ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

DETERMINATION OF THE STABILITY OF BEESWAX ENCAPSULATED RED BEETROOT EXTRACT IN KEFIR

M.Sc. THESIS

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Department of Food Engineering

Food Engineering Programme

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BALMUMU İLE KAPSÜLLENMİŞ KIRMIZI PANCAR EKSTRAKTININ KEFİR İÇERİSİNDEKİ STABİLİTESİNİN BELİRLENMESİ

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To my father and mother,

FOREWORD

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TABLE OF CONTENTS

Page

FOREWORD .... **ix**
TABLE OF CONTENTS .... **xi**
ABBREVIATIONS .... **xiii**
SYMBOLS .... **xv**
LIST OF TABLES .... **xvii**
LIST OF FIGURES .... **xix**
SUMMARY .... **xxi**
ÖZET .... **xxv**
1. INTRODUCTION **1**
1.1 Purpose of Thesis 2
1.2 Betalain Compounds 2
1.3 Phenolic Compounds 8
1.4 Red Beetroot Phenolic and Betalain Contents 14
1.5 Encapsulation 16
1.5.1 Encapsulation techniques 17
1.5.2 Wall materials used in encapsulation 21
1.5.3 Beeswax as a wall material 23
1.5.4 Red beetroot extract encapsulation 24
1.6 Hypothesis 25
2. MATERIALS AND METHODS **27**
2.1 Red Beetroot Samples 27
2.2 Chemicals 27
2.3 Red Beetroot Extraction Procedure 27
2.4 Encapsulation Procedure 28
2.5 Measurement of Particle Size Distribution and Zeta Potential 28
2.6 SEM (Scanning Electron Microscope) 28
2.7 Measurement of Total Phenolic Content 28
2.8 Measurement of Total Flavonoids Content 29
2.9 Measurement of Total Betalain Content 29
2.10 Determination of Encapsulation Efficiency 30
2.11 Measurement of Antioxidant Activity 30
2.11.1 Measurement of antioxidant activity using DPPH assay 30
2.11.2 Measurement of antioxidant activity using CUPRAC assay 31
2.12 HPLC (High Pressure Liquid Chromatography) Analysis 31
2.12.1 Determination of phenolic profile 31
2.12.2 Determination of betacyanin profile 31
2.13 Inclusion of the Capsules into Kefir Formulation 32
2.14 Storage Stability of Red Beetroot Extract and Capsules in Kefir 32
2.15 Color Measurements 32
2.16 Statistical Analysis 32
3. RESULTS AND DISCUSSION **33**
3.1 TPC, TFC and Betalain Content of Red Beetroot Extract 33

3.2 Characterization of Encapsulates 36
3.2.1 Particle size and polydispersity index 36
3.2.2 Zeta (ζ) potential 37
3.3 Encapsulation Efficiency 37
3.4 SEM Analysis 38
3.5 TPC, TFC and Betalain Content of Encapsulates 40
3.6 Antioxidant Activity of Red Beetroot Extract and Capsules 41
3.7 Storage Stability of Red Beetroot Extract and Capsules in Kefir 43
3.8 Color Measurements 51
4. CONCLUSION AND RECOMMENDATION 55
REFERENCES 59
APPENDICES 67
CURRICULUM VITAE 81

ABBREVIATIONS

HPLC	**:** High performance liquid chromatography
TPC	**:** Total phenolic content
TPF	**:** Total flavonoid content
GAE	**:** Gallic acid equivalent
CE	**:** Catechin equivalent
BE	**:** Betanin equivalent
VE	**:** Vulgaxanthin – I equivalent
DPPH	**:** 2,2 – diphenyl – 1 – picrylhydrazyl
CUPRAC	**:** Cupric ion reducing antioxidant capacity
K	**:** Kefir samples
KE	**:** Red beetroot extract added kefir samples
KC	**:** Encapsulated red beetroot extract added kefir samples
KB	**:** Empty capsules added kefir samples

SYMBOLS

M	: Molarity (mol / L)
mM	: Millimolar (mmol / L)
μM	: Micromolar (μmol /L)
nm	: Nanometer
mV	: Millivolt
μL	: Microliter
mL	: Mililiter
rpm	: Round per minute
w/w	: Weight per weight ratio
w/v	: Weight per volume ratio
p	: Statistical probability
s	: Second
min	: Minute

LIST OF TABLES

Page

Table 1.1: Betalain content of some plant species (Celli & Brooks, 2017)....................**3**
Table 1.2: Classification of phenolic compounds according to carbon number (Gutierrez-Uribe & Nollet, 2018)..**9**
Table 1.3: Phenolic contents of some foods (Gutierrez - Uribe & Nollet, 2018).......**13**
Table 1.4: Pigment content (g/kg) in Dutch (D) and Hungarian (H) varieties of redbeet (Neelwarne, 2013)..**16**
Table 1.5: Summarization of wall material used in encapsulation proces (Wandrey et al., 2010)...**23**
Table 1.6: Components of beeswax (Bognadov, 2004)..**24**
Table 3.1: Contents of red beetroot extract **33**
Table 3.2: Particle sizes of the prepared encapsulates...**36**
Table 3.3: Zeta potential of prepared encapsulates...**37**
Table 3.4: Calculated encapsulation efficiency according to different core / wall ratio **38**
Table 3.5: Contents of capsules **41**
Table 3.6: Antioxidant activity of red beetroot extract **42**
Table 3.7: Antioxidantactivity of the capsules **42**
Table 3.8: Change in TPC of different kefir samples during storage **43**
Table 3.9: Change in TFC of different kefir samples during storage **44**
Table 3.10: Change in betacyanin content of different kefir samples during storage **45**
Table 3.11: Change in betaxanthin content of different kefir samples during storage **45**
Table 3.12: Antioxidant activity of different kefir samples in terms of DPPH assay during storage **46**
Table 3.13: Antioxidant activity of different kefir samples in terms of CUPRAC assay during storage **47**
Table 3.14: Phenolic profile of the different kefir samples during storage **48**
Table 3.15: Betacyanin profile of different dekir samples during storage time…........**50**
Table 3.16: Color values of red beetroot extract and prepared encapsulates **51**
Table 3.17: Colors of different kefir formulations during storage period.....................**52**
Table 3.18: Changes in color values (L^*, a^* and b^*) of kefir samples during storage **53**

LIST OF FIGURES

Page

Figure 1.1: Biosynthesis of betalamic acid and cyclo – Dopa from tyrosine (Esquivel, 2016)……………………………………………………….**4**
Figure 1.2: Synthesis of betacyanin and betaxanthin pigments (Esquivel, 2016)…...**5**
Figure 1.3: Different betalain types structures (Esatbeyoğlu et al., 2015)……..……**5**
Figure 1.4: Structure of vulgaxanthin and indicaxanthin (Esatbeyoğlu et al., 2015)..**6**
Figure 1.5: Degradation pathways of betanin (Herbach et al., 2006)………………..**8**
Figure 1.6: Primary and secondary metobolism in plants (Giada, 2013)…………..**10**
Figure 1.7: Chemical structure of flavonoids, isoflavonoids and neoflavonoids (Gutierrez-Uribe & Nollet, 2018)………………………….………..**11**
Figure 1.8: Chemical structure of different flavonoids (Gutierrez-Uribe & Nollet, 2018)……………………………..…………………………..……**11**
Figure 1.9: Structures of different types of benzoic acids (Martins et al., 2011)…...**12**
Figure 1.10: Structures of different tyes of cinnamic acids (Martins et al., 2011)………………………………………………………...........**13**
Figure 1.11: Color diversity of beets; (1) shows absence of pigment, (2) shows dominant presence of betaxanthin, (3) sowhshigh betaxanthin + low betacyanin, (4) shows low betaxanthin + high betacyanin and (5) shows dominance of betacyanin (Neelwarne, 2013)..**15**
Figure 1.12: Bioactive contents of red beetroot (Clifford et al., 2015)…………….**16**
Figure 1.13: Spray drying system (Zuidam & Nedovic, 2010)…………………….**18**
Figure 1.14: Spray chilling / cooling system representation (Okuro et al., 2013).....**19**
Figure 1.15: Bottom and upward spraying type of fluid bed coating system representation (Zuidam & Nedovic, 2010)…………………………..**19**
Figure 1.16: Shematic view of extrusion process (Zuidam & Nedovic, 2010).........**20**
Figure 1.17: Shematic view of coacervation process (Salve & Changediya, 2016)……………………….........................**21**
Figure 3.1: SEM microphotographs of beeswax encapsulates at a magnification of 2000x, 4000x and 8000x…………………………………………….**39**
Figure A.1: Standard calibration curve for total phenolic analysis………………....**68**
Figure A.2: Standard calibration curve for total flavonoid analysis…………..……**68**
Figure A.3: Standard calibration curve for CUPRAC analysis……………………**69**
Figure A.4: Standard calibration curve for DPPH analysis………………………..**69**
Figure B.1: HPLC chromatogram of the red beetroot extract……………………...**70**
Figure B.2: HPLC chromatogram of the capsules…………………………………**71**
Figure B.3: HPLC chromatogram of the extract added kefir sample in 1st day……**72**
Figure B.4: HPLC chromatogram of the extract added kefir sample in 7th day……**73**
Figure B.5: HPLC chromatogram of the extract added kefir sample in 14th day…..**74**
Figure B.6: HPLC chromatogram of the extract added kefir sample in 21st day….**75**
Figure B.7: HPLC chromatogram of the capsule added kefir sample in 1st day…...**76**
Figure B.8: HPLC chromatogram of the capsule added kefir sample in 7th day…..**77**
Figure B.9: HPLC chromatogram of the capsule added kefir sample in 14th day….**78**
Figure B.10: HPLC chromatogram of the capsule added kefir sample in 21st day...**79**

DETERMINATION OF THE STABILITY OF BEESWAX ENCAPSULATED RED BEETROOT EXTRACT IN KEFIR

SUMMARY

Red beet is a plant belonging to the family of Amaranthaceae and is among 10 vegetables with high antioxidant activity. The antioxidant effect in the red beetroot is due to its phenolic and betalain compounds. Betalaines are water-soluble and nitrogen-containing color pigments that give a characteristic color of red beetroot. Betalains are separated into two main groups, these are betacyanins and betaxanthins. While betacyanins have red – violet color pigment, betaxanthins have yellow – orange color pigments. The reasons for this difference are due to the different chemical structures and formation pathway. They play an important role in the scavenging of free radicals and the prevention of oxidative stress-related diseases because of their high antioxidant potency. Betalaines can also be used as coloring agents in foods because they are suitable for use at a wide pH range (3 - 7) and can naturally provide the desired color. Especially in recent years, the use of betalaines has become widespread as consumers have preferred natural additives in food products. Taking all these into account, betalaines can be used for functional food production. However, the low stability of these substances limits its use in the food industry. The main factors affecting stability can be listed as follows; temperature, water activity, enzyme activity (polyphenol oxidase, peroxidase, glucosidase enzymes), presence of oxygen, light and metal ions. The most decisive factor is the temperature.

Encapsulation is a very effective method to stabilize betalaines because it can protect betalains from environmental factors which make them unstable. In this process, the active substance is trapped in a matrix having a protective structure. In betalain encapsulation, various materials can be used as coating material; these substances are generally classified as carbohydrates, proteins and oils / fats. In selecting appropriate coating material; the encapsulation technique to be applied, the targeted solubility characteristics, the process conditions and the food matrix are effective factors.

In this study, the red beetroot extracts were encapsulated into beeswax by using the emulsification method to enhance its stability. The reason of preferring beeswax as coating material is to prevent degradation by protecting the active ingredient from oxidation and hydrolysis.

For this purpose, red beetroot extracts were obtained by using a mixture of ethanol and water then it was lyophilized and stored at -20°C for further analysis. After that, the encapsulation was carried out and the capsules were prepared using different active ingredient / coating material ratios (1: 1, 1: 2, 1: 3 and 2: 3 w / w). In order to determine the contents of beet extracts and the capsules; total phenolic content, total flavonoid content and betalain amounts were determined by spectrophotometric and chromatographic methods. Antioxidant activity of the extract and capsules was also

determined by DPPH and CUPRAC analysis. Finally, color measurements were conducted for the extract and capsules.

Particle size analysis, zeta potentials and encapsulation activities of the capsules were determined to select the optimal encapsulation condition. The encapsulation efficiency was determined by using total phenolic content analysis. After the appropriate encapsulation rate was determined, the capsules were incorporated into the kefir formulation to examine the effect of the encapsulation on stability. The kefir samples were collected on 1st, 7th, 14th and 21st day of storage and the contents and antioxidant activities were controlled. Besides that, the color measurement was also conducted in order to detect color change during storage time. In addition to capsules added kefir (KC), plain kefir (K), extract added kefir (KE) and empty wax capsules added kefir (KB) samples were prepared as control samples. All kefir samples were stored at 4°C and in the dark during storage.

The content of the obtained extract is as follows; total phenolic content is 2448.38 ± 179.84 mg GAE / 100 g extract, total flavonoid content is 4001.45 ± 71.91 mg CE / 100 g extract, betacyanins content is 453.99 ± 4.12 mg BE / 100 g extract and betaxanthin content is 360.08 ± 2.44 mg VE / 100 g extract. Apart from spectrophotometric analysis, phenolic and betacyanin profile were also determined by HPLC, and the results show that the extract includes ferulic acid, hydroxybenzoic acid, vanillic acid, ethyl 3,4 dihydroxybenzoate, gallic acid, catechin, epigallocatechingallate, syringic acid, vanillin, hesperedin, caffeic acid, chlorogenic acid, p-coumaic acid, sinapic acid, rosmarinic acid, luteolin, quercetin, rutin, myricetin, epicatechin, betanin and isobetanin. The antioxidant activity of the extracts was determined as 42.29 ± 0.72 mM TE / g extract in DPPH analysis and 59.72 ± 1.12 mM TE / g extract in CUPRAC analysis. The color values of the extract were measured as L* = 31.30 ± 1.97, a* = 33.13 ± 1.08, b* = 8.11 ± 0.38, C* = 34.10 ± 0.34 and h° = 13.76 ± 0.59.

Both particle size and encapsulation efficiency were examined in capsules prepared at different ratios to determine the optimal encapsulation condition. According to the results, the particle size of the capsules was found to be between 265.9 ± 5.29 and 414.3 ± 28.87 nm and it was observed that the ratio of different active substance / coating material was significantly effective on the obtained particle size ($p < 0.05$). In addition to the particle size, the encapsulation efficiency was also determined and the highest efficiency was found in 1: 2 ratio as 67.16% while the lowest efficiency was found in 2:3 ratio as 42.18%. Besides that, it was observed that capsules prepared at different ratios have significant effect on the encapsulation efficiency ($p <0.05$). When all these are taken into consideration, the 1:2 ratio, in which the highest encapsulation efficiency is detected, has been chosen to carry out further studies.

The contents of the prepared capsules are as follows; total phenolic content is 101.68 ± 14.75 mg GAE / 100 g capsules, total flavonoid content is 103.12 ± 7.38 mg CE / 100 g capsules, betacyanins content is 26.95 ± 1.06 mg BE / 100 g capsules and betaxanthin content is 36.03 ± 0.35 mg VE / 100 g capsules. In addition to spectrophotometric assays, phenolic profile and betacyanin profile were also determined by HPLC. According to the results, the capsules include; hydroxybenzoic acid, vanillic acid, epigallocatechingallate, vanillin, caffeic acid, p-coumaric acid, ferulic acid, gallic acid, catechin, epicatechin, syringic acid, chlorogenic acid, betanin and isobetanin. The antioxidant activity of the capsules was determined as

3.05 ± 0.13 mM TE / g capsule in DPPH analysis and 2.40 ± 0.10 mM TE / g capsule in CUPRAC analysis. The color values of the extract were measured as L* = 68.93± 0.56, a* = 17.75± 0.57, b* = 16.11 ± 0.18, C* = 23.97± 0.19 and h° = 42.23± 0.65.

The capsules which are detected for encapsulation efficiency and contents were added to kefir formulation for stability study during storage time (21 days). There was no significant difference in the phenolic content of KC and KE samples after 21 days ($p > 0.05$) according to phenolic content results obtained. However, KC samples have higher phenolic content during storage period than phenolic content of KE sample ($p < 0.05$). The same results were also observed in total flavonoid conent of KE and KC samples. Although there are no significant differences in phenolic content of KE sample, the HPLC results showed that some individual phenolic compounds got lost during storage time. On the other hand, phenolic profile in KC sample remained stable during whole storage time ($p > 0.05$). The main reason of vanishing some phenolic compounds is thought as phenolic – milk protein interactions. In addition to phenolic and flavonoid content of the kefir samples, betacyanin and betaxanthin content were also measured in a specified time intervals. While both of betacyanin and betaxanthin content in KC could be preserved during 21 days, the initial betacyanin and betaxanthin content in KE sample are significantly different in 7^{th} day of storage ($p < 0.05$). The obtained results were corrected with HPLC analysis of betanin during storage. Therefore, beeswax encapsulation could preserve phenolic compounds and betanins during storage of kefir samples.

Apart from phenolic, flavonoid and betalain content analysis, antioxidant activity was also controlled during storage of the kefir samples by DPPH and CUPRAC assays. In DPPH assay, KC sample showed more antioxidant activity than KE sample ($p < 0.05$) and antioxidant activity in both sample did not show any significant difference during storage. In CUPRAC analysis, KE sample has more antioxidant activity than KC sample but this difference is not significant in 1^{st} and 21^{st} day of storage. The initial antioxidant activity of KE samples could not be protected until 7^{th} day and there is a significant decrease ($p < 0.05$). On the other hand, antioxidant activity of KC sample remained stable during 21 day. As a result, it appears that beeswax encapsulation can also protect antioxidant activity.

Lastly, color measurements were conducted to detect color changes during storage. The results show that there are no significant color change in KE sample but lightness, redness, yellowness and chroma value of KC sample increased significantly during storage. The reason of it may be releasing red beetrrot extract from beeswax into kefir.

Taking all these results into consideration, beeswax encapsulation showed positive effect on preserving of betalain and phenolic compounds. Nevertheless, the further stability studies are needed to fully observe the effect of the beeswax encapsulation on the stability.

BALMUMU İLE KAPSÜLLENMİŞ KIRMIZI PANCAR EKSTRAKTININ KEFİR İÇERİSİNDEKİ STABİLİTESİNİN BELİRLENMESİ

ÖZET

Kırmızı pancar, Amaranthaceae ailesine ait bir bitki türüdür ve yüksek antioksidan etkiye sahip 10 sebze arasında yeralmaktadır. Kırmızı pancardaki antioksidan etki içeriğindeki fenolik bileşenlerden ve özellikle de betalainlerden kaynaklanmaktadır. Betalainler kırmızı pancara özgü rengi veren suda çözünebilen ve nitrojen içeren renk pigmentleridir. Betasiyaninler ve betaksantinler olmak üzere iki ana gruba ayrılırlar. Betasiyaninler kırmızı – mor renk pigmentlerine sahipken betaksantinler sarı – turuncu renk pigmentlerine sahiptirler. Bu farklılığın sebebi ise kimyasal yapılarının ve oluşum yollarının farklı olmasından kaynaklanır. Sahip oldukları yüksek antioksidan etkiden dolayı serbest radikallerin tutulmasında ve oksidatif strese bağlı hastalıkların engellenmesinde etkili rol oynamaktadırlar. Bunun yanısıra, betalainler gıdalarda renklendirici olarak da kullanılabilmektedir çünkü hem geniş bir pH aralığında (3 – 7) kullanıma uygundurlar hem de istenilen rengi doğal olarak sağlayabilirler. Özellikle son yıllarda, tüketicilerin doğal katkı maddelerini tercih etmesiyle kullanımları yaygınlaşmıştır. Tüm bunlar göz önüne alındığında, betalainlerin fonksiyonel gıda üretiminde kullanılabileceği görülmektedir. Fakat bu maddelerin düşük stabiliteye sahip olması gıda endüstrisinde kullanımını kısıtlamaktadır. Stabiliteyi etkileyen başlıca faktörler şu şekilde sıralanabilir; sıcaklık, su aktivitesi, enzim aktivitesi (polifenoloksidaz, peroksidaz, glukosidaz enzimleri), oksijen, ışık ve metal iyonları varlığı. Bu etkenlerdenen belirleyici olan ise sıcaklıktır.

Enkapsülasyon işlemi betalainleri çevresel faktörlerden koruyarak daha stabil hale getirmek için oldukça etkili bir yöntemdir. Bu işlemde, aktif madde koruyucu yapıya sahip bir matriks içerisine hapsedilmektedir. Betalain enkapsülasyonunda, kaplama materyali olarak çeşitli maddeler kullanılabilmektedir, bu maddeler genel olarak sınıflandırıldığında karbonhidratlar, proteinler ve yağlardır. Uygun kaplama maddesinin seçilmesinde uygulanacak enkapsülasyon tekniği, hedeflenen çözünürlük özellikleri, proses koşulları ve gıda matrisi etkilidir.

Bu çalışmada kırmızı pancar ekstraktı stabilitesini artırmak için elde edilen ekstraklar balmumu ile emülsifikasyon metodu kullanılarak kapsüllenmiştir. Kaplama materyali olarak balmumunun tercih edilmesinin sebebi aktif maddeyi oksidasyon ve hidrolizden koruyarak degradasyona uğramasını engellenmesidir.

Bu amaç doğrultusunda, ilk olarak pancar ekstraktları etanol ve su karışımı kullanılarak elde edildi ve ileri çalışmalarda kullanılmak üzere liyofilize edilerek -20°C ‘de saklandı. Ardından kapsülleme işlemine geçildi ve farklı aktif madde / kaplama materyali oranları (1:1, 1:2, 1:3 ve 2:3 w/w) kullanılarak kapsüller hazırlandı. Elde edilen pancar ekstraklarının ve kapsüllerin içeriğini belirlemek amacıyla toplam fenolik madde, toplam flavonoid madde ve betalain miktarları spektrofotometrik ve kromatografik yöntemlerle belirlendi.

En uygun kapsülleme oranını tespit etmek için hazırlanan kapsüllerin boyut analizi, zeta potansiyelleri ve enkapsülasyon etkinlikleri belirlendi. Enkapsülasyon etkinliği toplam fenolik madde analizi kullanılarak gerçekleştirildi. Uygun kapsülleme oranı belirlendikten sonra, kapsüllerin stabilite üzerindeki etkisini incelemek için hazırlanan kapsüller kefir formülasyonu içerisine katıldı ve 1., 7., 14. ve 21. günlerde numuneler toplanarak kapsül içerikleri ve antioksidan aktiviteleri kontrol edildi. Bunların yanı sıra, renk stabilitesini tespit etmek için belirli aralıklarla renk ölçümleri de yapılmıştır. Kapsül eklenmiş kefirin (KC) yanı sıra, kontrol numunesi olarak sade kefir (K), ekstrak eklenmiş kefir (KE) ve boş balmumu kapsülü eklenmiş kefir (KB) örnekleri hazırlandı.

Elde edilen ekstrakların toplam fenolik içeriği 2448.38 ± 179.84 mg GAE / 100 g ekstrak, toplam flavonoid içeriği 4001.45 ± 71.91 mg CE / 100 g ekstrak, betasiyanin içeriği 453.99 ± 4.12 mg BE / 100 g ekstrak ve betaksantin içeriği 360.08± 2.44 mg VE / 100 g ekstrak olarak belirlenmiştir. Spektrofotometrik analizlerin dışında fenolik ve betasiyanin profili HPLC yöntemi ile belirlendi ve ekstraktın fenolik içeriğinin şu şekilde olduğu tespit edildi; ferulik asit, hidroksibenzoik asit, vanilik asit, etil 3,4 dihidroksibenzoat, gallik asit, kateşin, epigallokateşingallat, siringik asit, vanilin, hesperidin, kafeik asit, klorojenik asit, p-koumaik asit, sinapik asit, rosmarinik asit, luteolin, kuersetin, rutin, mirisetin, epikateşin, betanin ve isobetanin. Ekstrakların antioksidan aktivitesi ise DPPH analizinde 42.29 ± 0.72 mM TE / g ekstrak olarak belirlenirken, CUPRAC analizinde 59.72 ± 1.12 mM TE / g ekstrak olarak tespit edilmiştir. Yapılan renk ölçümleri sonucunda ise ekstrakların renk değerleri L^* = 31.30 ± 1.97, a^*= 33.13 ± 1.08, b^*= 8.11 ± 0.38, C^* = 34.10 ± 0.34 ve h°=13.76 ± 0.59 olarak ölçülmüştür.

