.282×10^{-4}	2.564×10^{-4}	5.128×10^{-4}
C4	6.87 ± 0.32	6.53 ± 0.40	94.53 ± 4.15
C6	1.84 ± 0.06		9.88 ± 0.43
C14	65.02 ± 2.56	62.98 ± 3.85	60.11 ± 2.64
C15	50.44 ± 1.98	40.59 ± 2.48	28.04 ± 1.23
C16	712.54 ± 27.85	606.29 ± 37.06	560.90 ± 24.64
C17	24.38 ± 0.96	20.32 ± 1.24	11.14 ± 0.49
C18:1	35.68 ± 1.42	26.82 ± 1.64	25.49 ± 1.12
C22			3.27 ± 0.15
C22:1		9.59 ± 0.59	5.90 ± 0.26
*C22:6 (DHA)	107.71 ± 4.88^{a}	87.69 ± 3.67^{b}	56.31 ± 3.00^{c}
C24	33.26 ± 1.33	30.06 ± 1.84	17.15 ± 0.75
*Total lipid content (%)	7.92 ± 0.08^{a}	7.51 ± 0.06^{b}	7.02 ± 0.02^{c}
	K_2HPO_4 (AG)		
	No. of moles of PO_4^{3-} (mol/L)		
FAMEs profile (mg.L^{-1})	1.282×10^{-4}	2.564×10^{-4}	5.128×10^{-4}
C4	6.29 ± 0.26	6.40 ± 0.29	95.86 ± 5.36
C6			11.02 ± 0.62
C14	58.36 ± 2.61	61.65 ± 2.78	69.65 ± 3.89
C15	44.85 ± 2.01	39.96 ± 1.80	34.04 ± 1.90
C16	622.06 ± 27.91	604.97 ± 27.27	613.02 ± 34.28
C18:1			29.23 ± 1.63
C22:1	8.71 ± 0.39	10.37 ± 0.47	
*C22:6 (DHA)	106.63 ± 7.21^{a}	89.60 ± 5.09^{b}	56.54 ± 4.15^{c}
C24	29.07 ± 1.29	29.54 ± 1.33	18.96 ± 1.06
*Total lipid content (%)	7.28 ± 0.03^{b}	7.16 ± 0.10^{b}	7.72 ± 0.11^{a}

Values are mean ± SD (n = 3).
*Values in a row followed by different letter(s) are significantly different ($p < 0.05$) using Tukey's test within the same phosphate salt.

8.4.2 Effects of working concentration of phosphate salts on microalgal growth and lipid production

In the second part of the study, we aimed to investigate the maximum working concentration of phosphate salt in the microalgal cultivation. The effect of same working concentration of KH_2PO_4 and K_2HPO_4-waste feedstock (0.005% (w/v)) in the cultivation of *A. limacinum* SR21 WT on the microalgal growth and lipid production was evaluated. The experiment was subsequently optimised with the chosen phosphate salt with different working concentrations.

As depicted in **Figure 8-2a**, with the replacement of 0.005% of KH_2PO_4 by 0.005% of K_2HPO_4-waste feedstock, the microalga grew rapidly over 6 days of cultivation. The microalgal cultivation with the K_2HPO_4-waste feedstock showed a slightly higher biomass concentration (13.43 ± 0.75 $g.L^{-1}$) compared to that of the KH_2PO_4 (12.93 ± 0.78 $g.L^{-1}$) on day 6. In addition, the cultivation using K_2HPO_4-waste feedstock produced significantly higher amounts of lipid and DHA content (8.29 ± 0.19% and 128.81 ± 9.93 $mg.L^{-1}$) (**Table 8-2**). As a result, K_2HPO_4-waste feedstock was chosen, and different working concentrations of the salt was further studied in the microalgal cultivation.