En uygun kapsülleme oranını tayin etmek için farklı oranlarda hazırlanan kapsüllerde hem parçacık boyutu hem de enkapsülasyon etkinliği incelendi. Elde edilen sonuçlara göre hazırlanan kapsüllerin parçacık boyutu 265.9 ± 5.29 ile 414.3 ± 28.87 nm arasında bulunmuştur ve uygulanan farklı aktif madde / kaplama materyali oranlarının elde edilen parçacık boyutu üzerinde anlamlı derecede etkili olduğu görülmüştür($p < 0.05$). Parçacık boyutuna ek olarak, enkapsülasyon etkinliği de belirlenmiştir ve en yüksek değer %67.16 olarak 1:2 oranında görülürken en düşük etkinlik değeri %42.18 olarak 2:3 oranındaki kapsüllerde görülmüştür. Ayrıca, farklı oranlarda hazırlanan kapsüllerin enkapsülasyon etkinliğini anlamlı bir şekilde etkilediği gözlenmiştir ($p < 0.05$). Tüm bunlar göz önüne alındığında, en yüksek etkinliğin görüldüğü 1:2 oranı ileri çalışmaları gerçekleştirmek üzere seçilmiştir.

Hazırlanan kapsüllerin toplam fenolik madde içeriği 101.68 ± 14.75 mg GAE / 100 g kapsül, toplam flavonoid madde içeriği 103.12 ± 7.38 mg CE / 100 g kapsül, betasiyanin içeriği 26.95 ± 1.06 mg BE / 100 g kapsül ve betaksantin içeriği 36.03 ± 0.35 mg VE / 100 g kapsül olarak belirlenmiştir. Spektrofotometrik analizlere ek olarak, fenolik profil ve betasiyanin profili HPLC yöntemiyle belirlenmiştir. Sonuçlara göre kapsüller; hidroksibenzoik asit, vanillik asit, epigallokateşin gallat, vanilin, kafeik asit, p-kumarik asit, ferulik asit, gallik asit, kateşin, epikateşin, siringik asit, klorojenik asit, betanin ve izobetanin içermektedir. Ayrıca kapsüllerin antioksidan aktivitesi ise DPPH analizinde 3.05 ± 0.13 mM TE / g kapsül olarak belirlenirken, CUPRAC analizinde 2.40 ± 0.10 mM TE / g kapsül olarak tespit edilmiştir. Hazırlanan kapsüllerde yapılan renk ölçümlerinin sonuçları ise şu şekildedir; L^* = 68.93 ± 0.56, a^*= 17.75 ± 0.57, b^*= 16.11± 0.18, C^* = 23.97± 0.19 ve h° = 42.23± 0.65.

Etkinliği ve içeriği belirlenen kapsüller kefir formülasyonuna katılarak 21 gün boyunca 4°C ‘de ve karanlıkta depolanarak stabilitesi incelenmiştir. Elde edilen fenolik içerik sonuçlarına göre 21. gün sonunda KC ve KE örneklerinin fenolik içeriğinde anlamlı bir fark bulunmadı ($p > 0.05$). Bununla birlikte, KC numuneleri depolama süresinde KE örneğinin fenolik içeriğinden daha yüksek fenolik içeriğe sahiptir ($p < 0.05$). Aynı sonuçlar KE ve KC örneklerinin toplam flavonoid içeriğinde de gözlenmiştir. KE numunesinin fenolik içeriğinde anlamlı bir farklılık olmamasına rağmen, HPLC sonuçları, bazı fenolik bileşiklerin depolama sırasında kaybolduğunu göstermiştir. Öte yandan, KC örneğindeki fenolik profil tüm depolama süresi boyunca sabit kalmıştır ($p > 0.05$). Bazı fenolik bileşiklerin yok olmasının temel nedeni fenolik - süt proteini etkileşimleri olarak düşünülmektedir. Kefir örneklerinin fenolik ve flavonoid içeriğine ek olarak, belirli bir zaman aralığındaki betasiyanin ve betaksantin içeriği de ölçüldü. KC örneğinde betasiyanin ve betaksantin içeriği 21 gün boyunca korunurken, KE örneğindeki başlangıçtaki betasiyanin ve betaksantin içeriği depolamanın 7. gününde anlamlı olarak azalmıştır ($p < 0.05$). Elde edilen sonuçlar aynı zamanda betaninin HPLC ile analizlenmesiylede doğrulanmıştır. Bu durumda, balmumu ile enkapsülasyon işlemi, kefir içerisindeki fenolik bileşikleri ve betaninleri koruyabilmiştir.

Kefir içeriklerine ek olarak, kefir örneklerindeki antioksidan aktivite de incelenmiştir. DPPH analizinde KC numunesi KE örneğinden daha fazla antioksidan aktivite göstermiştir ($p < 0.05$) ve her iki örnekte de antioksidan aktivite depolama sırasında anlamlı bir fark göstermemiştir. CUPRAC analizinde ise KE numunesinin KC örneğinden daha fazla antioksidan etkinliği vardır, ancak bu fark depolamadaki 1. ve 21. günlerde anlamlı değildir ($p > 0.05$). KE örneklerinin başlangıç antioksidan aktivitesi 7. güne kadar korunamamıştır ve anlamlı bir düşüş meydana gelmiştir ($p < 0.05$). Öte yandan, KC örneğinin antioksidan aktivitesi ise 21 gün boyunca sabit kalmıştır. Sonuçlara bakılarak, balmumu enkapsülasyonunun antioksida aktiviteyi de koruyabildiği görülmektedir.

Son olarak, depolama sırasında renk değişikliklerini tespit etmek için renk ölçümleri de yapılmıştır. Sonuçlar, KE numunesinde belirgin bir renk değişikliği olmadığını, ancak KC örneğinin açıklık, kırmızılık, sarılık ve renk değerinin depolama sırasında önemli ölçüde arttığını göstermektedir. Bunun nedeni kırmızı pancar ekstraktının balmumundan kefir içerisine salınımı olabilir.

Tüm bu sonuçlar göz önüne alınırsa, balmumu kapsüllemesinin betalain ve fenolik bileşikleri korumasıyla antioksidan aktivite üzerinde olumlu bir etki gösterdiği söylenebilir. Bununla birlikte, balmumu kapsüllemesinin stabilite üzerindeki etkisini tam olarak gözlemlemek için daha fazla ve ileri stabilite çalışmaları önerilmektedir.

1. INTRODUCTION

Red beetroot (*Beta vulgaris*) is known as one of the most powerful vegetables due to its valuable bioactive compounds. Especially, betalain compounds which are found in red beetroot and give its characteristic color are stand out due to their excellent antioxidant activity and coloring property. Betalains are water soluble, nitrogen containing color pigments and they have two different groups, these are red – violet colored betacyanins and yellow – orange colored betaxanthins (Kujala et al., 2000). Betalains have ability of preventing diseases originated from oxidative stress due to their high antioxidant activity. Therefore, betalains or red beetroot extract are good ingredients for producing functional food because of both ensuring desirable color at wide range of pH (3 – 7) and providing health benefits (Canadanovic-Brunet et al., 2011). However, there is a factor that limits the use of betalains in the food industry is their low stability.Betalains are affected from temperature, water activity, pH, enzymes (polyphenol oxidase, peroxidase and glucosidase), oxygen, light and presence of metal ions (Carmo et al., 2015; Esatbeyoğlu et al., 2015). Betalains are degraded during process or storage by isomerization, deglycosylation, hydrolysis, decarboxylation, and dehydrogenation reactions. As a result of these reactions, changes in color are observed (Esquivel, 2016).

Encapsulation process in which active compound are incorporated into a protective wall material is promising technique in order to increase betalain stability (Carmo et al., 2015; Rodriguez-Sanchez et al., 2017). Encapsulated betalains are protected from environmental conditions so their color and antioxidant activity remain stable during storageand this situation facilitates the use of betalains in the food industry (Gandia-Herrero et al., 2013). By incorporating the encapsulated betalains in food systems, the consumers can accessbetalain so the risk of oxidative diseases and cardiovascular diseases may be reduced (Canadanovic-Brunet et al., 2011). Besides that, stable color is also obtained naturally by encapsulated betalains (Ravichandran et al., 2014). The food products which contain natural additives are more attractive for consumers due to health aspects (Celli & Brooks, 2017).

In betalain encapsulation, various wall materials such as polysaccharides, proteins and lipids are preferred. Factors influencing the selection of coating materialscan be listed as follows; encapsulation technique, desired solubility (water or oil solubility), process conditions and food matrix (Ravichandran et al., 2014; Wandrey et al., 2010).

1.1 Purpose of Thesis

In the present study, red beetroot extract was encapsulated with beeswax by using melt emulsification technique in order to improve its stability during storage. For this purpose; total phenolic content, total flavonoid content, betacyanin content, betaxanthin content and antioxidant activity were measured in both of encapsulated and non – encapsulated forms of red beetroot extract. In order to detect storage stability, the obtained capsules were incorporated into kefir formulation and it was stored at 4°C in dark for 21 days and the same measurements were replicated at 1st, 7th, 14th and 21st days of storage. Non – encapsulated red beetroot extract was used as control sample and the results were compared with encapsulated extract. Besides that, color measurements were also conducted for the extract, capsules and kefir samples in order to detect color changes during storage.

1.2 Betalain Compounds

Betalains are water – soluble and nitrogen containing color pigments and they are produced by most plants which are belong to Caryophyllales plant order. They can be found in flowers, fruits, roots or plant tissues. Betalains are mostly found in red beet (*Beta vulgaris*), yellow beet, cactus pear, pricky pear (*Opuntia sp*.), red pithaya / red dragon fruit (*Hylocereus polyrhizus*), spiny amaranth (*Amaranthus sp*.), *Celosia sp.*, Globe amaranth (*Gomphrena globose*), mossrose (*Portulaca grandiflora*), *Bougainvillea sp.*, pokeberry (*Phytolacca americana*), goosefoot (*Chenopodium sp.*) and some mushrooms such as *Amanita*, *Hygrocybe* and *Hygrophorus* (Esquivel, 2016; Cardoso-Ugarte et al., 2014; Gengatharan et al.,2015; Sawicki et al., 2016).

Table 0.1.1: Betalain content of some plant species (Celli & Brooks, 2017).

Source	Total Betalain Content (mg/100g)*
Yellow beetroot (*Beta vulgaris L.*)	568[a]
Red beetroot (*B. vulgaris L.*)	40–77[b]
	2935–3969[a]
Swiss chard (*B. vulgaris L. spp. cicla*)	5.7–6.5[b]
Amaranth (*Amaranthus sp.*)	0.07–20.93[a]
Prickly pear	92.7[b]
Yellow prickly pear	3.78[b]
Red prickly pear (*Opuntia sp.*)	3[b]
Red prickly pear (*O. ficus-indica*)	10.24[b]
Purple prickly pear	30[b]
Pitaya peel (*Hylocereus sp.*)	101[a]
Pitaya peel (*H. polyrhizus*)	73[b]
Pigeon berry (*Rivina humilis L.*)	1700[a]
Facheiro	134[b]
Waterleaf flower (*Talinum triangulare*)	499.3[b]
Malabar spinach (*Basella rubra L.*)	143.8[b]
Quinoa (C. quinoa Willd. var. Pasankalla)	0.1[a]
Quinoa (C. quinoa Willd. var. Collana)	0.13[a]
Epiphitic cactus flower and fruits	782.1[a]
"Jiotilla"	20.8[b]
Garambullo fruit	214[a]

*Total betalain content expressed as mg/100 g of material on [a] dry weight or [b] fresh weight basis.

Betalains are synthesized from tyrosine amino acid and they are divided into two main groups according to the structure which are betacyanin (λ_{max} = 536 nm) and betaxanthin (λ_{max} = 476 nm). While betacyanins provide red – violet color, betaxanthinsprovide yellow – orange color. The reason of this difference is biosynthesis pathway of them. Betacyanins are formed by condensation of betalamic acid with cyclo – Dopa and various betacyanin derivatives occur via substitution of the hydroxyl group at C – 5 or C – 6 positions by glycosylation and acylation reactions (Tesoriere et al., 2009; Esquivel, 2016). There are four types of betacyanin; betanin, amaranthin, gomphrenin and 2 - descarboxybetanin (Figure 1.3) (Esatbeyoğlu et al., 2015). Betaxanthins are synthesized by condensation of betalamic acid and amines or amino acids, the color of them are determined by changes of amino acid type or amide side chains. Vulgaxanthin I – II and indicaxanthin are two types of betaxanthin (Figure 1.4) (Esatbeyoglu et al., 2015). The biosynthesis of Dopa and cyclo – Dopa are shown in Figure 1.1. In Figure 1.2 shows betacyanins and betaxantins biosynthesis (Esquivel, 2016).

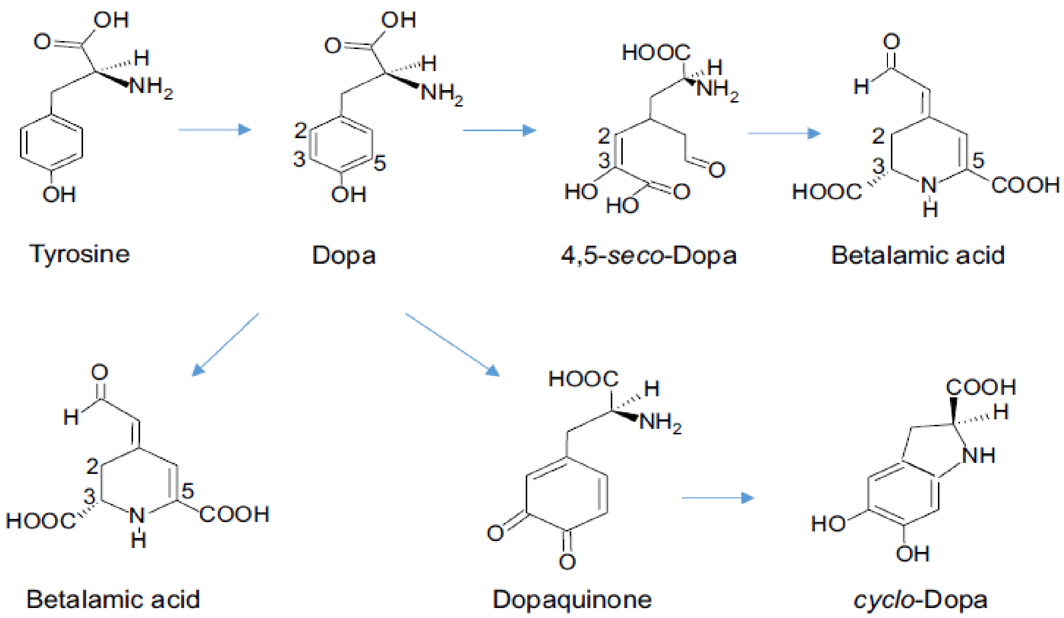

Figure 1.1: Biosynthesis of betalamic acid and cyclo – Dopa from tyrosine (Esquivel, 2016).

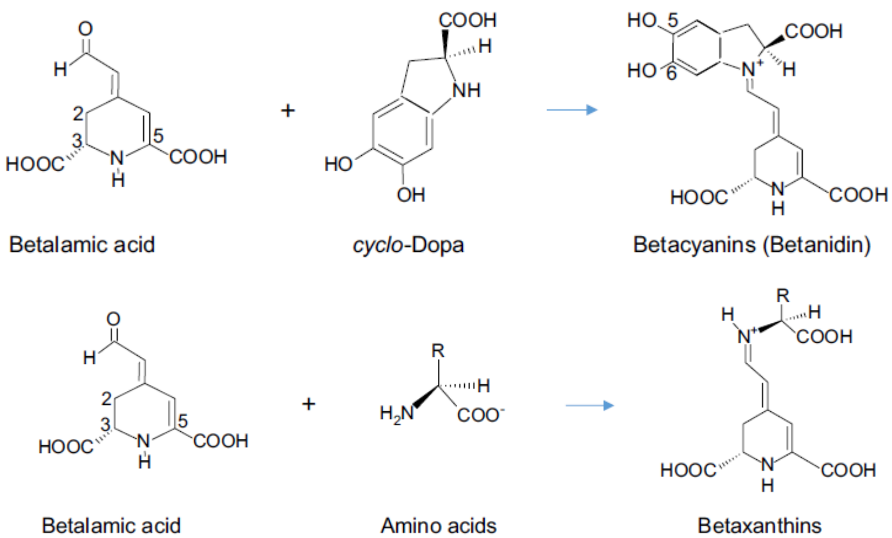

Figure 1.2: Synthesis of betacyanin and betaxanthin pigments (Esquivel, 2016).

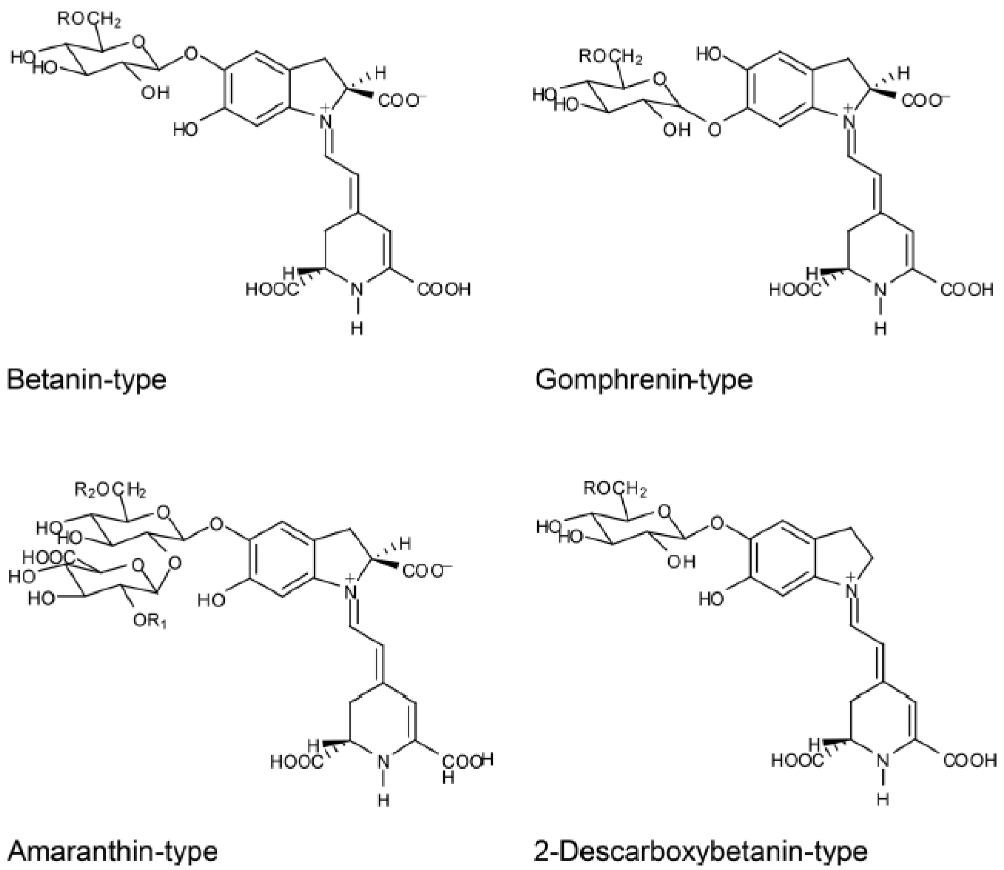

Figure 1.3: Different betalain types structures (Esatbeyoğlu et al., 2015).

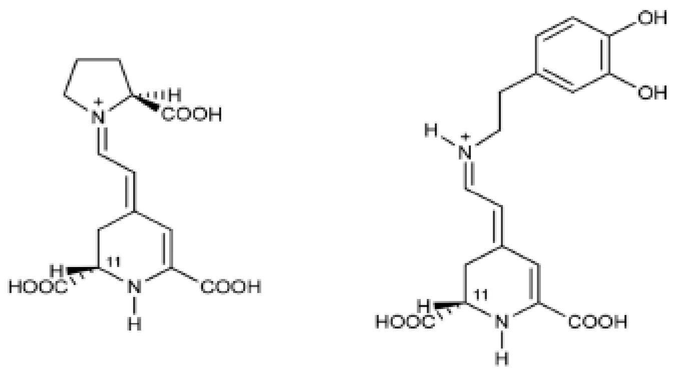

Figure 1.4: Structure of vulgaxanthin and indicaxanthin (Esatbeyoğlu et al., 2015).

Betalains, especially betanins, are preferred coloring agents in foods, cosmetics and pharmaceuticals because they provide desired color naturally and they can be used in a wide range of pH (between 3 and 7) (Esquivel, 2016). Especially in recent years, usages of natural food colorants have been gained importance as both of consumers' preference and toxic or allergic effects of synthetic colorants (Esquivel, 2016;

Esatbeyoğlu et al., 2015). In the current situation, betanin is permitted food colorant and it has been used with E162 code (Esatbeyoğlu et al., 2015).

In addition to desired color properties of betalain, they have also excellent health benefits. Betalains show antioxidant, anti – cancer, anti inflamatory, anti – lipidemic, antimicrobial, antiulcerogenic, antiproliferative and cardioprotective activity (Gengatharan et al., 2015; Stintzing et al., 2005; Celli & Brooks, 2017). Studies have shown that betalains have powerful ability of free radical scavenging and inhibiting oxidation reactions in cells. These abilities of betalains make it efficient anti – cancer compound. The studies that have been conducted for determination effect of betalain on ovarian cancer cells and cervical cancer cells have shown that betalain inhibited cancer cells growth significantly. The effect of the betalain has been found to be equivalent to a synthetic chemopreventive agent (Gengatharan et al., 2015). Besides that, betalain also have an effect on inhibition of cardiovascular diseases due to their anti – lipidemic effect. According to the studies, betalains prevented LDL (low density lipoproteins) oxidation by elimination lipoperoxyl radicals and they reduced LDL, cholesterol and trigliserid level in blood of rats (Allegra et al., 2007; Clemente & Desai, 2011). Apart from these effects of betalains, they are also effective against microorganisms. Betalains inhibit growth of both of Gram positive (*B. cereus*, *S. aureus*, *E. faecalis* and *Listeria monocytogenes*) and Gram negative (*E. coli*, *Pseudomonas aeruginosa*, *Citrobacter freundii*, *Citrobacter youngae*, *E. cloacae*, *Proteus mirabilis*, *Proteus vulgari*, *Salmonella typhimurium*, *P. aeruginosa*, *Yersinia enterocolitica* and *Enterobacter cloacae*) bacteria. Finally, they have also inhibitory effect on some yeasts (*Candida albicans* and *Rhizoctoniasolani*) and moulds (*Fusarium oxysporum*, *Cladosporium herbarum*, *Botrytis cinerea* and *Aspergillus flavus)* (Gengatharan et al., 2015).

The main factor that affects the usage of betalains is stability. Although betalains have desired coloring property and excellent health promoting effects, their unstable structure limits their usage as food colorant and antioxidant agent (Carmo et al., 2015; Gandia-Herrero et al., 2013). There are many factors that affect betalain stability can be sequenced in that chemical structure, pigment amount, enzymes (polyphenol oxidase, peroxidase and β - glucosidase), pH, heat, water activity, oxygen, light and metal ions. When betalains are exposed to such effects, they undergo some chemical changes by isomerization, hydrolysis, decarboxylation, dehydrogenation and deglycosylation reactions (Esquivel, 2016). The degradation pathways of betanin are shown in Figure 1.5. Isomerization is a type of structure modification and there are no color changes are observed.When betalain exposed to acidic or alkaline condition and thermal treatment, the structure of betalain and the ratio of betanin / isobetanin alters. In deglycosylation, the structure of betalain is degradaded by strong acidic / alkaline conditions at high temperature presence of β - glucosidase. As a result of deglycosylation deep violet color formation is observed by cleavage of glucose moiety of betanin. Sometimes, the violet color is wanted intentionally but deglycosylation makes betalain more susceptible to the oxidation. The other degradation reaction is hydrolysis that occurs at pH greater than 6 and presence of heat. In the consequence of hydrolysis, aldimine bond of betalain is cleavaged and betalamic acid and *cyclo* – Dopa are formed. The color of betalamic acid is light yellow and *cyclo* – Dopa is colorless compound. Therefore, betalains lose coloring effect. However, hydrolysis is reversible reaction and betalain can be formed when appropriate conditions are provided. The regeneration reaction of betalain is pH depended (pH 6). When decarboxylation takes place, the carboxyl group is cleaved from betanin, betanidin or indicaxanthin. The cleavage of carboxyl group may occur at C_2, C_{15} or C_{17} of betacyanins and C_{11} or C_{13} of betaxanthins. If decarboxylation occurs at C_2 or C_{15}, no color alteration is observed. The last degradation reaction is dehydrogenation which leads to yellow color formation when betanin or betanidin (red colored) is exposed to thermaltreatmentand oxidation. The degradation product is yellow colored neobetanin (Herbach et al., 2006).

In order to obtain more stabile betalain; exposure to heat, oxygen and light must be minimized, enzymes (polyphenol oxidase, peroxidase and glucosidase) must be inhibited, high water activity and metal ions must be prevented. For this purpose, betalain pigments may be stored as dried form or additional antioxidants may be added to betalain to protect it from oxidation. Besides that, encapsulation technology is a very promising method to obtain stable betalain pigments (Saenz et al., 2009). Betalains are protected from temperature, oxygen, light, enzyme activity and the other environmental factors that make betalains decomposed; therefore, encapsulation technology ensures stabilized betalain (Saenz et al., 2009; Esquivel 2016).

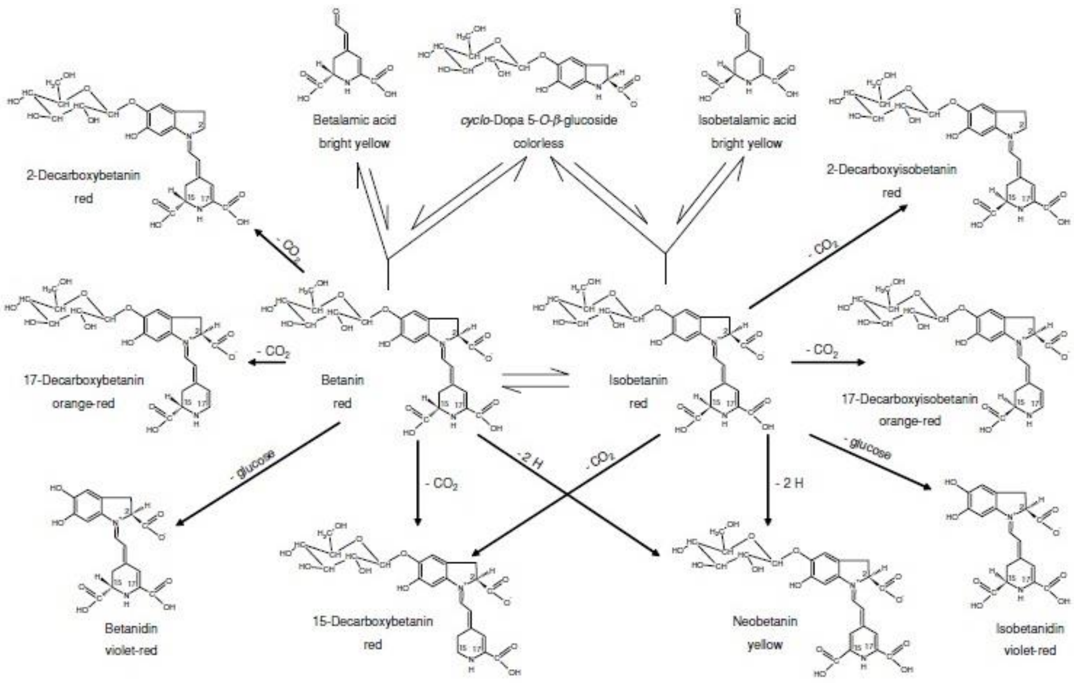

Figure 1.5: Degradation pathways of betanin (Herbach et al., 2006).

1.3 Phenolic Compounds

Phenolic compounds are produced as secondary metabolites in plants (Figure 1.6). They include at least one hydroxyl group which linked to aromatic ring. Phenolic compounds presence as bounded to other components such as sugar or proteins. Phenolic compounds have a wide variety and it has been reported that there are over 8000 species (Gutierrez-Uribe & Nollet, 2018). In order to classify the phenolic compounds, different approaches have been applied (Vermerris& Nicholson, 2008). Phenolic compounds can be grouped according to number of aromatic rings, carbon skeleton, chemical structure and being flavonoids or non – flavonoids. Table 1.2 shows phenolic compound classification accorfing to carbon number (Harborne &Simmonds, 1964; Gutierrez-Uribe & Nollet, 2018).

Table 1.2: Classification of phenolic compounds according to carbon number (Gutierrez-Uribe & Nollet, 2018).

Number of Aromatic Rings	Carbon Skeleton	Group Name
One	C_6	Simple phenols
		Benzoquinones
	$C_6 - C_1$	Phenolic acids
	$C_6 - C_2$	Phenolic aldehydes
		Acetophenones
		Phenylpropenes
		Chromones
	$C_6 - C_4$	Naphthoquinones
Two	$C_6 - C_1 - C_6$	Xanthones
	$C_6 - C_2 - C_6$	Stilbenes
		Anthraquinones
	$C_6 - C_3 - C_6$	Flavonoids
		Isoflavonoids
		Neoflavonoids
	$(C_6 - C_3)_2$	Lignans
		Neolignans
Three or more	$(C_6)_n$	Catechol melanins
	$(C_6 - C_3)_n$	Lignins
	$(C_6 - C_3 - C_6)_2$	Biflavonoids
	$(C_6 - C_3 - C_6)_n$	Condensed tannin (Proanthocyanidins)

In addition to these classification methods, phenolic compounds can be also classified according to frequency of presence, solubility and hydroxyl group number (Vermerris & Nicholson, 2008).

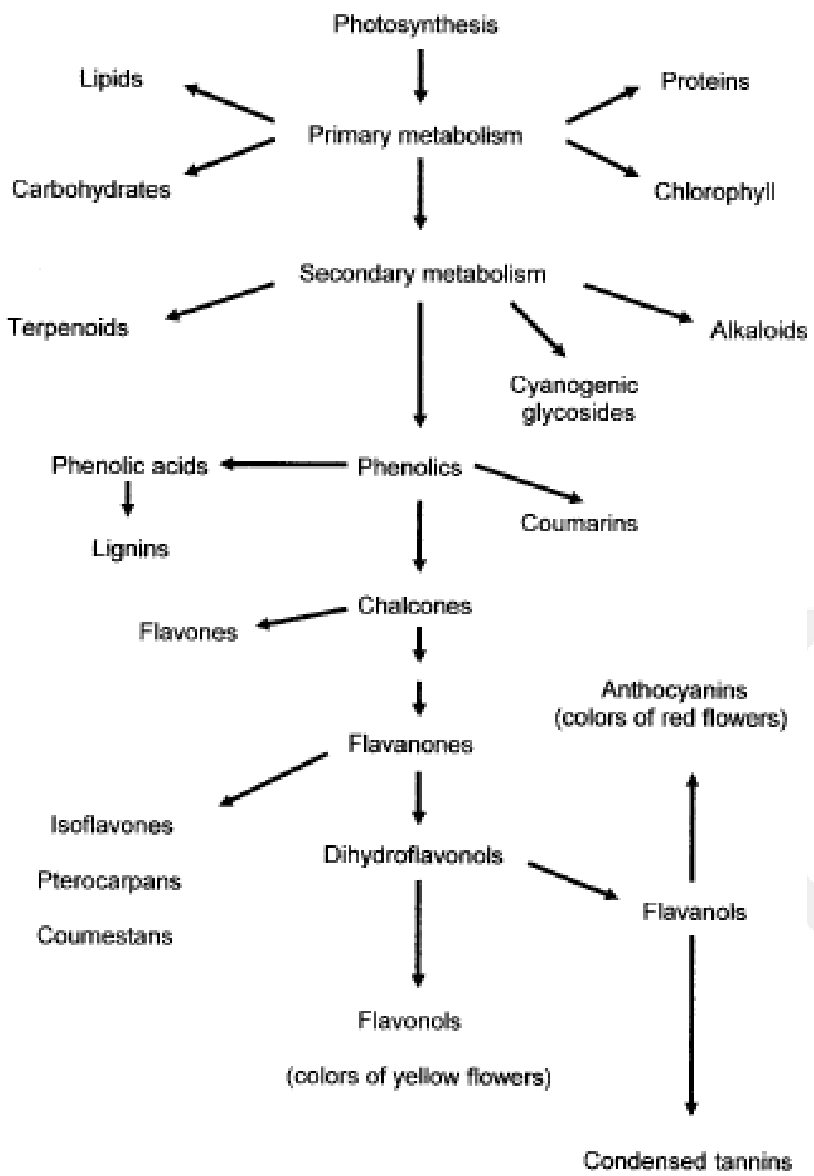

Figure 1.6: Primary and secondary metobolism in plants (Giada, 2013).

Flavonoids, tannins and phenolic acids are the main grous of phenolic compounds. Flavonoids have $C_6 - C_3 - C_6$ carbon skeleton structure and they are a structure formed by the combination of diphenyl propane and two benzene rings (Ring A and B) with a triple carbon chain. The triple carbon chain forms a closed form piran ring (Ring C) with one of the benzene rings. The flavonoids are classified according to the position of B ring as follows; flavonoids, isoflavonoids, and neoflavonoids (Figure 1.7). Besides that, flavonoids are divided into 8 group according to oxidation and saturation state of C ring in that flavanols, flavonols, flavones, isoflavones, flavanones, anthocyanidins, chalcones and aurones (Vermerris & Nicholson, 2008; Giada, 2013; Panche et al., 2016; Gutierrez-Uribe & Nollet, 2018).

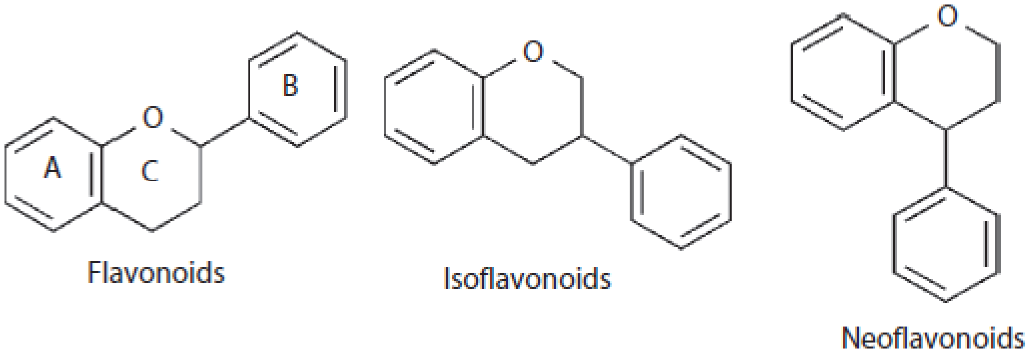

Figure 1.7: Chemical structure of flavonoids, isoflavonoids and neoflavonoids (Gutierrez-Uribe & Nollet, 2018).

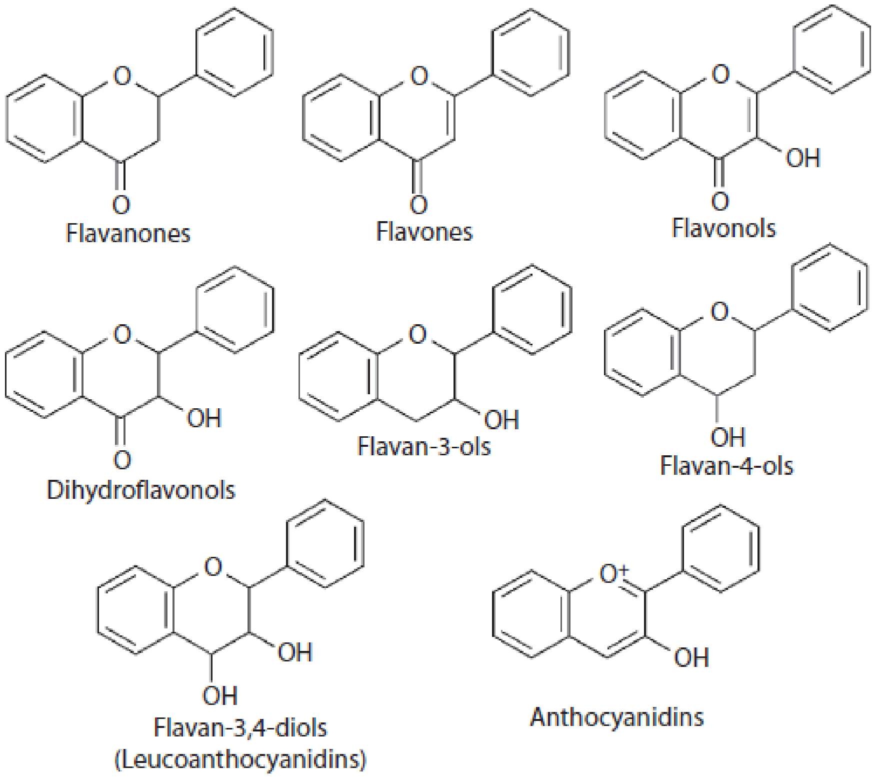

Figure 1.8: Chemical structure of different flavonoids (Gutierrez-Uribe & Nollet, 2018).

Tannins, which are high molecular weight substances, are produced by shikimic acid pathway and they are divided into three main groups; condensed tannins, hydrolysable tannins and complex tannins. Condensed tannins are catechin containing oligomeric or polymeric flavonoids. The second group of tannins is hydrolysable tannins; they have two subgroups; gallotannins and ellagitannins. Gallotannins are produced by condensation of the carboxyl group of gallic acid or polymeric derivatives of gallic acid with the hydroxyl groups of a monosaccharide. The monosaccharide group is mostly D – glucose. Another hydrolysable tannin is ellagitannins, they are occured from pentagalloylglucose and they contain extra C – C bond unlike gallotannins. Besides that, complex tannins have catechin unit which is linked with gallotannin or ellagitannin unit glycosidically (Gutierrez-Uribe & Nollet, 2018; Vermerris & Nicholson, 2008).

Phenolic acids comprise of benzene ring, carboxylic group, at least one hydroxyl group and / or methoxy group. Benzoic acids ($C_6 – C_1$), cinnamic acids ($C_6 – C_3$) and their derivatives are two main groups of phenolic acids. While phenolic acids generally presence in the form of esters, glycosides or amides, they uncommonly presence in free form. Derivatives of benzoic acids include gallic acid, vanillic acid, syringic acid, photocatechuic acids (Figure 1.9); cinnamic acids derivatives are ferulic acid, caffeic acid, *p*-coumaric acid and sinapic acids (Figure 1.10) (Khoddami et al., 2013).

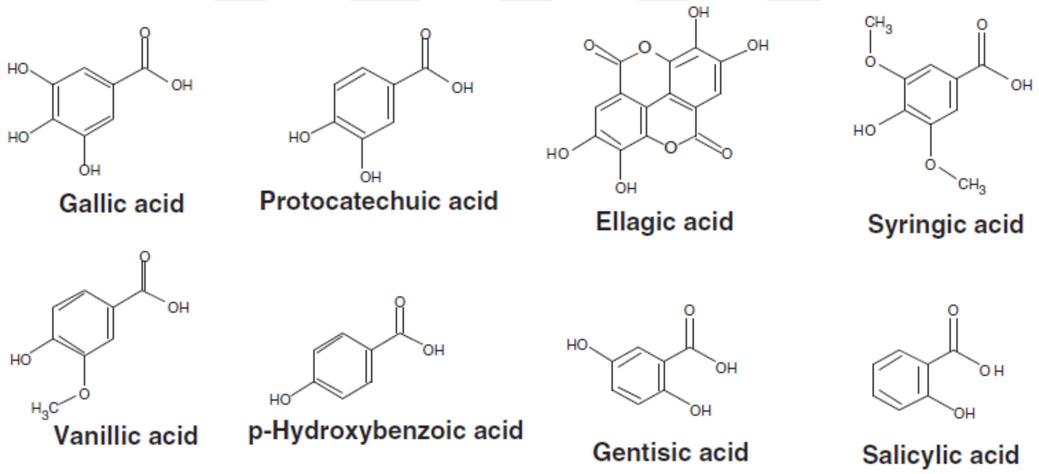

Figure 1.9: Structures of different types of benzoic acids (Martins et al., 2011).

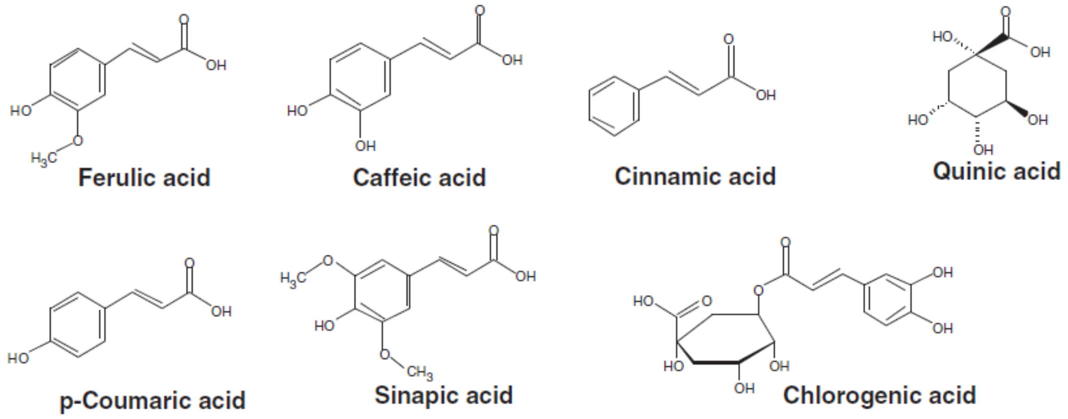

Figure 1.10: Structures of different tyes of cinnamic acids (Martins et al., 2011).

Phenolic compounds are found in many foodstuffs such as vegetables, grains, fruits, legumes and herbs (Table 1.3). They have antioxidant, anti-mutagenic, anti-allergenic, anti inflammatory, and antimicrobial effects; therefore, they can contribute human health and some of them are used in pharmacology (Giada, 2013; Martins et al., 2011). There are many studies that have shown the inhibitory or curing effects of phenolic compounds on cancer and cardiovascular diseases (Giada, 2013; Haminiuk et al., 2012).

Table 1.3: Phenolic contents of some foods (Gutierrez-Uribe & Nollet, 2018).

Group	Food Source	Phenolics[a]
Fruits	Blueberry	670.9
	Sour cherry	429.5
	Blackberry	355.3
	Strawberry	244.1
	Black grape	213.3
	White grape	184.1
	Raspberry	178.6
	Red apple	125.4
	Pear	124.7
	Green apple	118.1
	Yellow apple	99.7
	Sweet cherry	78.8
	Fig	59.0
	Peach	50.9
Nuts	Walnut	1404.0

Table 1.3 (Continued): Phenolic contents of some foods (Gutierrez-Uribe & Nollet, 2018).

Group	Food Source	Phenolics
	Western nut	1225.0
	Iranian pistachio	710.0
	Mexican pistachio	566.0
	Virginia peanut	457.0
	Spanish peanut	441.0
Legumes	Yellow bean	55.7
	Green bean	35.3
Vegetables	Green pepper	246.7
	Red pepper	173.2
	Brussel sprout	161.5
	Radish	160.0
	Red onion	154.1
	Red cabbage	139.3
	Lettuce	124.5
	Salad	116.2
	Celery leaf	113.0
	Broccoli	101.7
	Carrot	96.0
	Tomato	76.9
	Kohlrabi	44.9
Herbs	Mint	335.4
	Sage	316.4
	Lemonbalm	303.2
	Thyme	299.2
	Parsley	188.0

1.4 Red Beetroot Phenolic and Betalain Contents

Beetroot (*Beta vulgaris*) belongs to Amaranthaceae family and it has a wide kind according to the color contained (yellow to red). The difference in color arises from included pigment type and content (Figure 1.11). Red beetroot is the most common types for human consumption (Singh & Hathan, 2014; Neelwarne, 2013). Red beetroot which is known as one of the most powerful vegetables has excellent antioxidant activity due to its phenolic and betalain content (Saponjac et al., 2016). Because of high antioxidant effect, the consumption of red beetroot is influenced by the prevention of oxidation or inflammation– related diseases and cardiovascular diseases (Singh & Hathan, 2014; Clifford et al., 2015).

Figure 1.11: Color diversity of beets; (1) shows absence of pigment, (2) shows dominant presence of betaxanthin, (3) sowhshigh betaxanthin + low betacyanin, (4) shows low betaxanthin + high betacyanin and (5) shows dominance of betacyanin (Neelwarne, 2013).

Red beetroot comprises variety of bioactive compounds that are phenolics, ascorbic acid, carotenoids, nitrate and betalains (Figure 1.12). Total betalain content is between 40 – 200 mg / 100 g fresh beetroot and 0.4 – 20 mg / g dry beetroot. Different betalain content has been reported by conducted studies. Betalain content is affected from season; generally spring beets have higher betalain content than autumn beets (Neelwarne, 2013; Bucur et al., 2016). The main betalains in red beetroot are betanin, betanidin, isobetanin, isobetanidin, prebetanin and neobetanin (Kujala et al, 2000).

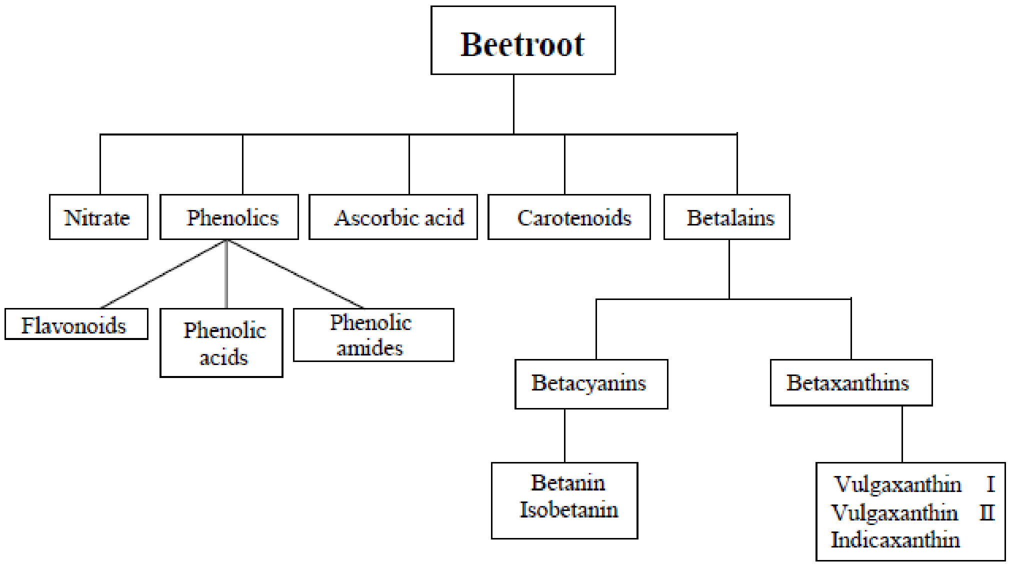

Figure 1.12: Bioactive contents of red beetroot (Clifford et al., 2015).

Red beetroot also includes phenolic compounds which are gallic acid, protocatechuic acid, p-Hydroxybenzoic acid, catechin, epicatechin, chlorogenicacid, caffeic acid, ferulicacid, sinapicacid, coumaricacid, myricetin, luteolin, quercetin and apigenin (Saponjac et al., 2016). The total phenolic content of red beetroot is between 250 – 400 mg / 100 g fresh weight (Guldiken et al., 2016; Kujala et al., 2000).

Table 1.4: Pigment content (g/kg) in Dutch (D) andHungarian (H) varieties of redbeet (Neelwarne, 2013).