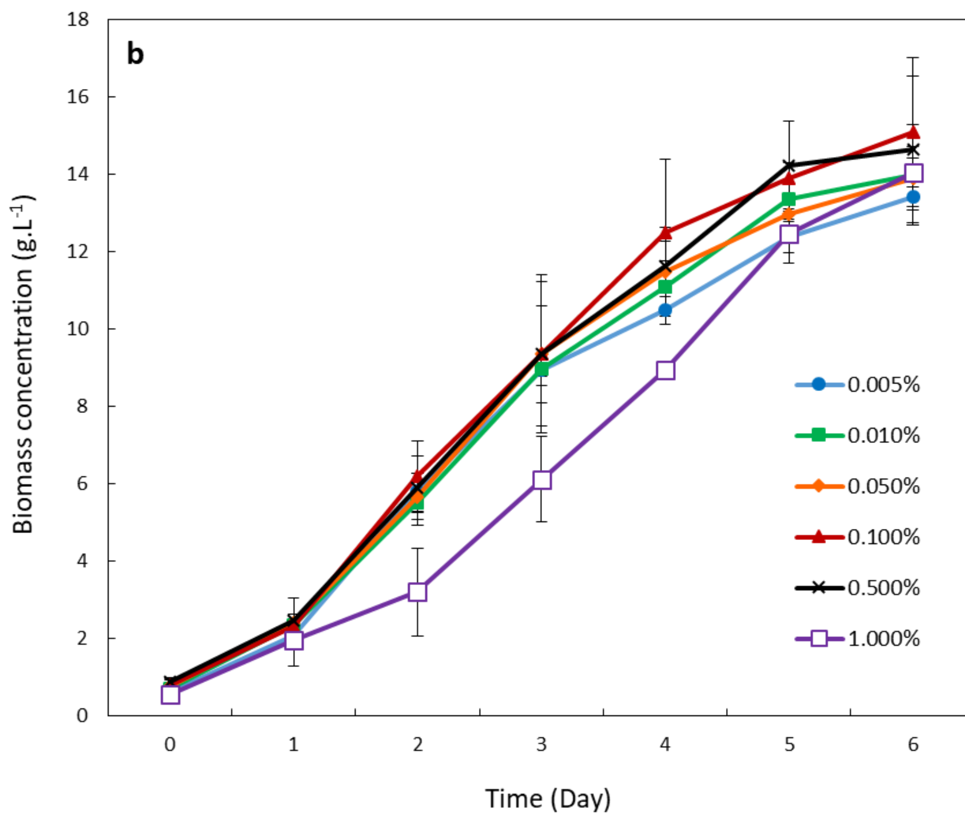


Figure 8-2: Effect of working concentration of different phosphate salts on biomass concentration of *A. limacinum* SR21 WT cultivation; (a) 0.005% (w/v) of KH_2PO_4 and K_2HPO_4-waste feedstock, (b) different working concentrations of K_2HPO_4-waste feedstock. Values are mean ± SD (n = 3).

Table 8-2: Lipid analysis of *A. limacinum* SR21 WT cultivation using different phosphate salts (based on working concentration).