	Betacyanins				Betaxanthins	
Variety	Betanin	Isobetanin	Betanidin	Isobetanidin	Vulgaxanthin - I	Vulgaxanthin - II
Bonel (D)	0.50	0.27	0.04	0.01	0.42	0.06
Nero (D)	0.41	0.13	0.03	0.01	0.32	0.03
Favorit (H)	0.49	0.24	0.05	0.02	0.41	0.02
Rubin (H)	0.46	0.25	0.07	0.03	0.37	0.03
Detroit (H)	0.44	0.21	0.05	0.01	0.37	0.04

1.5 Encapsulation

Entrapping of active compound into a protective shell (wall) is named as encapsulation. In food industry, encapsulation technology is mainly used for providing controlled release and preservation of food ingredient. Encapsulation can be divided into two groups according to the capsule size, which are microcapsules and nanocapsules. By using encapsulation technology, food products which meet consumer demands that are healthy, safe, delicious can be produced. The advantages of using encapsulation technology in food industry can be listed as follow (Zuidam & Nedovic, 2010; Nedovic, 2016);

– Easier handling of active ingredient (converting liquid ingredient to powder form)
– Providing more stable active material by protecting from environmental conditions such as oxygen, light and temperature
– Improving solubility of active compound
– Providing controlled release
– Increasing bioavailability
– Masking unpleasant odour

Most of active ingredient such as antioxidants, vitamins, minerals, fatty acids, phytochemicals, flavors, aroma compounds and probiotics can be encapsulated (Zuidam & Nedovic, 2010; Nedovic et al., 2011). In order to preparing capsules; polysaccharides, proteins, lipids, waxes and polymers can be preferred as wall material. Besides that, there are many techniques for preparing encapsulates, these are spray drying, spray chilling / cooling, freeze drying, fluid bed coating, extrusion, emulsification, coacervation and liposome entrapment. The appropriate method is preferred according tochemical / physical properties of active material, type of wall material and desired properties (Zuidam & Nedovic, 2010; Vladisavljević, 2012; Nedovic et al., 2011).

1.5.1 Encapsulation techniques

The most common method for encapsulation is spray drying. In this method, the powdered encapsulates are obtained by drying the liquid mixture that contains active ingredient and wall material. The liquid mixture may be found as emulsion, dispersion or solution. Figure 1.13 shows the spray drying system, emulsion meets with hot dry air at temperature 100 – 160°C so water is evaporated rapidly and solid capsules are obtained (Barbosa-Canovas et al., 2005; Gharsallaoui et al., 2007; Zuidam & Nedovic, 2010).

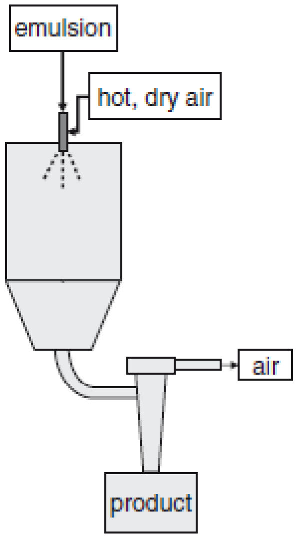

Figure 1.13: Spray drying system (Zuidam & Nedovic, 2010).

In spray drying process; inlet and outlet temperature, feeding rate and feed temperature are important parameters.

Spray chilling / cooling method is preferred for lipid wall materials such as fatty acids, triacylglycerols, hydrogenated vegetable oils and waxes. While spray chilling is appropriate for lipids that melt at 34 – 42°C, spray cooling is used for lipids that melt at higher tempretures. In this method, active ingreadient presence in melted lipid and the solution / emulsion (water/oil emulsion) is solidified by chilling or cooling process. The schematic representation is shown in Figure 1.14 (Gamboa et al., 2011; Okuro et al., 2013).

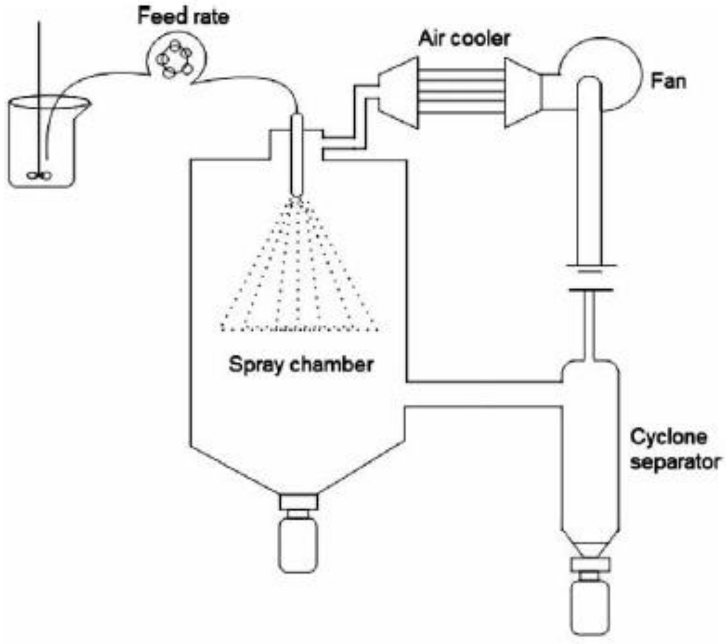

Figure 1.14: Spray chilling / cooling system representation (Okuro et al., 2013).

Freeze drying method includes lyophilization of capsules which are dissolved or dispersed in water. In this technique, the solution / emulsion must be frozen before drying process. Freeze drying is conducted at -40 - -90°C under vacuum (Wilkowska et al., 2016; Sanchez et al., 2013).

Encapsulation can also be conducted by fluid bed coating method. Encapsulation is achived by coating the fluidized particles. Particle fluidization is provided by air which has constant temperature. There are two types of technique that are bottom and upward spraying (Figure 1.15). In this process, spraying rate, temperature, water coating solution water content and air humidity are significant parameters (Zuidam & Nedovic, 2010).

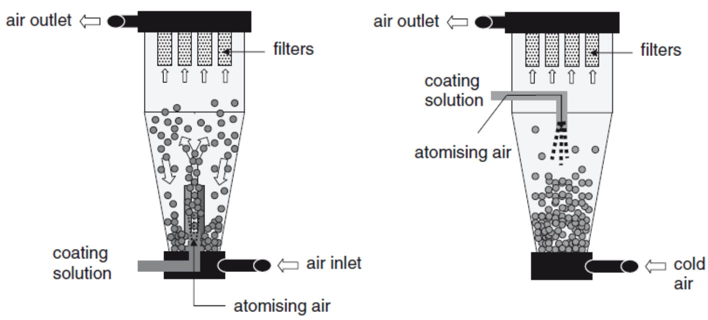

Figure 1.15: Bottom and upward spraying type of fluid bed coating system representation (Zuidam & Nedovic, 2010).

Encapsulation is performed by melt extrusion method in which the core material is dispersed into melted wall material and the mixture is forced through a die (Figure 1.16). The capsules are produced by cutting the formed threads. Generally, the sizes of the capsules are between 300 – 5000 μm (Nevodic et al., 201; Porzio, 2008).

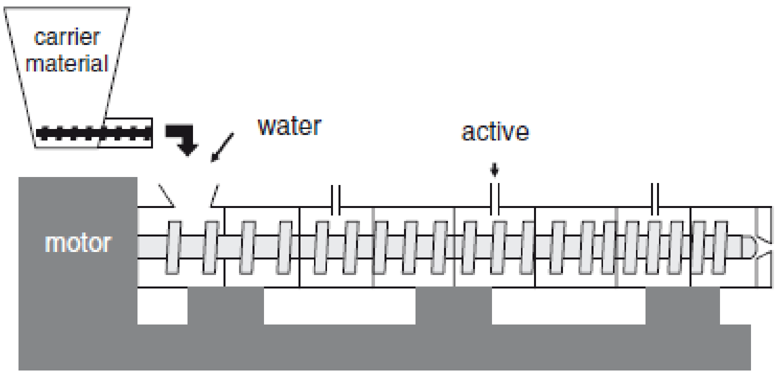

Figure 1.16: Shematic view of extrusion process (Zuidam & Nedovic, 2010).

Emulsification is also preferred for encapsulation. Emulsion is the mixture of two or more immiscible liquid in which one is presence as doplets distributed throughout the other. There are various types of emulsions that are oil in water (O/W), water in oil (W/O), water in oil in water (W/O/W) and oil in water in oil (O/W/O) (Söderberg 2013). Emulsions are obtained by applying high shear. High pressure homogenizers, ultrasound homogenizers or microfluidic devices can be used for providing high shear (Vladisavljević, 2012; Schuchmann, 2007). Emulsification technique can be applied for both of lipophilic and hydrophilic active compound encapsulation. After prepare the desired emulsion (O/W or W/O/W) system, it can be powdered by spray drying or freeze drying (Vladisavljević, 2012). The final particle size of the encapsulates are between 0.2 –5000 μm (Zuidam & Nedovic, 2010)

Coacervation method involves phase separation of the emulsion which contains core and wall material (Figure 1.17). Polymers are used as wall material in coacervation technique. After phase separation, coacervate (polymer rich) and solvent (polymer poor) phases are obtained. The formed encapsulates are found in coacervate. Coacervation method has two different types that are simple coacervation and complex coacervation. In simple coacervation, only one polymer is used and encapsulation occurs by decreasing solubility. However, at least two polymers which are opposite charged are used in complex coacervation and encapsulation occur via electrostatic attraction (complex formation) (Jun-Xia et al., 2011; Salve & Changediya, 2016). Particle size of the formed encapsulates is between 10 – 800 μm (Zuidam & Nedovic, 2010).

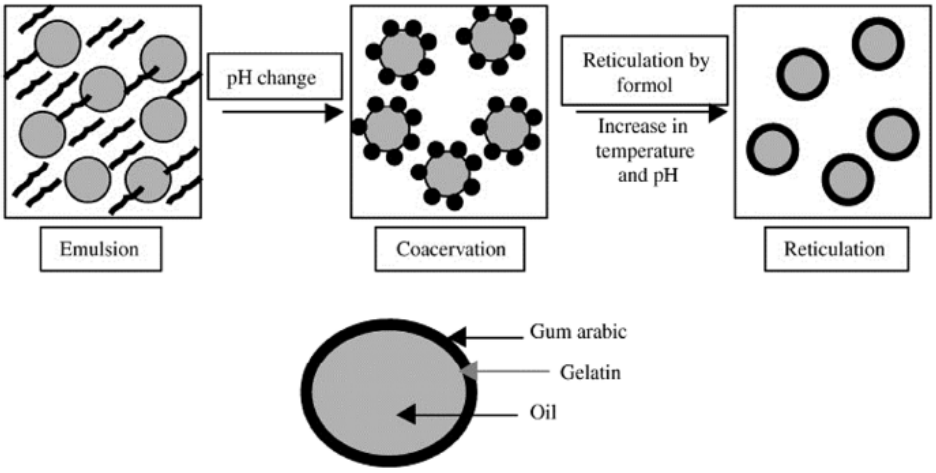

Figure 1.17: Shematic view of coacervation process (Salve & Changediya, 2016).

The last encapsulation technique is the liposome which is an amphiphilic molecule composed of phospholipids. The liposomes are obtained by dispersion of phospholipids in aqueous phase and applying high shear. Liposome encapsulation is suitable for both of lipophilic and hydrophilic active compounds due to its amphiphilic structure. After encapsulation process; desired particle size may be provided by homogenization with sonication and microfluidization. The particle size of the capsules is between 30 nm – 1000 μm (Socaciu et al., 2000; Zuidam & Nedovic, 2010; Nedovic et al., 2016).

1.5.2 Wall materials used in encapsulation

Wall or shell material used in encapsulation process can be divided into three main categories which are carbohydrates, proteins and lipids (Table 1.5). Each group has some advantages and disadvantages; therefore, selection of the suitable wall material is made according to the properties of encapsulated core material and encapsulation technique (Anandharamakrishnan & Ishwarya, 2015).

The carbohydrates provide a wide variety of wall materials in order to encapsulate the food ingredient. This group includes; starch derivative, cellulose derivatives, gum, polysaccharide, carrageenan, alginate, xanthan, gellan, dextran and chitosan (Anandharamakrishnan & Ishwarya, 2015). Starch derivatives (maltodextrin and dextrin) are appropriate for spray drying, freeze drying, fluidizied bed coating and extrusion techniques. Starch derivatives provide good oxygen, heat, light barrier and water soluble capsules so they are useful for flavors, fats, oils, vitamins, food colorants encapsulation (Nedovic et al., 2016). The second group of carbohydrates is cellulose and their derivatives which include cellulose, carboxymethylcellulose and methyl cellulose. Spray drying, extrusion, fluidized bed coating, freeze drying and emulsification methods can be applied for core materials that are covered with cellulose or cellulose derivatives. Gums are also highly preferred for food ingredient encapsulation. Especially; gum Arabic, guar gum and xanthan gum are used for encapsulation of the food materials such as flavors, oils and prebiotics. In order to obtain gum coated capsules; spray drying, freeze drying, fluidized bed coating, extrusion and coacervation are the most appropriate techniques (Wandrey et al. 2010; Zuidam & Nedovic, 2010).

The second group is protein originated wall materials. The source of protein may be animals or plants. Animal originated proteins are whey protein, casein and gelatin are used for encapsulation process. Besides that, plant proteins such as pea and soy proteins are also preferred as coating material (Wandrey et al., 2010). Protein type wall materials are appropriate for fluid bed coating, spray drying, freeze drying, spray chilling – cooling and emulsion encapsulation techniques. Proteins have amphiphilic structure so both of hydrophobic and hydrophilic bioactive compounds can be encapsulated (Nedovic et al., 2011; Prata et al., 2013).

The last coating material group is oils and lipids. The main property of this group is their hydrophobic structure and they include fatty acids, waxes and phospholipids. Spray chilling – cooling, emulsification methods are the most appropriate for lipid wall materials. The main advantage of lipid type wall materials is the moisture barrier they provide (Kapusniak & Tomasik, 2006; Wandrey et al., 2010; Quirós-Sauceda et al., 2014).

Table 1.5: Summarization of wall material used in encapsulation process (Wandrey et al., 2010)

Origin	Carbohydrate polymer	Protein	Lipid
Plant	Starch - Derivatives Cellulose - Derivatives Plant exudates - Gum arabic - Gum karaya - Mesquite gum Plant extracts - Galactomannans - Soluble soybean Polysaccharide	Gluten (corn) Isolates (pea, soy)	Fatty acids/alcohols Glycerides Waxes Phospholipids
Marine	Carrageenan Alginate		
Microbial/animal	Xanthan Gellan Dextran Chitosan	Caseins Whey proteins Gelatin	Fatty acids/alcohols Glycerides Waxes Phospholipids (Shellac)

1.5.3 Beeswax as a wall material

Beeswax is produced during honeycomb formation by worker honeybee. Beeswax has a very complex structure and it is esters of higher fatty acids and alcohol. Triacontylpalmitate, triacontylcerotinate, and paraffin are the main components of beeswax (Table 1.6). The melting point of beeswax is around 61 - 65°C at atmospheric pressure (Wandrey et al., 2010; Bognadov, 2004).

Table 1.6: Components of beeswax (Bognadov, 2004).

Component	Quantity (%)
Esters	67
Hydrocarbons	14
Free acids	12
Alcohols	1
Others	6

In encapsulation process, beeswax provides good moisture and oxygen barrier and it protects core material from oxidation and hydrolyzation so it provides more stable capsules (Soleimanian et al., 2018; Nedovic et al., 2016). Beeswax is appropriate wall material for fluidizied bed coating, emulsification and extrusion techniques (Nedovic et al., 2016). Beeswax is permitted as glazing agent and flavor encapsulation material in food systems by European Food Safety Authority (Aguilar et al., 2007). Beeswax can also be used as wall material by emulsification method for entrapping water soluble active materials (Mellema et al., 2006).

1.5.4 Red beetroot extract encapsulation

Encapsulation process is applied in order to provide more stable red beetroot extract by preserving it from envirionmental conditions or intrinsic changes. Encapsulated extracts may be used as food colorants or antioxidant agent in food systems. Therefore, storage stability is vital property for using in food matrix. The aim of the encapsulation of red beetroot extract is improving its usage area in food industry (Saikia et al., 2015; Otalora et al., 2016; Saponjac et al., 2016).

Many different wall materials were used in red beetroot extract encapsulation. In a conducted study, the extract was encapsulated by using chitosan and maltodextrin as wall material and storage stability of the capsules was detected. The results show that maltodextrin coated encapsulates are more stable than chitosan coated capsules at 20°C for 180 days. While the remaining extract content in maltodextrin capsules is 60%, there is no extract in chitosan capsules at end of the storage time (Gandía-Herrero, 2013). According to the study made by Pitalua et al. (2010), gum Arabic coated extract shows good chemical and physical (color) stability during 45 days at low water activities (0.1 – 0.5). In the other study, the effect of the encapsulation of red beetroot extracts with various wall materials (maltodextrin, gum Arabic, pectin, guar gum and xanthan gum) and techniques (freeze and spray drying) was investigated. The results indicated that the most stable capsules were providedby using xanthan gum / maltodextrin mixture as wall material and freeze drying technique (Ravichandran et al., 2014).

1.6 Hypothesis

The aim of the study is providing more stable red beetroot extract. Therefore, the extract was encapsulated by beeswax that preserves the extract from hydrolyzation and oxidation reactions due to its natural structure. In this study, it is expected that encapsulated extracts have more stability than free extracts during storage of the food product.

2. MATERIALS AND METHODS

2.1 Red Beetroot Samples

Red beetroot (*Beta vulgaris*) samples were obtained from a local market in Istanbul, Turkey. All samples were washed, peeled, cut into pieces and grounded by using analytical mill (IKA – A11, Staufen, Germany) with liquid nitrogen (- 80°C). Grounded red beetroot samples were collected and stored at - 20°C until further usage.

2.2 Chemicals

Aluminum chloride, copper (II) chloride dihydrate, neocuproine, sodium hydroxide, sodium nitrate, trolox, catechin, gallic acid, betanin andphenolic standards for HPLC were purchased from Sigma – Aldrich (Germany). Folin – Ciocalteau reagent, sodium carbonate and polysorbate 20 were from Merck KGaA (Darmstadt, Germany). 2,2 – diphenyl – 1 – picrylhydrazyl (DPPH) (Alfa Aesar, Karlsruhe, Germany), lecithin (soybean phospholipids, Rotel, Turkey) and beeswax was obtained from a local market in Istanbul, Turkey.

2.3 Red Beetroot Extraction Procedure

Extraction process was done according to method that recommended by Carmo et al. (2015) with some modifications. Firstly, sample of freezed red beetroot (20 g) was extrated with 50 % ethanol – water mixture (1:20 w/v) on ultrosonic bath at room temperature for 30 min. Then, the mixture was stirred at 400 rpm at room temperature for 2 h. After completed the stirring process, the mixture was centrifuged at 4000 rpm at 4°C for 30 min (Universal 32R, Hettich) and supernatants were separated. In order to obtain concentrated extract, ethanol was evaporated by using rotary evaporator (Rotavapor RII, BUCHI, Switzerland) at 40°C under vacuum. The concantrated supernatants were freeze dried at – 55°C (Alpha 1-2

LDPlus, CHRIST, Germany) for 18 h. The dried extract was stored at – 20°C for further analysis.

2.4 Encapsulation Procedure

Red beetroot extract encapsulation was prepared by melt emulsification technique and the process was conducted by using the method of Mellema et al. (2006) and Campos et al. (2015) with some changes. In encapsulation process, different core / wall ratio that are 1:1, 1:2, 1:3 and 2:3 w/w were prepared. Firstly, 3 grams of beeswax and 1.4 % (w/w) lecithin were weighed and melted at 65°C. After melting, beeswax and lecithin were mixed at 1000 rpm for 15 min. Then, desired amount of red beetroot extract which is diluted 1:10 (w/v) with phosphate buffer at pH 6 was added to the melted mixture drop by drop and the mixture was homogenized (Ultraturrax T18 basic, IKA) at 15000 rpm for 10 min. The homogenized mixture was added to distilled water (at 65°C) that contains 6% (w/v) of lecithin and polysorbate 20 mixture (1:4 w/w). Then, the mixture was homogenized at 15000 rpm for 15 min. After homogenization process, the formed emulsion was freeze dried at – 55°C for 18 hours.

2.5 Measurement of Particle Size Distribution and Zeta Potential

Particle size and zeta potential of empty and red beetroot extract loaded wax capsules which were diluted 0,005% (w/v) with distilled water were detected by light scattering using Zetasizer (NanoSizer 3000, Malvern Instruments, Malvern, UK) at 25°C

2.6 SEM (Scanning Electron Microscope)

SEM analysis was conducted (EVO LS 10, Zeiss, Germany) in order to examine the surface morphology of obtained wax capsules. The images were taken at different magnification.

2.7 Measurement of Total Phenolic Content

Total phenolic content of beetroot extract was detected by Folin – Ciocalteauspectrophotometric method. Before the phenolic content analysis, the beetroot extract was diluted 1:500 (w/v) by 50 % ethanol – water mixture. Phenolic content in beetroot extract was determined in that 15 μL of extract, 170 μL of distilled water, 12 μL of Folin – Ciocalteau's reagent which diluted ten foldand 30 μL of 20% sodium carbonate were mixed in a microplate. The microplate has been kept at dark for 1 hour at room temperature. The absorbance at 750 nm was detected by end of the time. Distilled water was used as blank and results were expressed as mg gallic acid equivalent (GAE) per g sample (Saponjac et al., 2016).

2.8 Measurement of Total Flavonoids Content

Total flavonoid contents in the extract and capsules were conducted by aluminium chloride colourimetric assay according to Herald et al. (2012) method. Firstly, 100 μL of distilled water, 10 μL of 50 g/L of $NaNO_2$ and 25 μL of sample solution were added and allowed to stand for 5 min. After that, 15 μL of 100 g/L $AlCl_3$ solution was added to the previous mixture. After 6 min, 50 μL of 1 M NaOH and distilled water were added and the plate was shaked for 30 s in reader. Finally, absorbances were read at 430 nm. Distilled water was used as blank. The results were expressed as mg catechin equivalent (CE) per gram sample.

2.9 Measurement of Total Betalain Content

The method that described by Saponjac et al. (2016) was used with minor modifications for total betalain content determination. 100 mg of dried sample was mixed with 50% ethanol – water mixture, vortexed for 1 min and centrifuged at 6000 rpm for 2 min. Then, 20 μL of sample and 230 μL phosphate buffer (0.05 M, pH 6.5) were added to the wells. Absorbances were read at 535 nm (betanin), 476 nm (vulgaxanthin-I) and 600 nm for correction. Phosphate buffer was used as blank. The results are given mg betanin equivalent for total betacyanins and mg vulgaxanthin-I equivalent for total betaxanthins by using the following equations (2.1, 2.2, 2.3 and 2.4):

$$x = 1.095 * (a - c) \tag{2.1}$$

$$y = b - z - (x/3.1) \tag{2.2}$$

$$z = a - x \tag{2.3}$$

$$C\ (mg/100\ mL) = x(y) * F * 1000/A \quad (2.4)$$

In these equations; x is corrected absorbance value for betanin, y is corrected absorbance value for vulgaxanthin-I, z is correction value, a is absorbance value at 535 nm, b is absorbance value at 476 nm and c is absorbance value at 600 nm.

2.10 Determination of Encapsulation Efficiency

Encapsulation efficiency (EE) was detected by total phenolic method. In order to calculate EE, surface (SPC) and core phenolic content (CPC) were detected. For SPC, 100 mg of capsules were mixed with 1 ml of 50 % ethanol / water mixture and vortexed for 1 min. Then, the mixture was centrifuged (EBA 20, Hettich) at 6000 rpm for 2 min and supernatant was used for phenolic analysis. For CPC, 100 mg capsules were mixed with 1 ml of 50 % acetone / water mixture and stirred for 3 hours. After that, the mixture was centrifuged at 6000 rpm for 2 min and supernatant was filtered by using 0.45 μm syringe filter and it was used for phenolic analysis. Finally, EE was calculated by following equation (2.5) (Saponjac et al., 2016);

$$EE\ (\%) = \left(\frac{(CPC - SPC)}{CPC}\right) \quad (2.5)$$

2.11 Measurement of Antioxidant Activity

Antioxidant activity of the samples was detected by 2, 2 – diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging methodandcupric reducing antioxidant capacity (CUPRAC).Trolox was used as standard for all antioxidant analysis.

2.11.1 Measurement of antioxidant activity using DPPH assay

The method recommended by Kumaran et al. (2006) was adapted to 96 well microplate. Firstly, 250 μL of DPPH solution was added to 15 μL of diluted sample solution and the mixture was stored at dark for 50 min. The absorbance was measured at 515 nm by the end of time. Methanol was used as blank and the result was given as % scavenging activity (SA %) and mM Trolox equivalent.

2.11.2 Measurement of antioxidant activity using CUPRAC assay

CUPRAC assay was conducted by using Riberio et al. (2011) method. Firstly, 50 μL of 10 mM copper chloride solution was added to the well; secondly, 50 μL of 1 M ammonium acetate buffer at pH 7 was added and lastly 50 μL of 7.5 mM neocuproine solution was added. After adding all reaction medium, 100 μL of diluted sample solution was added and the plate was stored at dark for 30 min. The absorbance was read at 450 nm at the end of time. Distilled water was used as blank and the results were given as mM Trolox equivalent.

2.12 HPLC (High Pressure Liquid Chromatography) Analysis

The HPLC system **(**Shimadzu Corporation, Kyoto, Japan**)** comprises from a micro vacuum degasser (DGU-20A$_3$, Shimadzu, an auto sampler (SIL-20A HT, Shimadzu), a column oven (CBM-20A, Shimadzu), a controller and a PDA detector (SPD M20A, Shimadzu). The column properties are ACE C18 column (250 mm x 4.6 mm, 3 μm) with guard column (4.0 x 10 mm x 2).

2.12.1 Determination of phenolic profile

The samples which were dissolved in methanol were filtered through a 0.45 μm syringe filter and were injected to HPLC system with PDA detector. Phenolic compound profile was detected by using two mobile phases (A and B). Phase A is asetonitrile and phase B is 1% formic acid. In HPLC analysis, A and B were used at a flow rate of 1 mL / min and the following gradient profile was used; 0 – 10 min from 10% to 25% A, 10 – 20 min linear rise up to 60% A, 20 – 30 min linear rise up to 70% A, followed by 10 min reverse to initial 10% A with additional 5 min equilibrium time (Saponjac et al., 2016).

2.12.2 Determination of betacyanin profile

The samples which were dissolved in Milli-Q water were filtered through a 0.45 μm syringe filter and were injected to HPLC system with PDA detector. The temperature of column is set to 30°C. In betanin analysis, phase A is acetonitrile and phase B is water with 0.5% formic acid. Flow rate is 0.5 mL / min and the gradient profile is that initial 100% B, linear gradient to 100 % B in 3 min, linear gradient to 25 % B in

25 min, linear gradient to 25 % B in 30 min and linear gradient to 100 % B in 35 min (Saponjac et al., 2016).

2.13 Inclusion of the Capsules into Kefir Formulation

Obtained beeswax encapsulated red beetroot extract was incorporated into kefir formulation at 1:10 (w/w) ratio. In order to control the effect of encapsulation process, 3 different control samples which are kefir (K), kefir with crude extract (KE) and kefir with empty capsule (KC) were also prepared at the same ratio.

2.14 Storage Stability of Red Beetroot Extract and Capsules in Kefir

Storage stability has been conducted for 21 days. Kefir samples were stored at 4°C in dark and the samples were collected in 1^{st}, 7^{th}, 14^{th} and 21^{st} days for storage stability analysis. In order to determine stability; total phenolic content, total flavonoid content, betalain content, antioxidant activity assays and color measurement were achieved for each group and obtained results were compared with the initial values.

2.15 Color Measurements

Color measurement of the extract, capsules and the fortified food product was detected by CIE Lab method (Minolta Colorimeter CR – 400, Japan). In order to color measurement; L^* (lightness), a^*(red – green) and b^*(blue – yellow) values were detected. Color measurements were conducted at initial time and during storage period (Carmo et al., 2015).

2.16 Statistical Analysis

All experimental measurements were conducted in triplicate and the results were given as mean ± standard deviation. Data analysis was performedby analysis of variance (ANOVA) and Tukey test. The value of $p < 0.05$ was considered statistically significant. Statistical analysis were conducted by Minitab 18 sofware (Minitab Inc., USA).

3. RESULTS AND DISCUSSION

3.1 TPC, TFC and Betalain Content of Red Beetroot Extract

Red beetroot extract contents were expressed for 100 g extract and red beetroot contents were expressed for 100 g fresh red beetroot. Total phenolic content (TPC), total flavonoid content (TFC), betacyanin content, betaxanthin content, phenolic profile and betacyanin profile were determined. The results are presented in Table 3.1. TPC and TFC were determined as 338.37 ± 24.85 mg GAE / 100 g sample and 553 ± 9.94 mg CE / 100 g sample resectively. TPC of red beetroot are determided as 255 ± 48 mg GAE / 100 g sample and 393.42 ± 51 mg GAE / 100 g sample in different studies (Guldiken et al., 2016; Kujala et al., 2000). The result obtained from the present study is similar to literature values. In addition to TPC, TFC are detected as 546 ± 27 mg CE / 100 g beet and 610.34 mg CE / 100 g beet in literature (Guldiken et al., 2016; Arruda Ramos et al., 2016). When the result obtained from this study is compared with the literature values, it is observed that the obtained results are consistent with the literature.

Table 3.1: Contents of red beetroot extract.

Compounds	Content (per 100 g extract)	Content (per 100 g red beetroot)
Total Phenolics[a]	2448.38 ± 179.84	338.37 ± 24.85
Total flavonoids[b]	4001.45 ± 71.91	553.00 ± 9.94
Betacyanins[c]	453.99 ± 4.12	62.74 ± 0.57
Betaxanthins[d]	360.08 ± 2.44	49.76 ± 0.34
Ferulic Acid[e]	0.159 ± 0.016	0.022 ± 0.002
4 (p-) Hydroxybenzoic acid[e]	0.219 ± 0.052	0.030 ± 0.001

Table 3.1 (Continued): Contents of red beetroot extract.

Compounds	Content (per 100 g extract)	Content (per 100 g red beetroot)
Vanillic Acid[e]	0.329 ± 0.06	0.045 ± 0.001
Ethyl 3,4 dihydroxybenzoate[e]	2.454 ± 0.012	0.339 ± 0.001
Gallic Acid	35.12 ± 2.828	4.854 ± 0.39
Catechin[e]	1.42 ± 0.01	0.196 ± 0.001
Epigallocatechingallate (EGCG) [e]	5.329 ± 0.045	0.736 ± 0.001
Syringic Acid[e]	0.376 ± 0.021	0.052 ± 0.002
Vanilin[e]	0.419 ± 0.075	0.058 ± 0.01
Hesperidin[e]	0.219 ± 0.007	0.03 ± 0.001
Caffeic Acid[e]	0.441 ± 0.061	0.061 ± 0.008
Chlorogenic Acid[e]	2.190 ± 0.041	0.303 ± 0.006
p-Coumaric Acid[e]	0.377 ± 0.138	0.052 ± 0.02
Sinapic Acid[e]	2.719 ± 0,009	0.376 ± 0.001
Rosmarinic Acid[e]	0.388 ± 0.040	0.054 ± 0.005
Luteolin[e]	0,003 ± 0.00	-
Quercetin[e]	0.124 ± 0.001	0.017 ± 0.00
Rutin[e]	0.063 ± 0.003	0.009 ± 0.00
Myricetin[e]	0.095 ± 0.00	0.013 ± 0.00
Epicatechin[e]	0.545 ± 0.006	0.075 ± 0.001
Betanin[e]	574.5 ± 24.33	79.396 ± 3.362
Isobetanin[e]	38.50 ± 0.64	5.32 ± 0.088

*Data indicates the mean value ± standart deviation (SD). [a] expressed as mg gallic acid equivalents; [b] expressed as mg catechin; [c] expressed as mg betanin equivalents, [d] expressed as mg vulgaxanthin – I equivalents and [e] expressed as mg.

In this study, betacyanin and betaxanthin contents were determined as 62.74 ± 0.57 mg BE / 100 g beet and 49.76 ± 0.34 mg VE / 100 g beet respectively. The betacyanin content is significantly more than betaxanthin content ($p < 0.05$). In literature, total betalain of red beetroot is between 40 – 200 mg / 100 g beet (wet basis) (Sanchez-Gonzales et al., 2013; Neelwarne, 2013). The obtained result from the present study is 112.5 mg / 100 g red beetroot and it is accordance with literature values. According to Bucur et al (2016), autumn and spring red beetroot have different betalain content. The beetroot growing in the spring contains approximately two times more betalain content in dry basis than the beetroot growing in the autumn. In this study, autumn red beetroot samples were used.

Besides TPC, TFC, total betacyanin and betaxanthin contents, HPLC analysis was conducted for identifying individual phenolics and betalains. The results are shown in Table 3.1. According to the results, the red beetroot sample includes ferulic acid, hydroxybenzoic acid, vanillic acid, ethyl 3,4 dihydroxybenzoate, gallic acid, catechin, epigallocatechingallate, syringic acid, vanillin, hesperidin, caffeic acid, chlorogenic acid, p-coumaic acid, sinapic acid, rosmarinic acid, luteolin, quercetin, rutin, myricetin and epicatechin. In different studies, the phenolic profile of red beetroot is described as follows; caffeic acid, ferulic acid, p-coumaric acid, hydroxybenzoic acid, gallic acid, sinapic acid, catechin, protocatechuic acid, chlorogenic acid, vanillic acid, cinnamic acid, rutin and hesperidin (Saponjac et al., 2016; Kujala et al., 2000; Kujala et al., 2002; Georgiev et al., 2010; Ravichandran et al., 2012; Szekely et al., 2014; Koubaier et al., 2014). When the obtained phenolic profile is compared with literature, the result is mostly consistent with the literature.

In addition to phenolic compounds, individual betacyanins were also detected by HPLC system. Betanin content was determined as 79.396 mg / 100 g beet and isobetanin content was determined as 5.31 mg / 100 g beet. Kujala et al. (2002) reported betanin content is between 37.32 – 66.92 mg / 100 g beet in flesh part isobetanin content is between 0.257 – 5.148 mg / 100 g beet in flesh part. While the betanin content in the present study was found more than the reported value, isobetanin content is accordance with reported range. The difference in betanin content may be attributed to many intrinsic or extrinsic factors such as genotype, harvesting season or soil type.

3.2 Characterization of Encapsulates

3.2.1 Particle size and polydispersity index

The average particle size (PS) and polydispersity index (PDI) of encapsulates that have different core : wall ratio were measured. The obtained results are shown in Table 3.2. PDI indicates the size distribution of the particles and it is in the range 0.2 (high homogenity) to 0.5 (medium homogenity) (Campos et al., 2015). According to the results, the measured PDI values are between 0.205 – 0.368 so all analysis meets the homogenity criteria.

Particle size is important parameter for particle characterization. In this section, particle size of empty, 1:1, 1:2, 1:3 and 2:3 encapsulates were measured. The results show that particle sizes of empty capsules are significantly different from extract loaded capsules ($p < 0.05$). Drug loaded encapsulates have larger particle size than empty capsules. While there are no significant difference ($p > 0.05$) between 1:1 – 1:2 and 1:2 – 2:3; 1:1 – 1:3, 1:1 – 2:3, 1:2 – 1:3 and 1:3 – 2:3 formulations are significantly different ($p < 0.05$) from each other. It indicates that different drug / wall ratio affects the particle size of the capsules. The smallest particle size was observed in capsules prepared at a ratio of 1: 1 and the largest particle size was observed in 1:3 core / wall ratio.

Table 3.2: Particle sizes of the prepared encapsulates.

Core : Wall Ratio (w/w)	PS (nm)	PDI
Empty	218.9 ± 5.29^{a}	0.226
1 :1	265.9 ± 13.43^{b}	0.234
1 :2	290.0 ± 5.53^{bd}	0.224
1 :3	414.3 ± 28.87^{c}	0.368
2 :3	318.4 ± 15.50^{d}	0.205

*Different letters indicate the significant difference ($p < 0.05$).

In a study conducted by Campos et al. (2015), the particle size of carnauba wax and witepsol H15 wax nanoparticles were measured in a range 408 – 1001 nm and 286 – 332 nm. In other study, particle size of capsules which contain water soluble drug was detected between 159.5 and 246.7 nm (Shah et al., 2012). When these values are taken into consideration, it is observed that the data obtained from the present study are compatible with the literature values.

3.2.2 Zeta (ζ) potential

Zeta potential of empty and drug loaded encapsulates are also measured. The results are shown in Table 3.3. Zeta potential gives information about surface charge of the particles in liquid system and it is vital parameter to detect dispersion stability (Shah et al., 2012). The higher zeta potential provides the more stable dispersion. According to the results, there are no significant differences between empty and drug loaded particles. Although different core / wall ratio has no significant effect ($p < 0.05$) on zeta potential, the maximum zeta potential was observed in formulation that prepared with a ratio of 2:3.

Table 3.3: Zeta potential of prepared encapsulates.

Core :Wall Ratio (w/w)	Zeta Potential (mV)
Empty	-53.3 ± 7.03^{a}
1 : 1	-48.1 ± 6.56^{a}
1 : 2	-44.7 ± 4.93^{a}
1 : 3	-48.2 ± 5.92^{a}
2 : 3	-41.8 ± 12.4^{a}

3.3 Encapsulation Efficiency

After formed the capsules, encapsulation efficiency (EE) was calculated for different capsule formulations. The EE results are shown in Table 3.4. EE was observed between 42.18% and 67.16% and the highest EE was determined in 1:2 core / wall ratio. According to the study conducted by Campos et al. (2015), EE was calculated between 79.1 – 87.4% for sage and savoury extract encapsulation with witepsol H15 wax and 79.8 – 87.2% sage and savoury encapsulation into carnauba wax. In another study conducted by Shah et al. (2012), ciprofloxacin HCl that is a water soluble drug was encapsulated in oil phase (cetyl palmitate and glyceryl stearate) and EE was determined in a range of 39.1 – 28.4%. Therefore, it can be concluded that the EE of the present study is among these literature values.

Table 3.4: Calculated encapsulation efficiency according to different core/wall ratio.

Core : Wall Ratio (w/w)	Encapsulation Efficiency (EE %)
1 : 1	44.00^{a}
1 : 2	67.16^{b}
1 : 3	53.58^{c}
2 : 3	42.18^{d}

*Different letters indicate the significant difference ($p < 0.05$).

The results show that different core / wall ratio has effect on EE because the EE of the capsules which have different core / wall ratio are significantly different from each other ($p < 0.05$).
As a result, capsules prepared at a 1:2 ratio, which showed the best EE, were selected for further studies.

3.4 SEM Analysis

The SEM microphotographs at a magnification of 2000x, 4000x and 8000x of the capsules are shown in Figure 3.4. In microphotographs, it can be seen that the capsules are located agglomerated form and they are irregular in shapes. The reasons of this situation may be nature structure of beeswax and applying freeze drying process. The agglomeration was also reported by Choi et al. (2010) during freeze drying process and irregular shapes of the capsules were also detected in freeze drying of maltodextrin coated microcapsules (Saikia et al., 2015). In addition to agglomerated structure, the capsules have inward dents and shrunken structure. According to Gowda et al. (2011), the drying process and rate of dyring have significant effet on the appearance of final capsules.

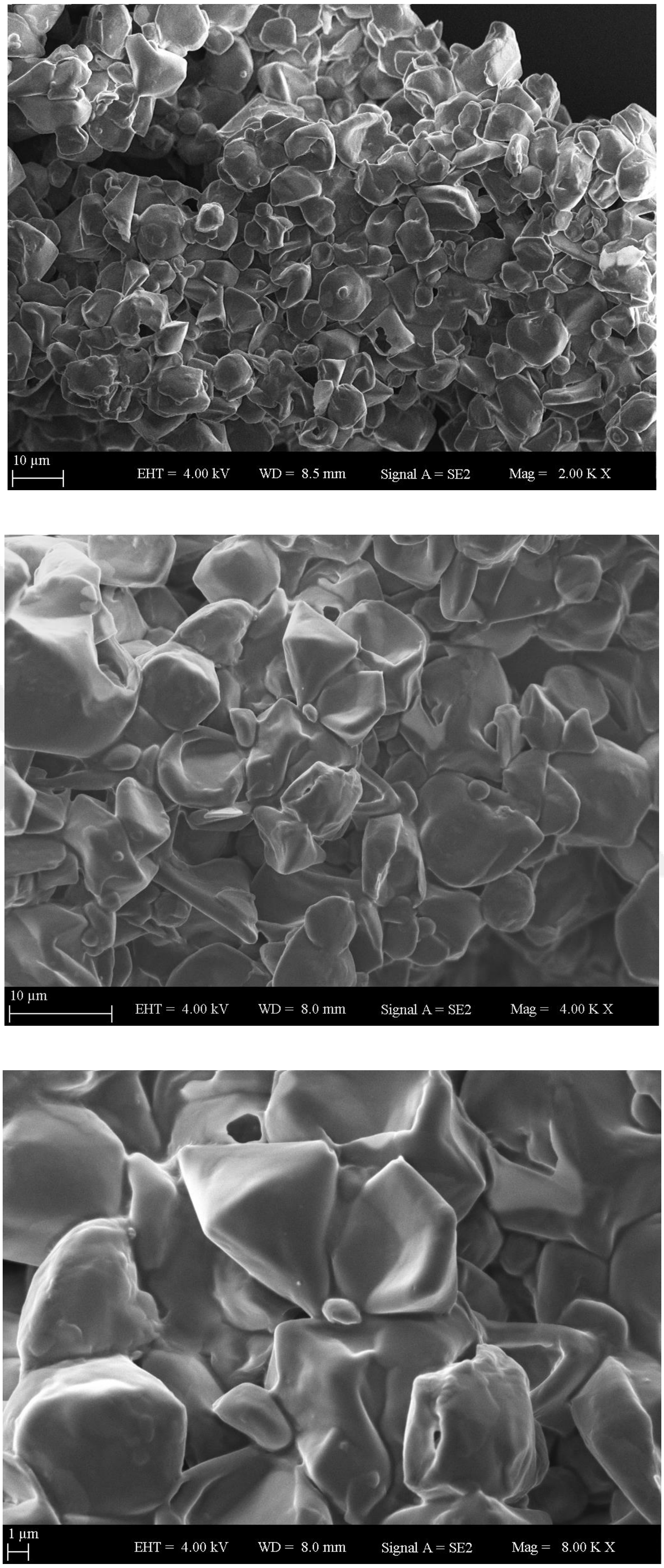

Figure 3.1: SEM microphotographs of beeswax encapsulates at a magnification of 2000x, 4000x and 8000x.

3.5 TPC, TFC and Betalain Content of Encapsulates

The contents of TPC, TFC, betacyanin, betaxanthin, individual phenolic compounds and individual betayanins of prepared capsules are presented in Table 3.5. Total phenolic content was determined as 101.68 ± 14.75 mg GAE / 100 g capsules, total flavonoid content was determined as 103.12 ± 7.38 mg CE/ 100 g capsules, total betacyanin content was determined as 29.95 ± 1.06 mg / 100 g capsules and total betaxanthin content was determined as 36.03 ± 0.35 mg / 100 g capsule. Betaxanthin content is significantly different from betacyanin content in the capsules ($p < 0.05$). The phenolic profile of the capsules was determined as follows; hydroxybenzoic acid, vanillic acid, epigallocatechingallate, vanillin, caffeic acid, p-coumaric acid, ferulic acid, gallic acid, catechin, epicatechin, syringic acid, chlorogenic acid. Apart from total betacyanin and betaxanthin content, betanin and isobetanin content were also detected by HPLC. According to the results, betanin content is 29.789 ± 2.545 mg / 100 g capsule and isobetanin content is 3.628 ± 0.336 mg / 100 g capsules. The reason of less phenolic and betalain content in the capsules is that the diluted extract (1:10 w/v) was used for encapsulation.

Table 3.5: Contents of capsules.

Compounds	Content (per 100 g capsule)
Total Phenolics[a]	101.68 ± 14.75
Total Flavonoids[b]	103.12 ± 7.38
Betacyanins[c]	26.95 ± 1.06
Betaxanthins[d]	36.03 ± 0.35
4 (p-)Hydroxybenzoic Acid	0.024 ± 0.007
Vanillic Acid	< 0.01
Epigallocatechingallate (EGCG)	0.878 ± 0.043
Vanilin	0.02 ± 0.006
Caffeic Acid	0.375 ± 0.007
p-Coumaric Acid	< 0.01
Ferulic Acid	0.017 ± 0.002
Gallic Acid	9.356 ± 0.102
Cathechin	0.38 ± 0.04
Epicathechin	< 0.01
Syringic Acid	0.129 ± 0.033
Chlorogenic Acid	0.235 ± 0.086
Betanin[e]	29.789 ± 2.545
Isobetanin[e]	3.628 ± 0.336 mg

*Data indicates the mean value ± standart deviation (SD).[a] expressed as mg gallic acid equivalents; [b] expressed as mg catechin; [c] expressed as mg betanin equivalents, [d] expressed as mg vulgaxanthin – I equivalents and [e] expressed as mg.

3.6 Antioxidant Activity of Red Beetroot Extract and Capsules

Antioxidant activity of the extract and the capsules were determined by 2,2-diphenyl-2-picrylhydrazyl (DPPH) and cupric ion reducing antioxidant capacity (CUPRAC) assays and the results are represented in Table 3.6 and Table 3.7 for extract and capsules respectively. DPPH antioxidant value was detected as 42.29 ± 0.72 mM TE / g extract, CUPRAC antioxidant activity was determined as 59.72 ± 1.12 mM TE / g extract and radical scavenging activity was 38.90 ± 0.66%. The radical scavenging activity of red beetroot extract is reported as 14.2 ± 0.7% (Georgiev et al., 2010).

Besides that, the antioxidant capacity of cactus pear extract is determined between 4.16 and 29.85 mM TE (Castro-Munoz et al., 2014). The result of the present study is more than literature values; there are many reasons in order to explain this difference. It may be arised from using different sources, different extraction process and different experimental design.

Table 3.6: Antioxidant activity of red beetroot extract.

Antioxidant Assay	Antioxidant Effect
DPPH	42.29 ± 0.72^{a}
DPPH Scavenging Activity	38.90 ± 0.66^{b}
CUPRAC	59.72 ± 1.12^{a}

[a] express antioxidant activity mM trolox equivalent per gram extract and [b] express antioxidant activity as % scavenging acitivity.

When antioxidant activity of the crude extract and the capsules were compared with each other, it is observed that while the extract has more antioxidant activity in terms of CUPRAC than in terms of DPPH, the capsules show more antioxidant activity in DPPH assay than antioxidant activity in CUPRAC. The differences between DPPH and CUPRAC assays are statistically significant for both of extract and capsules ($p < 0.05$).

Table 3.7: Antioxidant activity of the capsules.

Antioxidant Assay	Antioxidant Effect
DPPH	3.05 ± 0.13^{a}
DPPH Scavenging Activity	28.02 ± 1.19^{b}
CUPRAC	2.40 ± 0.10^{a}

[a] express antioxidant activity mM trolox equivalent per gram capsule and [b] express antioxidant activity as % scavenging acitivity.

3.7 Storage Stability of Red Beetroot Extract and Capsules in Kefir

In order to determine storage stability of red beetroot extract and capsules in kefir formulation, the kefir samples were stored at 4°C for 21 days in dark and the samples were collected and analyzed for TPC, TFC, betalain content and antioxidant activity in the 1st, 7th, 14th and 21st days. Four different kefir samples were prepared, these are kefir (K), extract added kefir (KE), capsule added kefir (KC) and empty capsule added kefir (KB). K and KB samples were used as control sample.

Table 3.8 shows the TPC for each kefir formulation in specified time of storage. The results show that there were no significant changes at the end of storage time in TPC of KE and KC samples ($p > 0.05$). However, TPC in KC samples are 1.5 fold more than TPC in KE samples on 7th, 14th and 21st day of storage ($p < 0.05$). Even though there are no significant changes in TPC of KE sample, there is a decline in TPC between 1st and 7th day of storage. When all results are taken into account, it can be said that beeswax encapsulation both providing higher TPC and protecting TPC in kefir during storage.