FAMEs profile ($mg.L^{-1}$)	Working concentration of phosphate salt (%, w/v)						
	KH_2PO_4	K_2HPO_4-waste feedstock					
	0.005	0.005	0.010	0.050	0.100	0.500	1.000
C4	32.00 ± 2.00		141.63 ± 13.58	66.73 ± 4.23	81.54 ± 10.43	90.07 ± 11.09	
C6	5.26 ± 0.33			30.64 ± 1.94	14.32 ± 1.86	8.10 ± 0.97	4.19 ± 0.11
C14		72.23 ± 2.37	91.54 ± 8.65	37.32 ± 2.37	29.42 ± 3.89	12.96 ± 1.56	10.73 ± 0.28
C15		53.43 ± 3.12	57.99 ± 5.30	26.08 ± 1.65	18.43 ± 2.37	7.79 ± 0.93	5.94 ± 0.15
C16	19.87 ± 1.24	734.77 ± 25.95	805.17 ± 76.07	338.83 ± 21.65	254.98 ± 32.42	101.26 ± 12.15	82.63 ± 2.28
C17	0.34 ± 0.59	23.93 ± 1.01	25.53 ± 2.44	11.03 ± 0.70	8.09 ± 1.02	3.62 ± 0.43	0.35 ± 0.61
C18:1	1.13 ± 1.96	37.48 ± 1.61	45.34 ± 4.29	18.94 ± 1.23	16.60 ± 2.11	7.99 ± 0.96	8.02 ± 0.21
C21		7.85 ± 0.34					
C22	1.13 ± 1.96	7.80 ± 0.28	15.18 ± 1.44	1.28 ± 2.21	1.51 ± 2.62	1.15 ± 2.00	1.18 ± 2.05
C22:1		10.65 ± 0.46	11.14 ± 1.11	4.92 ± 0.32		3.61 ± 0.43	3.56 ± 0.10
*C22:6 (DHA)	10.27 ± 0.50[d]	128.81 ± 9.93[a]	81.54 ± 8.98[b]	53.11 ± 3.12[c]	51.48 ± 6.80[c]	14.77 ± 1.06[d]	2.15 ± 0.06[d]
C24		35.32 ± 1.54	31.50 ± 3.00	17.40 ± 1.22	16.86 ± 2.14	6.56 ± 0.79	
*Total lipid content (%)	0.54 ± 0.03[g]	8.29 ± 0.19[b]	9.34 ± 0.03[a]	4.36 ± 0.03[c]	3.27 ± 0.01[d]	1.76 ± 0.02[e]	0.85 ± 0.02[f]

Values are mean ± SD (n = 3).
*Values in a row followed by different letter(s) are significantly different ($p < 0.05$) using Tukey's test.

As shown in **Figure 8-2b**, the biomass concentration on day 6 increases as the working concentration of K_2HPO_4-waste feedstock increased from 0.005 to 0.100% (from 13.43 ± 0.75 to 15.09 ± 1.92 g.L^{-1}), which then decreases slightly for the subsequent working concentration up to 1.000% (14.05 ± 0.37 g.L^{-1}). However, the cultivation with different working concentrations of K_2HPO_4-waste feedstock ranged from 0.005 to 1.000% (w/v) demonstrated a non-significant result on the biomass concentrations after 6 days. The different working concentrations showed no remarkable difference among themselves with the biomass concentration ranging from 13.43–15.09 g.L^{-1}.

On the other hand, an increase in the working concentration of K_2HPO_4-waste feedstock from 0.005 to 0.010% greatly increased the lipid production from 8.29 ± 0.19% to 9.34 ± 0.03%, but a further increment in the working concentration up to 1.000% seem to cause a reduction in the lipid content. In contrast, increasing in the working concentration from 0.005–1.000% significantly decreased the DHA production. The highest DHA content was noted in the cultivation with 0.005% of K_2HPO_4-waste feedstock, with a value of 128.81 ± 9.93 mg.L^{-1}, whereas the lowest was observed with the use of 0.500% and 1.000% of K_2HPO_4-waste feedstock (both showed no significant difference), with values of 14.77 ± 1.06 and 2.15 ± 0.06 mg.L^{-1}, respectively (**Table 8-2**). In addition, **Table 8-2** also presents a FAMEs profile obtained from the microalgal cultivation using different phosphate salts which based on working concentration.

Overall, the cultivation of *A. limacinum* SR21 WT with replacement of KH_2PO_4 by K_2HPO_4-waste feedstock affected the lipid and DHA production. Collectively, 0.005% of the K_2HPO_4-waste feedstock (containing 1.565×10^{-4} mol/L of PO_4^{3-}) provided the

highest DHA production and a higher lipid accumulation, although its effect on biomass concentration was minimal. Approximately 12-folds higher DHA production and 15-folds higher lipid content in the cultivation using salt-rich waste feedstock were achieved compared to the use of the original culture medium.