Table 3.8: Changes in TPC of different kefir samples during storage.

	TPC (mg GAE / 100 g kefir)			
	1	7	14	21
K	5.75 ± 1.50^{aA}	4.05 ± 0.34^{bC}	4.04 ± 0.42^{bD}	5.06 ± 0.21^{abBC}
KE	7.10 ± 1.94^{aA}	5.32 ± 0.84^{aBC}	5.73 ± 0.50^{aC}	5.87 ± 0.52^{aB}
KC	6.52 ± 0.27^{aA}	7.77 ± 1.50^{aA}	7.81 ± 0.15^{aA}	6.98 ± 0.59^{aA}
KB	4.71 ± 0.14^{bA}	6.65 ± 0.05^{aAB}	6.60 ± 0.05^{aB}	4.02 ± 0.08^{cC}

*K: Kefir sample, KE: Extract added kefir sample, KC: Capsule added kefir sample and KB: Empty capsule added kefir. The values are presented as mean values ± standard deviation. Different lower case letters in the the rows represent statistically significant differences and different upper case letters in the columns represent statistically significant differences ($p < 0.05$).

TFC of the various kefir formulations in different times are represented in Table 3.9. TFC in KC samples is significantly more than TFC in KE samples during whole storage period ($p < 0.05$). TFC in KC samples has maximum value in 7th and 14th day and TFC values in 7th day are significantly different from TFC in 21st day ($p < 0.05$). However, there was no significant difference in TFC of KC sample between 1st and

21^{st} day of storage. The same situation was also observed in the KB sample in 7^{th} and 14^{th} day of storage.

Table 3.9: Change in TFC of different kefir samples during storage.

	TFC (mg CE / 100 g kefir)			
	1	7	14	21
K	1.68 ± 0.44^{aC}	1.54 ± 0.55^{aC}	$0,82 \pm 0.04^{aC}$	1.02 ± 0.08^{aB}
KE	2.84 ± 0.24^{aB}	2.72 ± 0.82^{aBC}	2.33 ± 0.68^{aBC}	2.25 ± 0.53^{aB}
KC	3.92 ± 0.46^{abA}	5.40 ± 0.11^{aA}	4.80 ± 0.55^{abA}	3.56 ± 0.66^{bA}
KB	2.20 ± 0.14^{bBC}	3.21 ± 0.06^{aB}	3.18 ± 0.45^{aAB}	2.43 ± 0.47^{abAB}

* K: Kefir sample, KE: Extract added kefir sample, KC: Capsule added kefir sample and KB: Empty capsule added kefir. The values are presented as mean values ± standard deviation. Different lower case letters in the the rows represent statistically significant differences and different upper case letters in the columns represent statistically significant differences ($p < 0.05$).

In addition to TPC and TFC, betacyanin and betaxanthin contents were also controlled during storage time and the results are represented in Table 3.10 and Table 3.11. In the first day of storage, there are no significant difference in betacyanin content between KC and KE samples ($p > 0.05$). However, betacyanin content of KC sample is significantly higher than betacyanin content of KE samples until 14^{th} day ($p < 0.05$). The initial betacyanin content of KE sample is significantly more than betacyanin content in further days of storage ($p < 0.05$) and there are no significant difference in betacyanin content in 7^{th}, 14^{th} and 21^{st} day of storage ($p > 0.05$) so it shows that the initial betacyanin content in KE samples could not be protected during whole storage period. On the other hand, betacyanin content in KC samples remained stable during storage ($p > 0.05$).

Table 3.10: Change in betacyanin content of different kefir samples during storage.

	Betacyanin Content (mg BE/100 g kefir)			
	1	7	14	21
K	nd*	nd*	nd*	nd*
KE	0.46 ± 0.01^{aA}	0.31 ± 0.00^{bB}	0.30 ± 0.01^{bB}	0.30 ± 0.02^{bA}
KC	0.36 ± 0.03^{aA}	0.36 ± 0.00^{aA}	0.33 ± 0.01^{aA}	0.30 ± 0.01^{aA}
KB	nd*	nd*	nd*	nd*

* K: Kefir sample, KE: Extract added kefir sample, KC: Capsule added kefir sample and KB: Empty capsule added kefir. The values are presented as mean values ± standard deviation. Different lower case letters in the the rows and different upper case letters in the columns represent statistically significant differences ($p < 0.05$). nd*, not determined.

Apart from betacyanin content, Table 3.11 shows betaxanthin content in different samples during storage. According to the results, initial betaxanthin content in KE and KC sample is not significantly different from each other ($p > 0.05$) but betaxanthin content of KE sample is significantly less than initial value in 7th, 14th and 21st day of storage time ($p < 0.05$). In KC sample, betaxanthin content can be protected during storage period ($p > 0.05$) and its betaxanthin content approximately 2 times grater than betaxanthin content of KE sample. According to the stability results of betacyanin and betaxanthin, beeswax encapsulation has also protective effect on betalain stability.

Table 3.11: Change in betaxanthin content of different kefir samples during storage.

	Betaxanthin Content (mg VE/100 g kefir)			
	1	7	14	21
K	nd*	nd*	nd*	nd*
KE	0.38 ± 0.01^{aA}	0.27 ± 0.02^{bB}	0.24 ± 0.05^{bB}	0.23 ± 0.02^{bB}
KC	0.41 ± 0.03^{aA}	0.41 ± 0.00^{aA}	0.40 ± 0.01^{aA}	0.40 ± 0.01^{aA}
KB	nd*	nd*	nd*	nd*

* K: Kefir sample, KE: Extract added kefir sample, KC: Capsule added kefir sample and KB: Empty capsule added kefir. The values are presented as mean values ± standard deviation. Different lower case letters in the the rows and different upper case letters in the columns represent statistically significant differences ($p < 0.05$). nd*, not determined.

The changing in antioxidant activity was also controlled during storage time and the results are shown in Table 3.12 and Table 3.13. In order to detect antioxidant capacity, DPPH and CUPRAC analysis were conducted in a specified time interval. According to the DPPH results, KE and KC samples have significantly different antioxidant activity ($p < 0.05$) in 1st and 7th day of storage. Although, KC sample has more antioxidant activity than KE sample during storage, there are no significant difference between them 14th and 21st day of storage ($p > 0.05$). There are no significant differences in antioxidant activity of all samples during storage time ($p > 0.05$).

Table 3.12: Antioxidant activity of different kefir samples in terms of DPPH assay during storage.

	DPPH (mM TE / 100 g kefir)			
	1	7	14	21
K	$1.07 \pm 0,19^{aC}$	1.08 ± 0.19^{aD}	0.72 ± 0.20^{aB}	0.84 ± 0.27^{aB}
KE	6.68 ± 0.60^{aB}	5.75 ± 0.41^{aB}	6.20 ± 0.45^{aA}	6.76 ± 0.15^{aA}
KC	8.63 ± 0.33^{aA}	8.85 ± 0.51^{aA}	8.27 ± 1.94^{aA}	8.57 ± 1.72^{aA}
KB	2.07 ± 0.19^{aC}	2.03 ± 0.21^{aC}	1.84 ± 0.30^{aB}	2.02 ± 0.46^{aB}

* K: Kefir sample, KE: Extract added kefir sample, KC: Capsule added kefir sample and KB: Empty capsule added kefir. The values are presented as mean values ± standard deviation. Different lower case letters in the the rows and different upper case letters in the columns represent statistically significant differences ($p < 0.05$). nd*, not determined.

In addition to DPPH analysis, the results of CUPRAC analysis are shown in Table 3.13. According to the results, initial antioxidant value of KE and KC samples are not significantly different from each other ($p > 0.05$) but their antioxidant activity are significantly different from two control samples (K and KB) ($p < 0.05$). Antioxidant activity in KC sample could be remained stable during 21 days ($p > 0.05$). However, there is a significant decline in antioxidant value of KE sample between 1st and 7th day of storage. According to Mehanna et al. (2014), phenolic and milk proteins interactions may lead to decreasing in antioxidant activity by forming complex structure. The study reported that gallic acid, catechin, chlorogenic, rosmarinic acid, quercetin, caffeic acid and vanillin can form complex structure with casein and whey protein and this complex structure reduces antioxidant activity (Mehanna et al., 2014). The protein – phenolic complex can be formed by both of non – covalent forces (hydrogen bond, Van der Waals forces, hydrophobic binding) and covalent bonds (covalent linkages and cross-linking) (Özdal et al., 2013). Although there are no significant difference in antioxidant activity of KE and KC sample in 1st and 21st day, antioxidant activity of KE sample is greater than KC sample. This result is accordance with antioxidant activity of extract and capsules because capsules show significantly more antioxidant capacity in terms of DPPH assay and show less antioxidant activity in terms of CUPRAC assay than crude extract ($p < 0.05$).

Table 3.13: Antioxidant activity of different kefir samples in terms of CUPRAC assay during storage.

	CUPRAC (mM TE / 100 g kefir)			
	1	7	14	21
K	9.08 ± 0.97^{aB}	6.10 ± 0.23^{bD}	6.31 ± 0.16^{bD}	3.84 ± 0.50^{cC}
KE	17.64 ± 0.66^{aA}	15.56 ± 0.66^{bA}	16.29 ± 0.31^{abA}	16.20 ± 0.26^{abA}
KC	14.76 ± 2.36^{aA}	13.52 ± 0.59^{aB}	15.10 ± 0.23^{aB}	15.02 ± 0.60^{aA}
KB	10.57 ± 0.23^{aB}	10.69 ± 0.11^{aC}	9.66 ± 0.33^{bC}	6.79 ± 0.10^{cB}

* K: Kefir sample, KE: Extract added kefir sample, KC: Capsule added kefir sample and KB: Empty capsule added kefir. The values are presented as mean values ± standard deviation. Different lower case letters in the rows represent statistically significant differences and different upper case letters in the columns represent statistically significant differences ($p < 0.05$).

Besides TPC, TFC and betalain content; phenolic and betacyanin profile of all kefir samples were also analysed by HPLC. The phenolic profiles of the samples are represented in Table 3.14.

Table 3.14: Phenolic profile of the different kefir samples during storage.

Day	1				7				14				21			
Compounds	K	KE	KC	KB	K	KE	KC	KB	K	KE	KC	KB	K	KE	KC	KB
Gallic Acid	0.00	0.174 ± 0.01^{aA}	0.152 ± 0.04^{bA}	0.00	0.00	0.200 ± 0.02^{aA}	0.216 ± 0.041^{aA}	0.00	0.00	0.219 ± 0.02^{aA}	0.181 ± 0.04^{aA}	0.00	0.00	0.205 ± 0.02^{aA}	0.120 ± 0.04^{bA}	0.00
Catechin	0.00	0.060 ± 0.00^{bB}	0.487 ± 0.02^{aB}	0.00	0.00	0.219 ± 0.06^{bA}	0.470 ± 0.01^{aB}	0.00	0.00	0.227 ± 0.04^{bA}	0.468 ± 0.01^{aB}	0.00	0.00	0.288 ± 0.043^{bA}	1.176 ± 0.24^{aA}	0.00
4 (p-) Hydroxybenxoic Acid	0.00	0.00	< 0.01	0.00	0.00	0.00	< 0.01	0.00	0.00	0.00	< 0.01	0.00	0.00	0.00	< 0.01	0.00
Vanilic Acid	0.00	< 0.01	< 0.01	0.00	0.00	0.00	< 0.01	0.00	0.00	0.00	< 0.01	0.00	0.00	0.00	< 0.01	0.00
Syringic Acid	0.00	0.020 ± 0.00^{c}	0.084 ± 0.004^{aA}	0.062 ± 0.00^{b}	0.00	0.00	0.012 ± 0.01^{B}	0.00	0.00	0.00	< 0.01	0.00	0.00	0.00	0.093 ± 0.01^{A}	0.00
Chlorogenic Acid	0.00	0.023 ± 0.00^{bA}	0.040 ± 0.00^{aA}	0.00	0.00	0.023 ± 0.00^{bA}	0.056 ± 0.02^{aA}	0.00	0.00	0.00	0.027 ± 0.018^{A}	0.00	0.00	0.00	0.060 ± 0.015^{A}	0.00
Caffeic Acid	0.00	< 0.01	< 0.01	0.00	0.00	< 0.01	< 0.01	<0.01	0.00	< 0.01	< 0.01	<0.01	0.00	< 0.01	< 0.01	0.00
Ferulic Acid	0.00	0.00	< 0.01	< 0.01	0.00	0.00	< 0.01	0.00	0.00	0.00	< 0.01	0.00	0.00	0.00	< 0.01	0.00
Rosmarinic Acid	0.00	0.00	0.089 ± 0.01^{aA}	0.077 ± 0.02^{a}	0.00	0.00	0.077 ± 0.00^{A}	0.00	0.00	0.00	0.085 ± 0.01^{A}	0.00	0.00	0.00	0.086 ± 0,00^{A}	0.00
p-Coumaric Acid	0.00	< 0.01	< 0.01	< 0.01	0.00	< 0.01	0.00	<0.01	0.00	< 0.01	< 0.01	<0.01	0.00	< 0.01	< 0.01	< 0.01

[*] K: Kefir, KE: Extract added kefir, KC: Capsule added kefir and KB: Empty capsule added kefir. The results are given as mean ± standard deviation and they are expressed in mg/100 g kefir. Different lower case letters in rows show significant difference between samples and diffrenent upper case letters in rows show significant difference between days ($p < 0.05$).

In first day, KE and KC has nearly same phenolic profile. While KE sample include gallic acid, catechin, vanilic acid, syringic acid, chlorogenic acid, caffeic acid and p-coumaric acid; KC sample includes hydroxybenzoic acid, ferulic acid and rosmarinic acid additionally. The predominant phenolic compounds in KE and KC sample were identified as gallic acid and catechin in the first day and the contents of gallic acid, catechin, syringic acid and chlorogenic acid in KE and KC samples are significantly different from each other ($p < 0.05$). While gallic acid content is higher in KE sample, content of the other phenolic compounds are higher in KC sample in 1st day.

When phenolic profile of the KE sample is examined, it can be seen that phenolic profile has been changed during storage period. Although gallic acid and catechin are predominant phenolic compounds during storage; syringic, vanilic acid chlorogenic acid could not be detected after 1st and 7th day of storage. The reasons of absence syringic, vanilic acid and chlorogenic acid may be that biodegradation of phenolic compounds by lactic acid bacteria which found in kefir. Rodriguez et al. (2009) reported that, chlorogenic acid can be degraded by lactobacilli bacteria. Besides microbial action, the phenolic compounds may be also affected from phenolic – milk protein interactions because forming protein – phenolic complex have an effect on phenolic content (Özdal et al., 2013). When phenolic profile of the KC sample is examined, it can be seen that almost all individual phenolic compounds are avaible in kefir at the end of 21st day of storage. Gallic acid and catechin are also detected as predominant phenolic compounds in KC sample. The significant difference in the content of them was not observed during storage time ($p > 0.05$). Besides gallic acid and catechin, there are also no significant differences in the content of syringic, rosmarinic and cholorogenic acid between 1st and 21st day of storage. The phenolic profile of the KC samples also includes hydroxybenzoic acid, vanillic acid, caffeic acid, ferulic acid and p-coumaric acid in trace level during storage period.

In addition to phenolic profile of the samples, betacyanin profile was also determined during 21 days. The obtained results are represented in Table 3.15. The betanin content of KE sample decreased significantly from 1st day to 21st day of storage ($p < 0.05$). The betanin degradation may be affected from high water activity (0.875 ± 0.028) in kefir (Setyawardani & Sumarmono, 2015; Herbach et al., 2006). High water activity triggers hydrolytic reactions and leads to cleavage of aldimine bond in betalain structure (Herbach et al., 2006). Although initial betanin content of KE sample is more than KC sample, betanins could not be protected from degradation. On the other hand, no significant change in the betanin content of the KC sample has been observed at the end of 21st day ($p > 0.05$). When the results of isobetanin content change during storage are examined, it is seen that the degradation rate is more than betanin degradation for KE sample. More than 50% of initial isobetanin content of KE was degraded in 7 days whereas isobetanin content in KC sample could remain more stable until 14th day ($p > 0.05$). In that point, it can be commented that the beeswax encapsulation of red beetroot extract could protect both of the betacyanins and phenolics in kefir during storage.

When all stability results are taken into account, it can be said that beeswax encapsulation has positive effect on both of preserving betalains and phenolics in kefir samples. The antioxidant activity could be protected by this effect. Nevertheless, some further analysis may be proposed for detecting betalain preservation by beeswax encapsulation against light, temperature and oxygen.

Table 3.15: Betacyanin profile of different kefir samples during storage time.

	Betanin	Isobetanin
	Day 1	
KE	0.426 ± 0.022^{aA}	0.073 ± 0.002^{aA}
KC	0.305 ± 0.00^{bD}	0.072 ± 0.00^{aD}
	Day 7	
KE	0.312 ± 0.004^{aB}	0.031 ± 0.007^{bB}
KC	0.267 ± 0.02^{bD}	0.066 ± 0.001^{aDE}
	Day 14	
KE	0.299 ± 0.031^{aB}	0.033 ± 0.005^{bB}
KC	0.248 ± 0.05^{aD}	0.059 ± 0.006^{aDF}
	Day 21	
KE	0.192 ± 0.021^{aC}	0.024 ± 0.005^{bB}
KC	0.237 ± 0.03^{aD}	0.052 ± 0.00^{aF}

*KE: Extract added kefir and KC: Capsule added kefir. The results are given as mean value ± standard deviation and they are expressed as mg / 100 g kefir. Small letters in row shows significant difference and capital letters in columns shows significant difference ($p < 0.05$).

3.8 Color Measurements

Color measurements were conducted and the results are shown in Table 3.16, Table 3.17 and Table 3.18. L^* values indicates the lightness and it is in a range of 0 – 100 (black to colorless), a^* values shows green ($a^* < 0$) / red ($a^* > 0$) color, b^* represents blue ($b^* < 0$) / yellow ($b^* > 0$) color, C^* indicates chroma and h° is hue angle and shows the color. The color is determined as follows (Carmo et al., 2015);

- 0° or 360° = Red

- 90° = yellow

- 180° = green

- 270°= blue

In Table 3.16, the color values of crude extract and the encapsulated extract are represented. According to the results, crude extract has darker red color than the capsules. In a study conducted by Chethana et al. (2007), the color of crude extract was determined as L^*= 7.60, a^* = 25.21 and b^* = 3.76. When the results are compared with each other, it is observed that color of the extract are lighter, redder and more yellow than the literature color but they are in accordance in general.

Table 3.16: Color values of red beetroot extract and prepared encapsulates.

Color Paramaters	Red Beetroot Extract	Dried Capsules
L^*	31.30 ± 1.97	68.93 ± 0.56
a^*	33.13 ± 1.08	17.75 ± 0.57
b^*	8.11 ± 0.38	16.11 ± 0.18
C^*	34.10 ± 0.34	23.97 ± 0.19
h°	13.76 ± 0.59	42.23 ± 0.65

In addition to determine color of the extract and the capsules, color measurements were also conducted for kefir samples during storage period. The obtained results are represented in Table 3.18. When the color change of KE sample is examined, there are no significant changes in lightness, redness, chroma and hue angle but the yellowness increased significantly at the end of 21st day. The same situation was also seen in kefir sample so increasing yellowness in KE sample may be arised from kefir not red beetroot extract. In KC sample, there are significant increase in lightness, redness, yellowness and chroma ($p < 0.05$). Besides that, a decline has been seen in hue angle but it is not statistically significant change ($p > 0.05$). Therefore, it can be seen that redder KC sample was obtained at the end of storage time. The reason of it may be releasing red beetroot extract from beeswax capsules. The real colors are shown in Table 3.17.

Table 3.17: Colors of different kefir formulation during storage period.

Day / Sample	1	7	14	21
K				
KE				
KC				
KB				

*K: Kefir, KE: Extract added kefir, KC: Capsule added kefir and KB: Empty capsule added kefir.

Table 3.18: Changes in color values (L^*, a^* and b^*) of kefir samples during storage.

	1					7					14					21				
	L^*	a^*	b^*	C^*	h°	L^*	a^*	b^*	C^*	h°	L^*	a^*	b^*	C^*	h°	L^*	a^*	b^*	C^*	h°
K	86.64 ± 0.45^{a}	-2.22 ± 0.09^{b}	1.81 ± 0.22^{b}	2.87 ± 0.21^{ab}	140.92 ± 2.48^{a}	81.76 ± 0.2^{b}	-1.61 ± 0.04^{a}	1.51 ± 0.07^{b}	2.07 ± 0.24^{b}	132.41 ± 7.72^{ab}	87.51 ± 0.38^{a}	-2.76 ± 0.16^{c}	3.78 ± 0.46^{a}	4.18 ± 0.50^{a}	118.94 ± 4.11^{b}	87.43 ± 0.50^{a}	-2.58 ± 0.19^{c}	3.37 ± 0.47^{a}	3.68 ± 0.88^{a}	128.28 ± 5.95^{ab}
KE	58.95 ± 0.05^{b}	31.50 ± 0.09^{ab}	6.3 ± 0.02^{b}	32.12 ± 0.10^{a}	11.31 ± 0.01^{a}	58.08 ± 0.06^{c}	30.15 ± 0.04^{c}	5.44 ± 0.04^{d}	30.47 ± 0.29^{b}	9.97 ± 0.45^{b}	60.00 ± 0.52^{a}	30.56 ± 0.70^{cb}	5.71 ± 0.12^{c}	30.74 ± 0.57^{b}	9.36 ± 0.17^{b}	59.14 ± 0.12^{b}	31.70 ± 0.17^{a}	6.48 ± 0.02^{a}	32.09 ± 0.47^{a}	11.35 ± 0.35^{a}
KC	79.07 ± 0.17^{b}	6.28 ± 0.08^{b}	9.49 ± 0.02^{bc}	11.37 ± 0.05^{b}	56.49 ± 0.31^{a}	79.38 ± 0.23^{b}	6.41 ± 0.07^{b}	9.05 ± 0.15^{c}	11.09 ± 0.16^{b}	54.69 ± 0.15^{a}	80.60 ± 0.10^{a}	7.81 ± 0.28^{ab}	10.08 ± 0.18^{ab}	12.75 ± 0.31^{a}	52.24 ± 0.53^{a}	80.34 ± 0.41^{a}	8.17 ± 0.11^{a}	10.26 ± 0.47^{a}	12.99 ± 0.15^{a}	50.83 ± 6.58^{a}
KB	87.58 ± 0.62^{b}	-5.25 ± 0.23^{a}	12.17 ± 1.13^{b}	13.26 ± 1.12^{b}	113.40 ± 1.07^{a}	89.38 ± 0.51^{a}	-5.68 ± 0.19^{b}	16.9 ± 0.86^{a}	17.83 ± 0.88^{a}	108.59 ± 0.31^{b}	89.28 ± 0.34^{a}	-5.56 ± 0.02^{ab}	15.66 ± 0.51^{a}	16.62 ± 0.48^{a}	109.56 ± 0.53^{b}	89.49 ± 0.26^{a}	-5.69 ± 0.05^{b}	17.11 ± 0.57^{a}	18.03 ± 0.53^{a}	108.41 ± 0.71^{b}

[*]K: Kefir, KE: Extract added kefir, KC: Capsule added kefir and KB: Empty capsule added kefir.

4. CONCLUSION AND RECOMMENDATION

In the present study, red beetroot extract was encapsulated with beeswax in order to improve its stability during storage time of the food product. The red beetroot extract has high antioxidant activity due to its phenolic and betalain content but its stability is affected from temperature, oxygen, light, enzyme activity, metal ions and water activity so it has poor stability in normal form. Therefore, encapsulation is convenient method to improve stability of red beetroot extract because of the protective feature it provides. In the study, the beeswax capsules were prepared at different core / wall ratios which are 1:1, 1:2, 1:3 and 2:3 w/w in order to determine highest encapsulation efficiency. Apart from encapsulation efficiency, particle size of the capsules was also determined. According to the results, particle size of the capsules are in a range of 265.9 – 414.3 nm and it was observed that different wall / core ratio has an significant effect on particle size of the capsules ($p < 0.05$). Besides particle size, encapsulation efficiency was also affected from various core / wall ratio and each groups of capsules have significantly different encapsulation efficiency ($p < 0.05$). The highest encapsulation efficiency that is 67.16% was observed in 1:2 ratio and the lowest encapsulation efficiency that is 42.18% was belonged to 2:3 ratio. Therefore, the further studies were conducted by using the capsules that are prepared in 1:2 ratio.

Before inclusion the capsules and the extract into kefir formulation, the contents of the capsules and the contents of extract were determined by spectrophotometric and chromatographic methods. The content of the capsules was detected in that TPC is 101.68 ± 14.75 mg GAE / 100 g capsule, TFC is 103.12 ± 7.38 mg CE / 100 g sample and total betalain is 362.98 ± 1.41 mg / 100 g capsule. The total betalain content is comprised from betacyanins and betaxanthins parts. The content of betacyanins is 26.95 ± 1.06 mg BE / 100 g capsule and the content of betaxanthins is 36.03 ± 0.35 mg VE / 100 g capsule. The content of betacyanins and betaxanthins are significantly different from each other ($p < 0.05$). In addition to spectrophotometric method, phenolic and betacyanin profile were also detected by chromatographic method. The results showed that the capsules include hydroxybenzoic acid, vanillic acid, epigallocatechingallate, vanillin, caffeic acid, coumaric acid, ferulic acid, gallic acid, catechin, epicatechin, syringic and cholorogenic acid as phenolic compounds. Besides that, betanin and isobetanin content were detected as 29.789 ± 2.545 mg / 100 g capsules and 3.628 ± 0.336 mg / 100 g capsule respectively. The content of the extract was detected in that TPC is 2448.38 ± 179.84 mg GAE / 100 g extract, TFC is 4001.45 ± 71.91 mg CE / 100 g extract, betacyanin and betaxanthin content are 453.99 ± 4.12 mg BE / 100 g extract and 360.08 ± 2.44 mg VE / 100 extract. The extract includes ferulic acid, hydroxybenzoic acid, vanillic acid, ethyl 3,4 dihydroxybenzoate, gallic acid, catechin, epigallocatechingallate, syringic acid, vanillin, hesperedin, caffeic acid, chlorogenic acid, p-coumaic acid, sinapic acid, rosmarinic acid, luteolin, quercetin, rutin, myricetin and epicatechin as phenolic compounds. In addition to these, the betanin and isobetanin content were also determined as 574.5 ± 24.33 mg / 100 g extract and 38.50 ± 0.64 mg / 100 g extract respectively. After determined the contents of the capsules and extract, antioxidant activity was also detected by DPPH and CUPRAC methods. The radical scavenging activity was determined as 28.02 ± 1.19% in capsules and 38.90 ± 0.66% in extract. Antioxidant activity was determined as 3.05 ± 0.13 mM TE/g capsule and 42.29 ± 0.72 mM TE/g extract in terms of DPPH. Antioxidant activity was detected as 2.40 ± 0.10 mM TE/g capsule and 59.72 ± 1.12 mg/g extract in terms of CUPRAC. While tha capsules shows more antioxidant activity in DPPH assay than CUPRAC assay, the extract shows more antioxidant activity in CUPRAC assay than DPPH assay.

Finally, the capsules and the extract were added to kefir formulation at a ratio of 1:10 w/w to observe the effect of encapsulation process on stability of red beetroot extract in kefir. In order to detect storage stability, 4 different kefir formulations were prepared, these are kefir (K), crude extract added kefir (KE), capsule added kefir (KC) and empty capsule added kefir (KB). The samples were collected in 1st, 7th, 14th and 21st day of storage time. Phenolic content, flavonoid content, betalain content, phenolic profile, betacyanin profile and antioxidant acitivity were controlled during storage.

The results of TPC stability show that the significant difference was not be observed in both of KE and KC sample during storage ($p > 0.05$) but TPC of KC sample are significantly more than TPC of KC sample in 7th, 14th and 21st day of storage although the initial content of them is similar. In that point, it can be commented that beeswax encapsulated extract have more TPC and it can be protected during whole storage time. When the results of TFC stability are examined, it can be seen that the same situation which occur in TPC stability is also valid for TFC in KE and KC. While there are no significant difference in TFC of KE and KC sample during storage period ($p > 0.05$), TFC in KC sample is significantly more than TFC in KE sample suring storage ($p < 0.05$). In addition to TPC and TFC, betacyanin and betaxanthin contents were also analysed in 1st, 7th, 14th and 21st day of storage and the results indicate that betacyanins and betaxanthins in KC sample remained stable during storage period but the betalain content in KE sample was significantly different between 1st and the other days of storage ($p < 0.05$). Therefore, it can be said that the initial betacyanin and betaxanthin content could not be protected in KE sample and the reason of it may be high water activity in kefir because it leads to hydrolysis of the pigments.

Antioxidant activity was also determined during storage pediod by DPPH and CUPRAC assays. According to DPPH results, KC sample has more antioxidant activity than KE sample, the difference is significant in 1st and 7th day of storage ($p < 0.05$) but insignificant in 14th and 21st of storage ($p > 0.05$). However, there are no significant differences in antioxidant activity of KE and KC samples during storage ($p > 0.05$). When results of CUPRAC is examined, KE and KC samples have similar antioxidant value in 1st day of storage but the antioxidant activity in KE sample has a decline in 7th day of storage ($p < 0.05$) and antioxidant activity in KC sample did not show any significant change during storage. The decline in antioxidant acitivity of KE sample may be arised from phenolic – milk protein interactions because the phenolic – protein complexes has a negative effect on antioxidant effect.

Additionally, phenolic and betacyanin profile of the samples were detected by HLPC. The results show that predominant phenolic compounds are gallic acid and catechin in both samples and they could remain stable during storage period. However, the other phenolic compounds which presence in KE sample vanished during storage time. On the other hand, phenolic profile could be protected in KC sample during storage period and there are no significant differences in content of individual phenolic content ($p > 0.05$). In addition to phenolic profile, betacyanin profile was also detected and the results are accordance with betacyanin results. The initial betanin and isobetanin content in KE sample could not remain stable in 7th day of storage but the initial betanin and isobetanin could be protected and remain stable during storage period ($p > 0.05$).

Finally, color measurements were conducted for crude extract, capsules and kefir samples. The color values (lightness, redness and yellowness) of crude extract are compatible with literature value. The results of color stability in kefir samples show that there are no significant differences in color of KE sample during storage ($p > 0.05$) but there are significant increases in lightness, redness and yellowness value in KC sample at the end of the storage time ($p < 0.05$). As a result, redder color was observed in KC sample and it may be originated from extract releasing from beeswax during storage.

Consequently, beeswax encapsulated red beetroot extract could protect phenolic compounds and betalains during storage of kefir. TPC and TFC were also did not change in KE sample but KC samples have more TPC and TFC than KE sample during storage. The main effect of beeswax encapsulation were seen in betanin and individual phenolic compounds protection because both of betanin / isobetanin and individual phenolic compounds were degraded in KE sample but this effect have not been seen in KC sample during storage time. Therefore, it can be commented that beeswax encapsulation may be effective technique for preserving betalains and phenolics in food products and further different studies are also needed to see detailed effect of beeswax encapsulation.

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APPENDICES

APPENDIX A: Calibration graphics.

APPENDIX B: HPLC chromatograms.

APPENDIX A

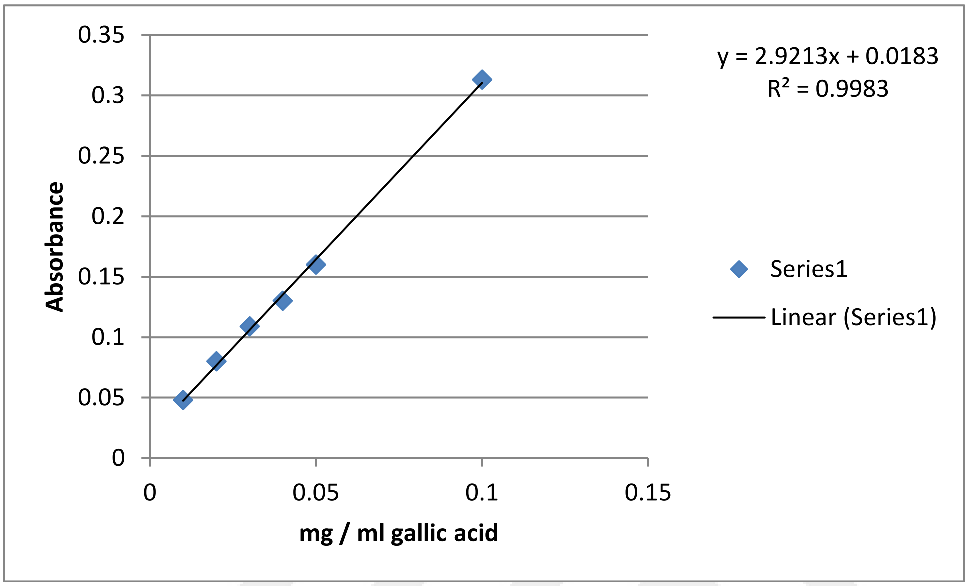

Figure A.1: Standard calibration curve for total phenolic analysis.

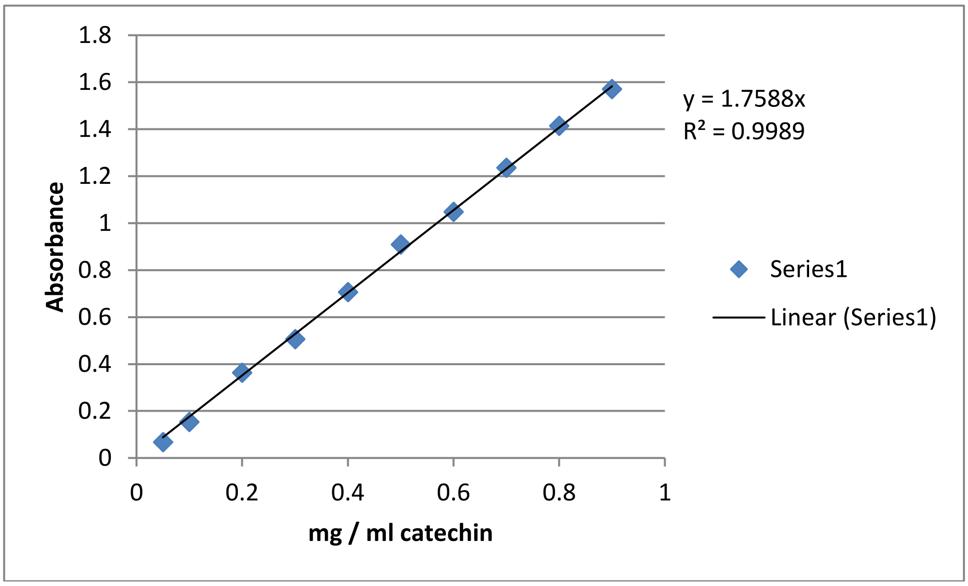

Figure A.2: Standard calibration curve for total flavonoid analysis.

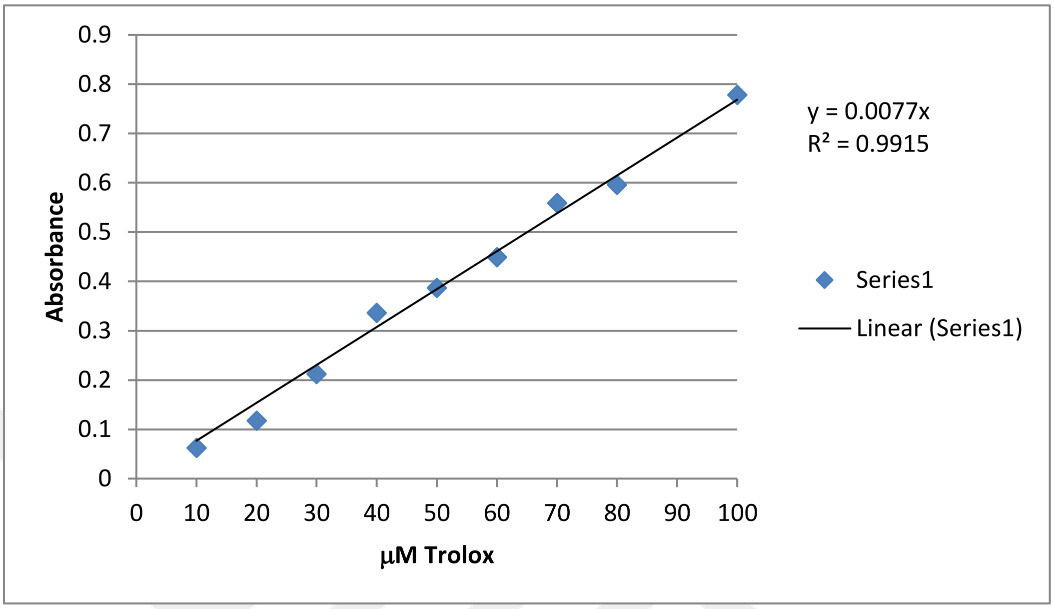

Figure A.3: Standard calibration curve for CUPRAC analysis.

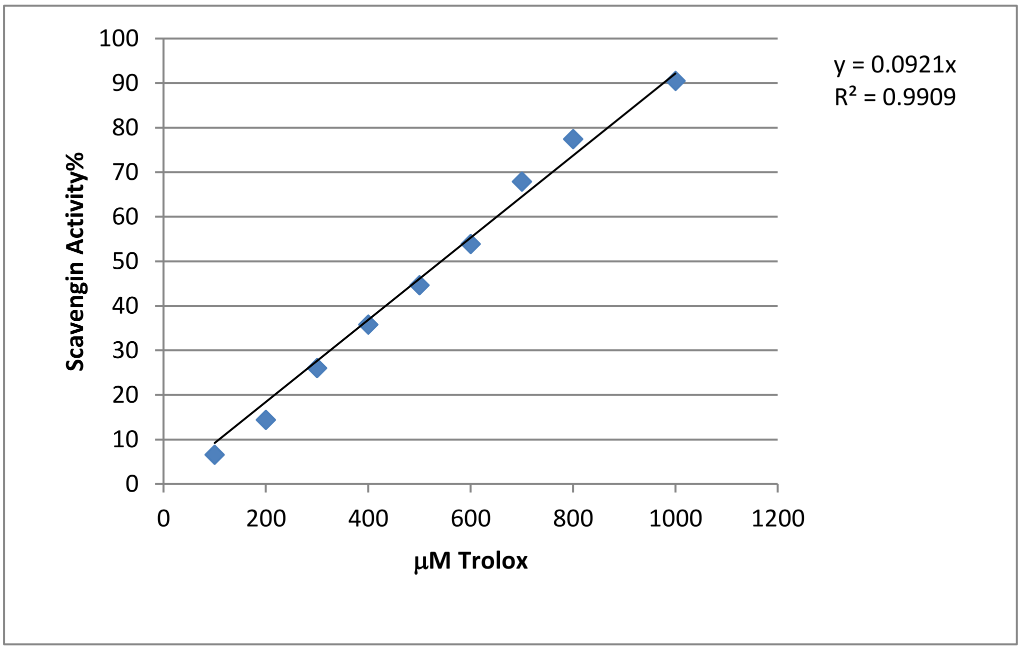

Figure A.4: Standard calibration curve for DPPH analysis.

APPENDIX B

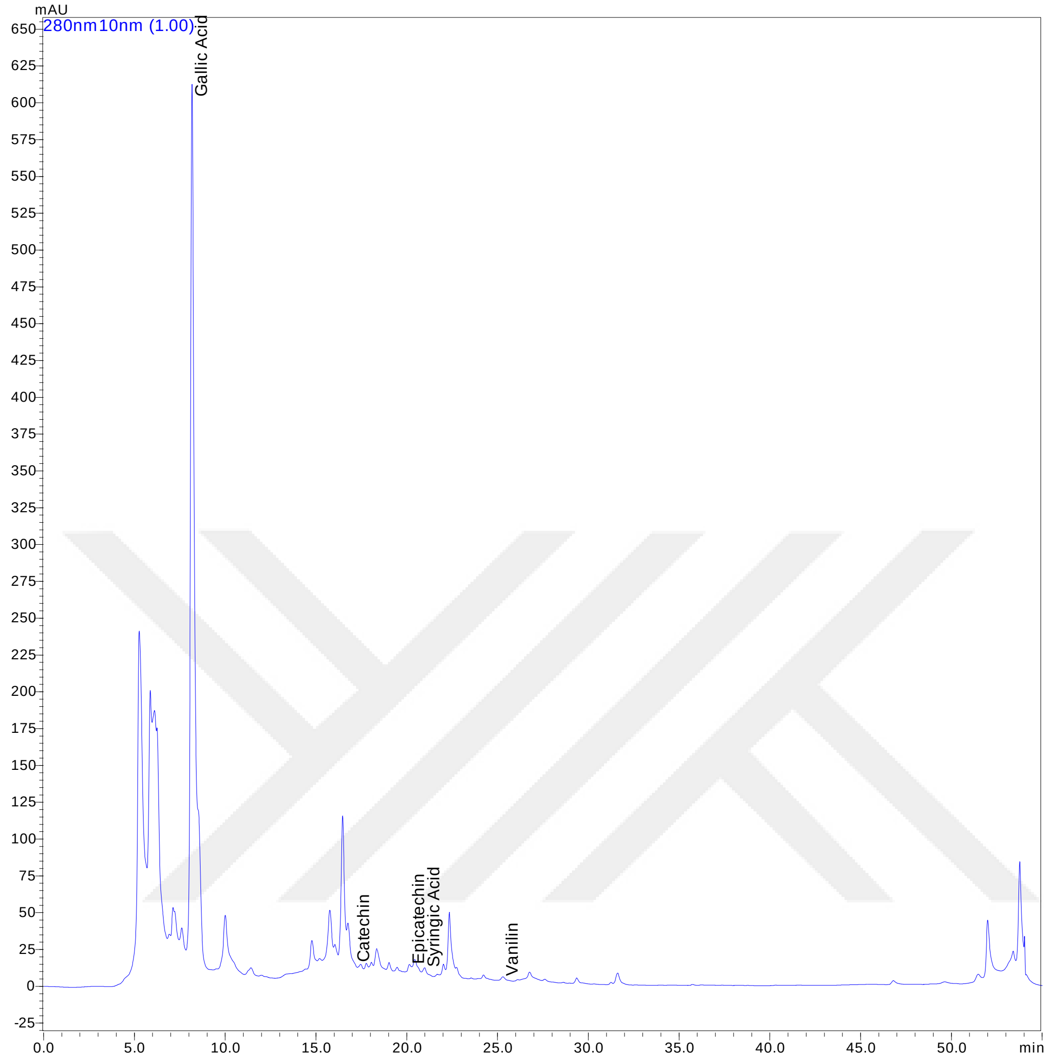

Figure B.1: HPLC chromatogram of the red beetroot extract.

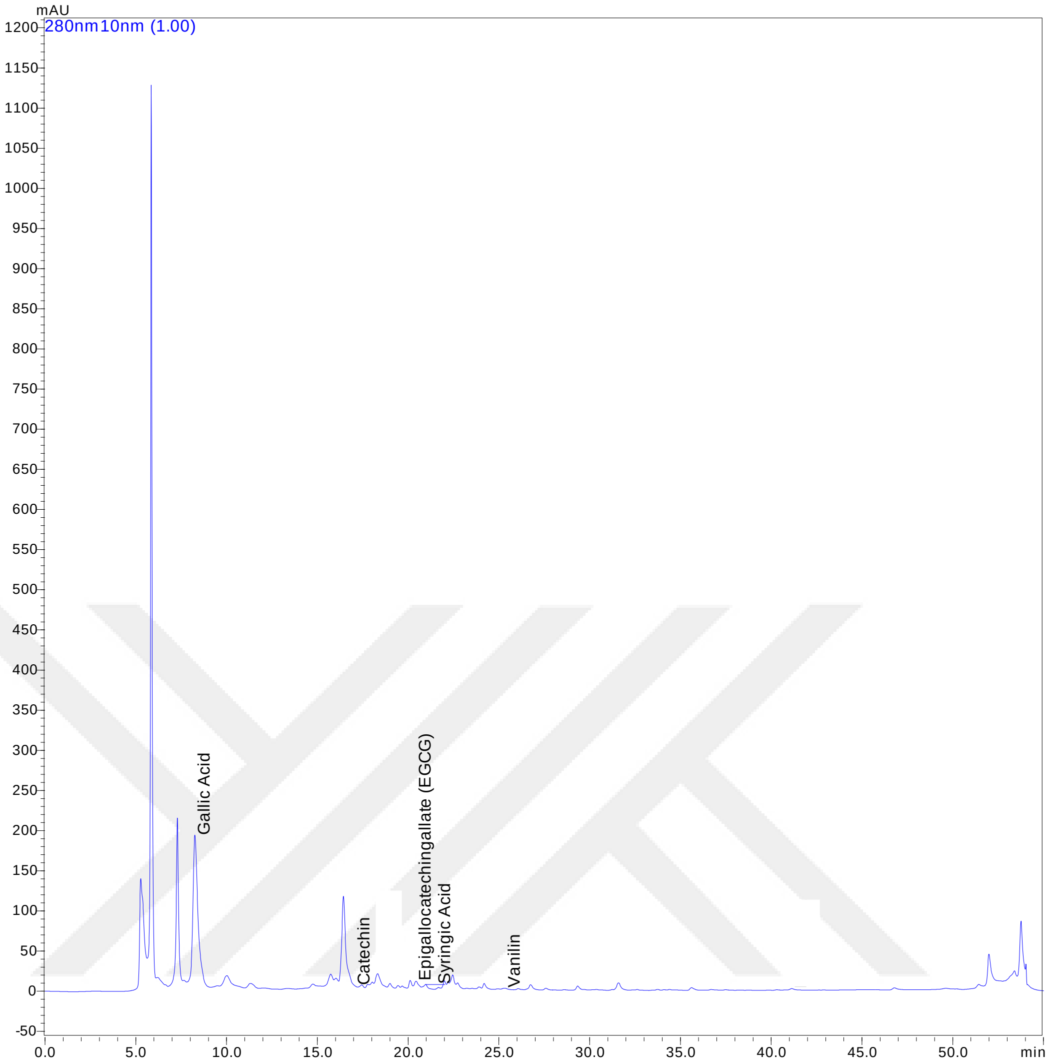

Figure B.2: HPLC chromatogram of the capsules.