Although our experimental results on the biomass concentration as well as lipid and DHA production are lower compared to the literatures (Chi et al., 2007; Ethier et al., 2011; Gao et al., 2013; Huang et al., 2012; Li et al., 2015; Lung et al., 2016; Rosa et al., 2010; Yokochi et al., 1998), there is a possibility to enhance the concentrations of biomass, lipid and DHA with further optimisation of the culture conditions. In addition, with the utilisation of salt-rich waste feedstock to replace KH_2PO_4, we can reduce the cultivation cost and achieve improvements on the microalgal lipid and DHA production compared to the original culture medium.

Conforming to literatures (Brennan and Owende, 2010; Cheah et al., 2018; Chew et al., 2017; Tan et al., 2016), the lipid accumulation in microalgae culture can be increased by manipulating several growth determining conditions, such as temperature, salt concentration, light intensity, carbon source, nitrogen concentration etc. The lipid productivity increases with the simultaneous increment in biomass productivity and lipid accumulation. Therefore, to further improve our biomass and lipid production, we can optimise the growth determining elements. Last but not least, this study renews attention toward the potentiality of microalgae biorefinery in respect to economic and environment evaluations as well as sustainability using salt-waste feedstock.

8.4.3 Hard candy production using natural plant colourants

In this experiment, we utilised our natural plant pigment products, i.e. peel and flesh extract of red-purple pitaya containing stabilised betacyanins (Leong et al., 2018b) as natural colourants for hard candy production. Both the peel and flesh extract showed a red-violet colouration. We have successfully applied them in the making of hard candy, as shown in **Figure 8-3**. The candy made with the peel and flesh extract showed different variations, saturations and intensities of red colour. The candy with the peel extract showed a red-yellowish colour (positive values of a^* and b^*), while the candy with the flesh extract demonstrated a red-bluish colour (positive value of a^* and negative value of b^*) (**Table 8-3**). In addition, literature had reported on the utilisation of stabilised betanin in gummy candy production (Amjadi et al., 2018). These applications could be useful in the food industries as to replace artificial food colourants, in addition to the utilisation in nutraceuticals, medicals and cosmetics fields.

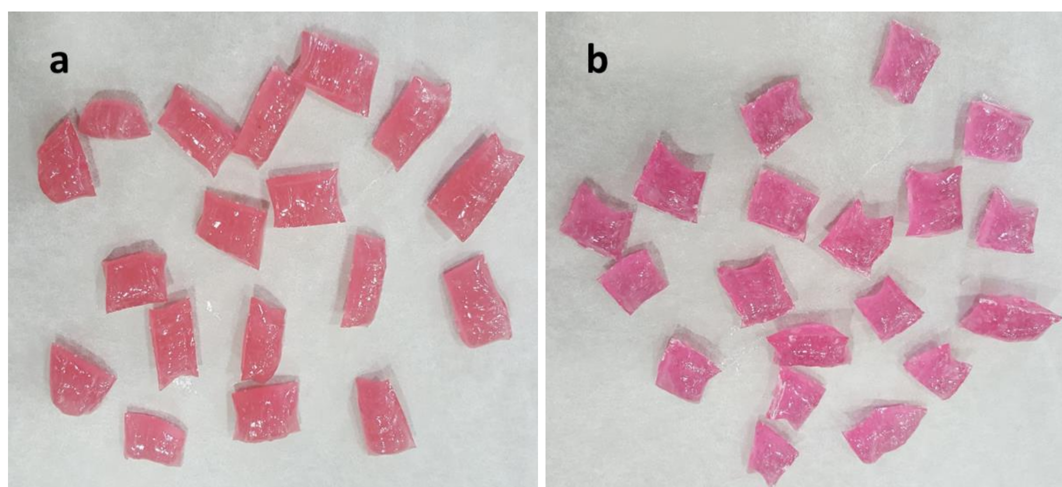


Figure 8-3: Hard candy made with natural red colouring agents obtained from (a) peel and (b) flesh extract of red-purple pitaya.

Table 8-3: Colour analysis of hard candy made with natural colourants obtained from the red-purple pitaya extract.

Colour parameter	Hard candy with peel extract	Hard candy with flesh extract
L^*	24.53 ± 3.43	26.13 ± 1.44
a^*	16.35 ± 1.96	18.02 ± 2.07
b^*	1.02 ± 0.22	-2.47 ± 0.69
C^*	16.38 ± 1.97	18.17 ± 2.01
h°	3.60 ± 0.72	352.00 ± 2.96

Values are mean ± SD (n = 6).

8.5 Concluding remarks

In summary, it was found that 0.005% (w/v) of K_2HPO_4-waste feedstock (20%, w/v) comprises 1.565×10^{-4} mol/L of PO_4^{3-} can replace 0.005% (w/v) of KH_2PO4 in the *Aurantiochytrium limacinum* SR21 WT cultivation, due to the highest DHA production and a higher lipid accumulation. This study reveals that there is a potentiality to develop microalgal cultivation using salt-rich waste feedstock for production of high value-added products. In addition, further studies and eventually scale-up such cultivation are definitely worth to investigate as it could reduce the cultivation cost. Furthermore, natural plant pigment products containing stabilised betacyanins were successfully applied as natural food colourants.

CHAPTER 9: General Conclusions and Future Prospective

With the vast evolution of biotechnology in the last few decades, many efforts have been used to upgrade the downstream processing, especially in the field of food science. With regard to this matter, the innovative and effective bioseparation technologies have been developed. Liquid biphasic system represents an effective and green separation approach for many biotechnological and natural products. In this research, LBF and LBPS (i.e. type of liquid biphasic system) were successfully applied to extract betacyanins from the peel and flesh of red-purple pitaya. To seek for further improvement on the betacyanins extraction efficiency of these methods, we integrated electricity treatment to these systems (i.e. LBEF and LBEPS), which improved the betacyanins extraction efficiency. **Table 9-1** summarises the results of betacyanins extraction by various liquid biphasic systems and the conventional solvent extraction approaches in this dissertation. It can be concluded that, by the application of liquid biphasic systems, a significant amount of betacyanins was achieved as compared to that of the conventional extraction methods.

Additionally, two types of applications were successfully accomplished in the present study. The salt-rich waste feedstock obtained from the liquid biphasic systems was used in *Aurantiochytrium limacinum* SR21 wild type cultivation, and a higher lipid accumulation and DHA production were noted. Whereas, the natural plant pigment products containing the stabilised betacyanins were applied as natural red colourants in the making of hard candy. This dissertation presents a sustainable management on the waste feedstock and utilisation of natural colouring agents in food products preparation.

The suggestions on the further research works include (1) solvent recycling processes in the liquid biphasic systems, (2) the study on kinetics, thermodynamics and also on the development of theoretical models for various liquid biphasic systems, (3) the complete modelling of these processing methods due to it is essential for a rapid assessment of the outcome of diverse process parameters as to further scale-up of these systems for extraction and purification of biomolecules, (4) integration process with liquid biphasic system for a superior downstream process, (5) further studies on the microalgae cultivation for value-added products and (6) application of natural plant pigment products as natural food colourant. These innovative and green ideas are interesting and useful for food industries, and they create new opportunities for microalgae and natural plant pigment applications.

Table 9-1: Results of several extraction techniques on betacyanins extraction from (a) peel and (b) flesh of red-purple pitaya in this dissertation.

(a)

Extraction approach	Betacyanins extraction from peel				
	C_t (%)	K	E (%)	V_r	TBC (mg of BEs/ 100 g of crude extract)
LBF system	95.989 ± 0.479	24.168 ± 2.949	88.361 ± 1.708		7.566 ± 0.580
LBPS	98.080 ± 0.051	51.097 ± 1.354		1.667	180.654 ± 2.239
LBEF system	99.014 ± 0.074	100.814 ± 7.324	98.383 ± 0.215		8.067 ± 0.420
LBEPS	99.256 ± 0.014	133.433 ± 2.566		1.667	156.877 ± 2.655
Conventional extraction with purified water					100.027 ± 0.028
Conventional extraction with 80% (w/w) ethanol					21.961 ± 0.145

(b)

Extraction approach	Betacyanins extraction from flesh				
	C_t (%)	K	E (%)	V_r	TBC (mg of BEs/ 100 g of crude extract)
LBF system	95.488 ± 0.213	21.195 ± 1.030	94.886 ± 0.060		23.005 ± 1.161
LBPS	96.256 ± 0.207	25.764 ± 1.525		2.167	139.645 ± 0.198
LBEF system	96.132 ± 0.154	24.883 ± 1.052	96.576 ± 0.083		15.649 ± 0.226
LBEPS	97.189 ± 0.172	34.665 ± 2.253		2.167	147.840 ± 3.038
Conventional extraction with purified water					97.363 ± 0.022
Conventional extraction with 80% (w/w) ethanol					44.350 ± 0.068

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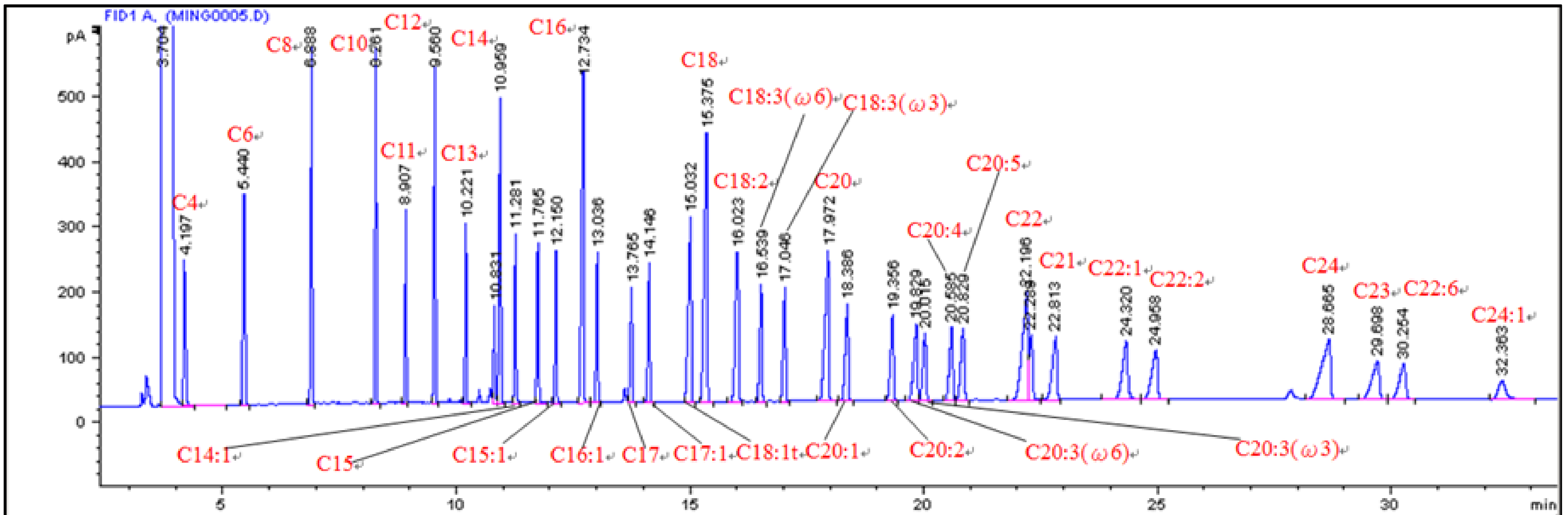
Appendix

Apendix A: Gas chromatography analysis of fatty acid methyl ester standard mixture.



In this analysis, the sample showed a range of short carbon chain (C4:0) to a medium long carbon chain (C24:1) saturated and unsaturated fatty acids.