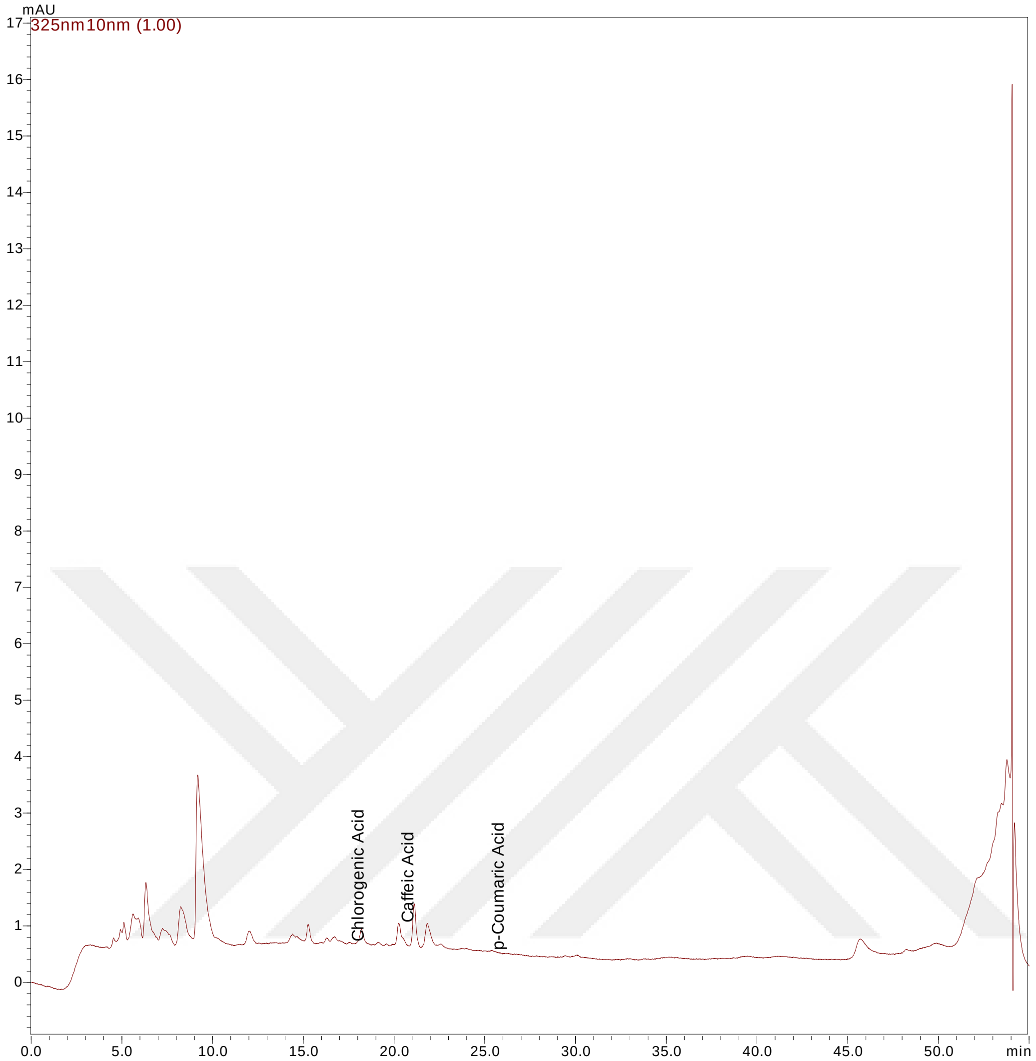

Figure B.3: HPLC chromatogram of the extract added kefir sample in 1st day.

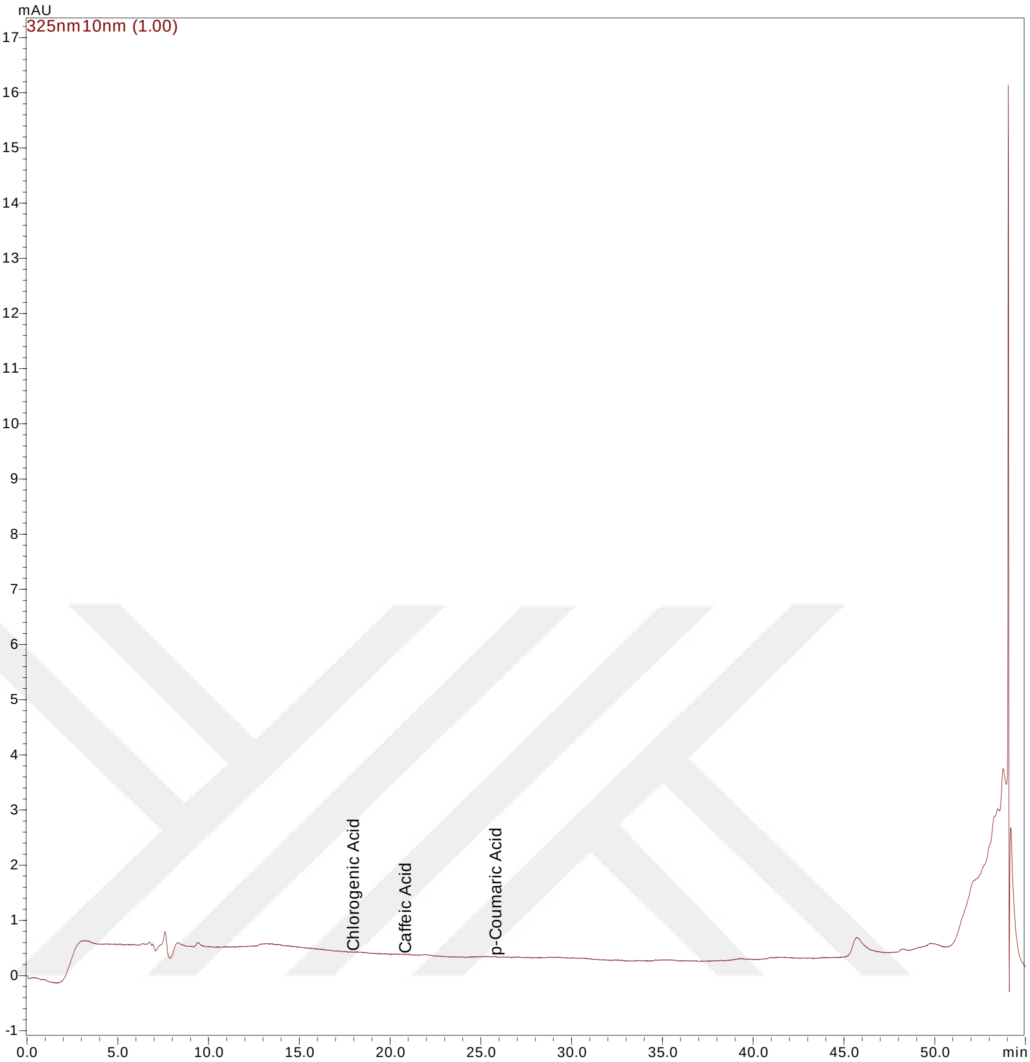

Figure B.4: HPLC chromatogram of the extract added kefir sample in 7th day.

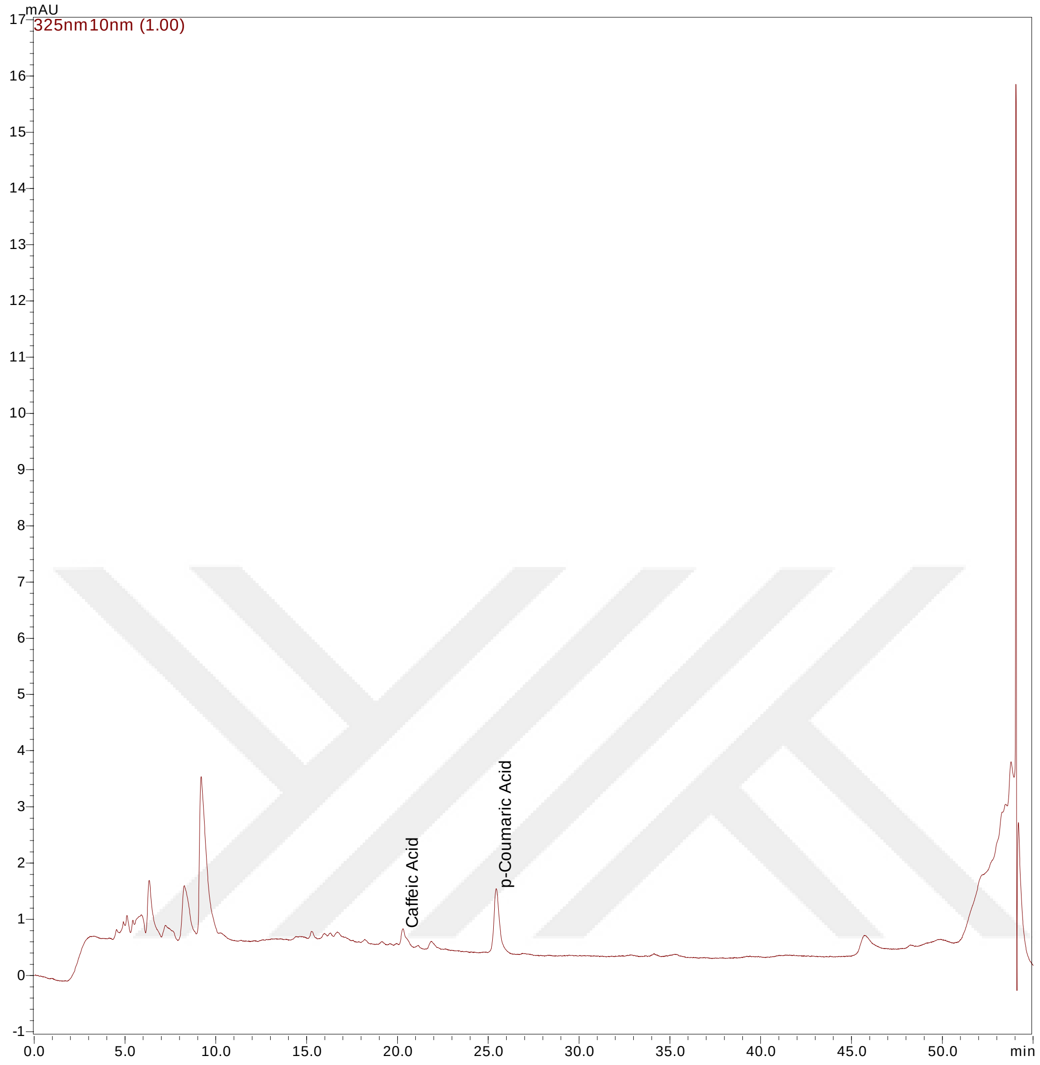

Figure B.5: HPLC chromatogram of the extract added kefir sample in 14th day.

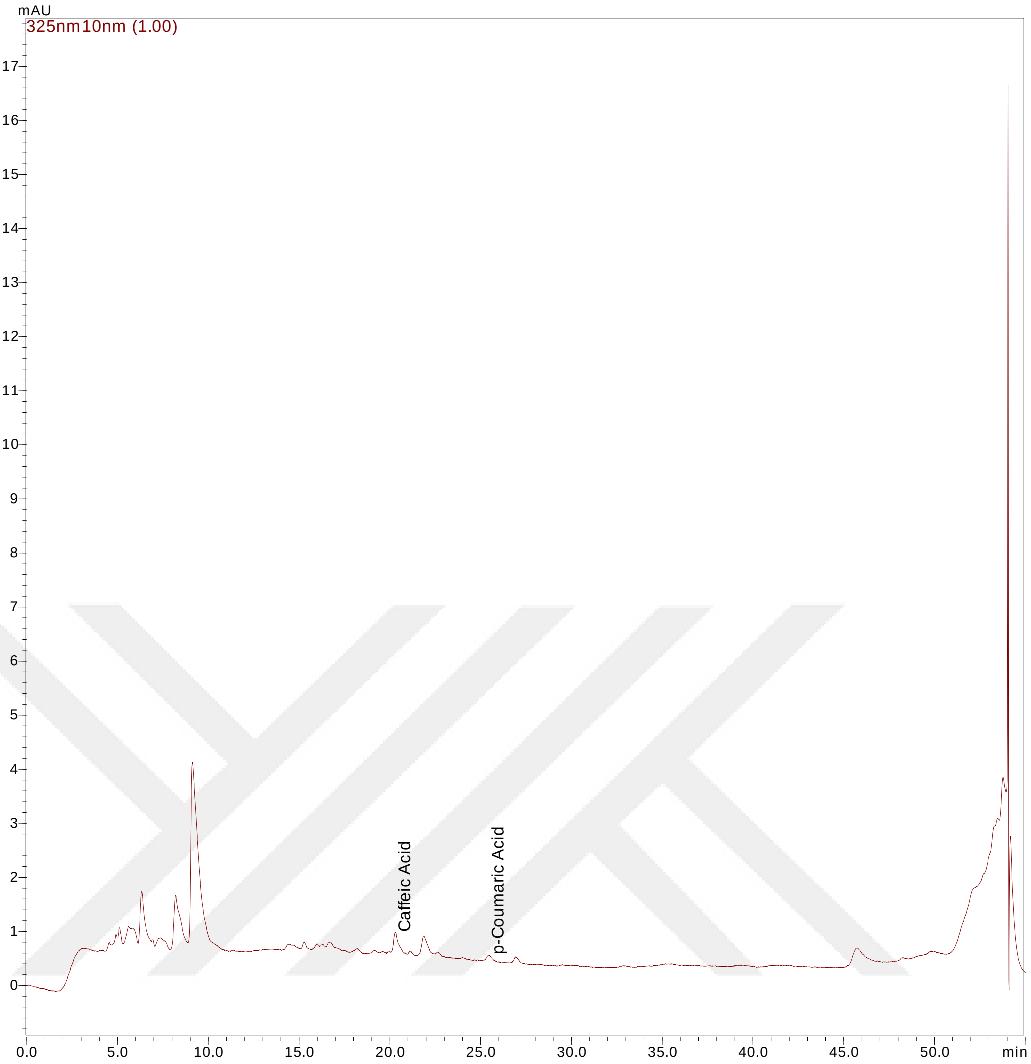

Figure B.6: HPLC chromatogram of the extract added kefir sample in 21st day.

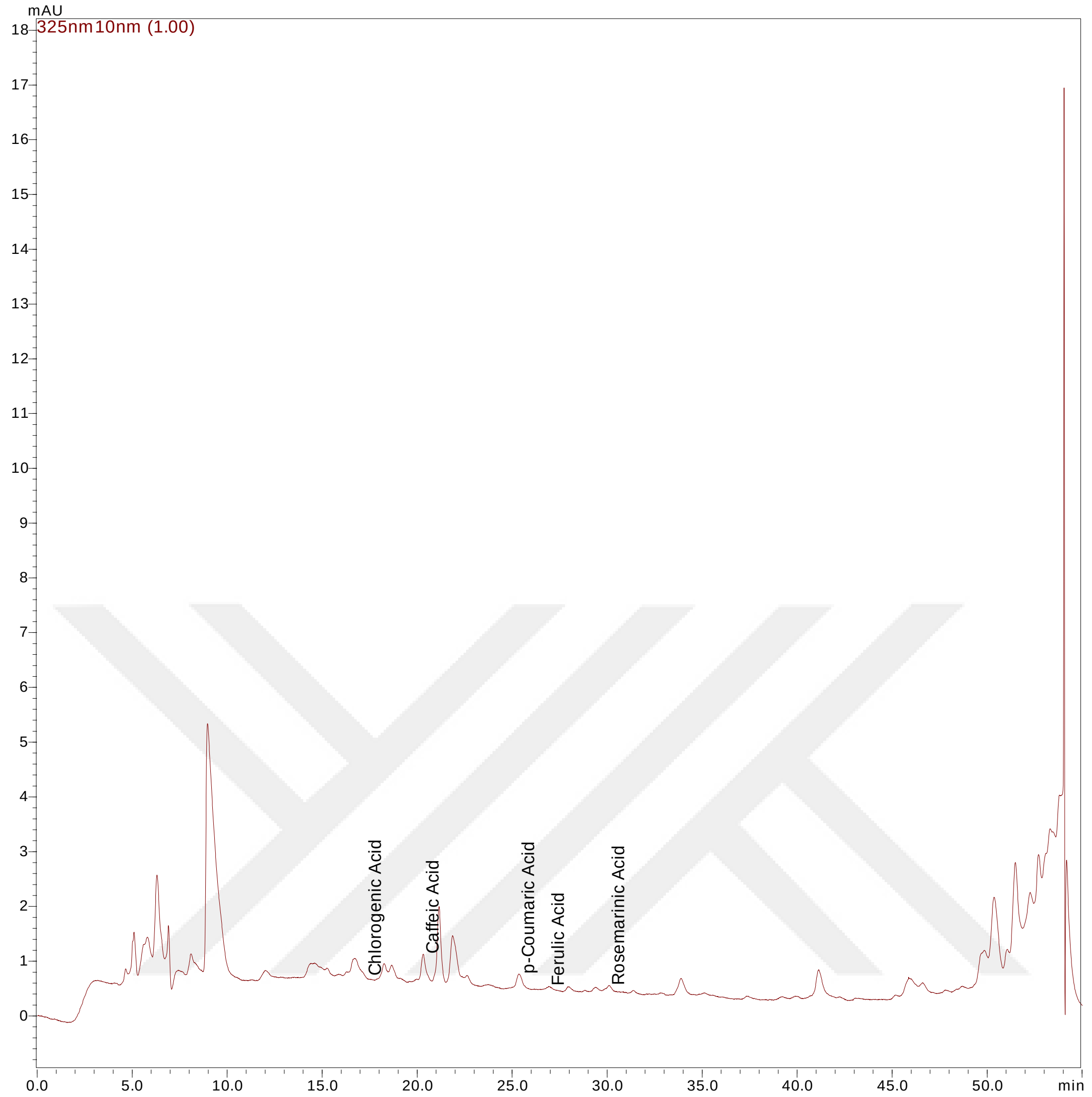

Figure B.7: HPLC chromatogram of the capsule added kefir sample in 1st day.

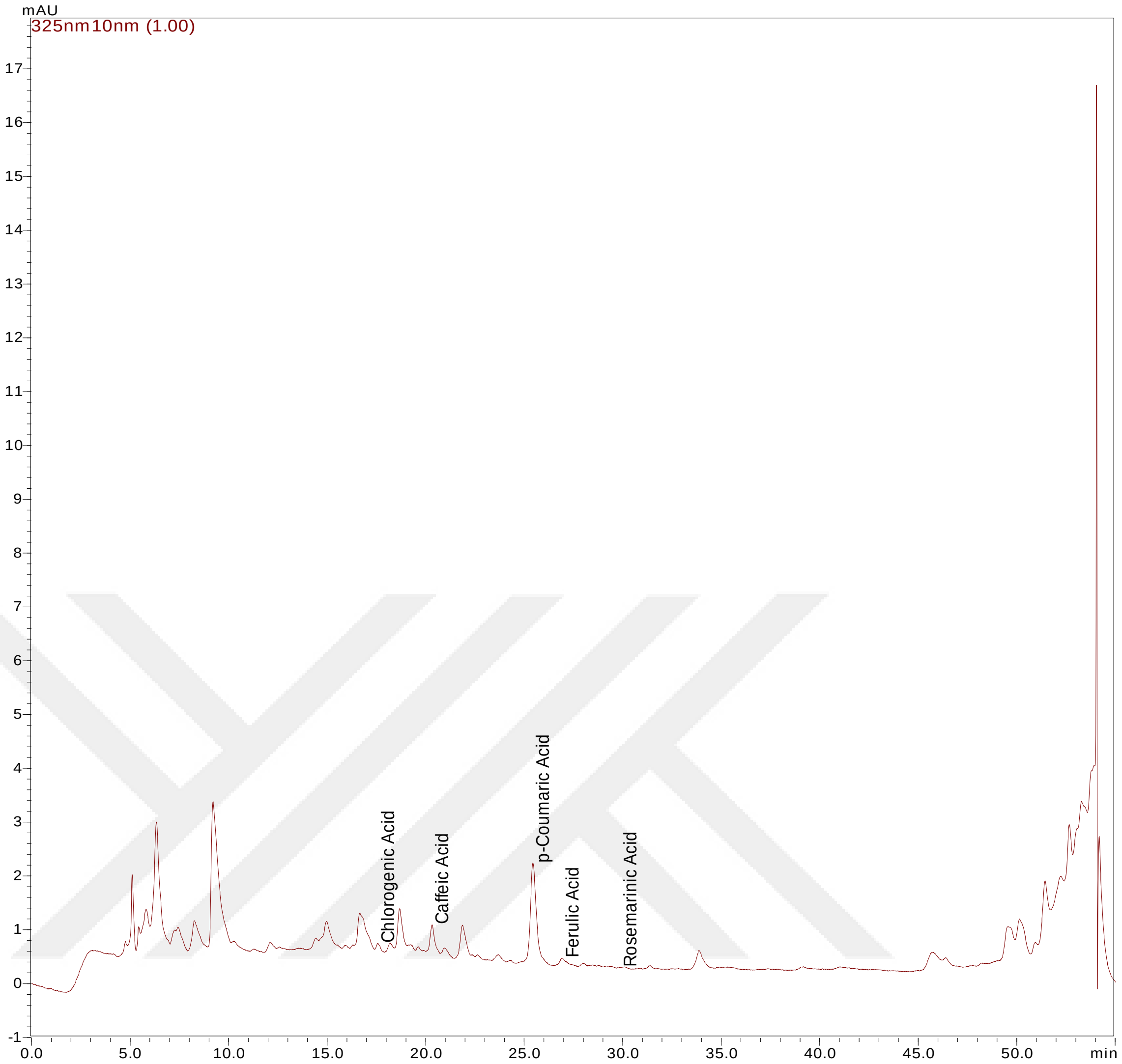

Figure B.8: HPLC chromatogram of the capsule added kefir sample in 7th day.

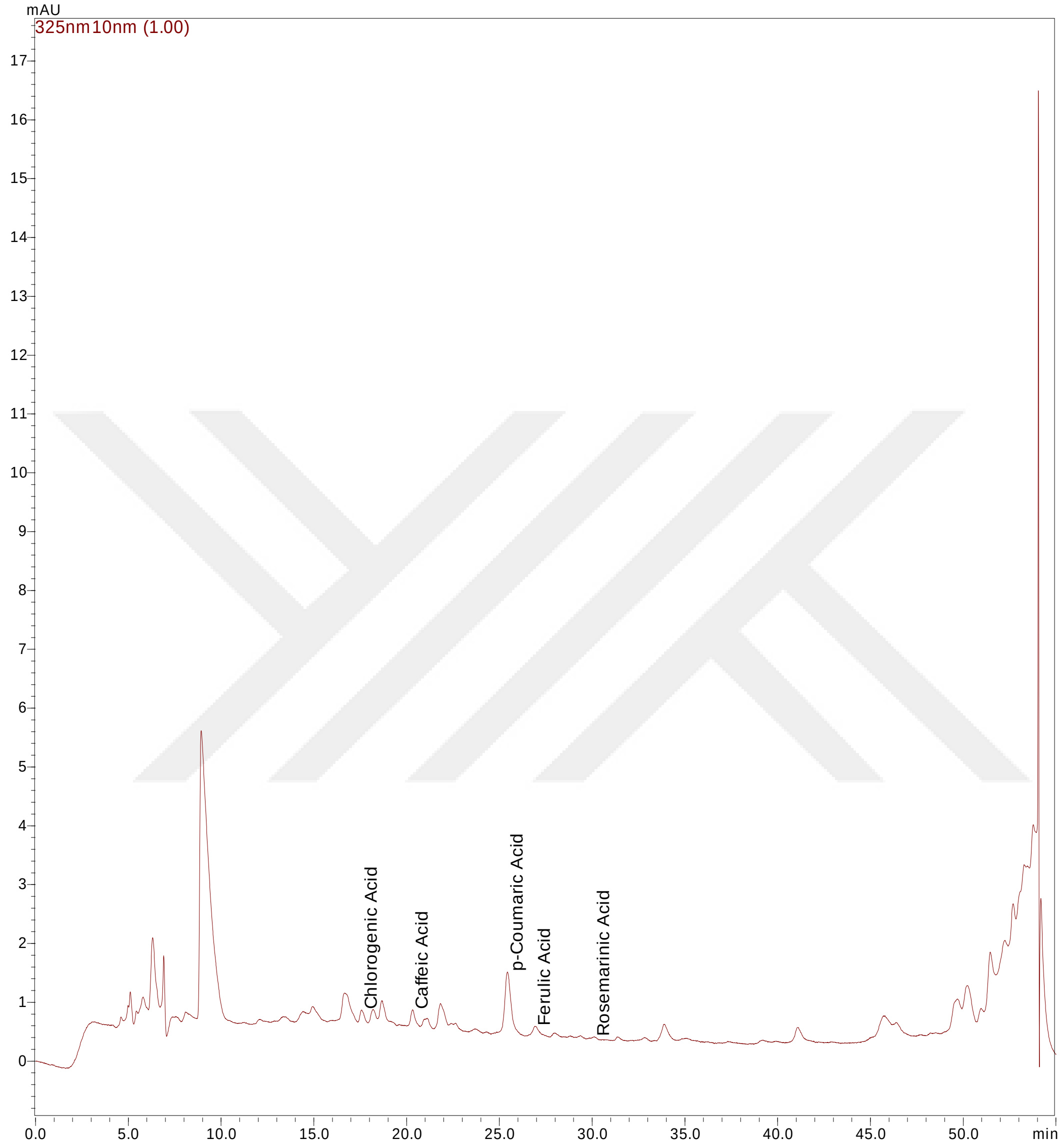

Figure B.9: HPLC chromatogram of the capsule added kefir sample in 14th day.

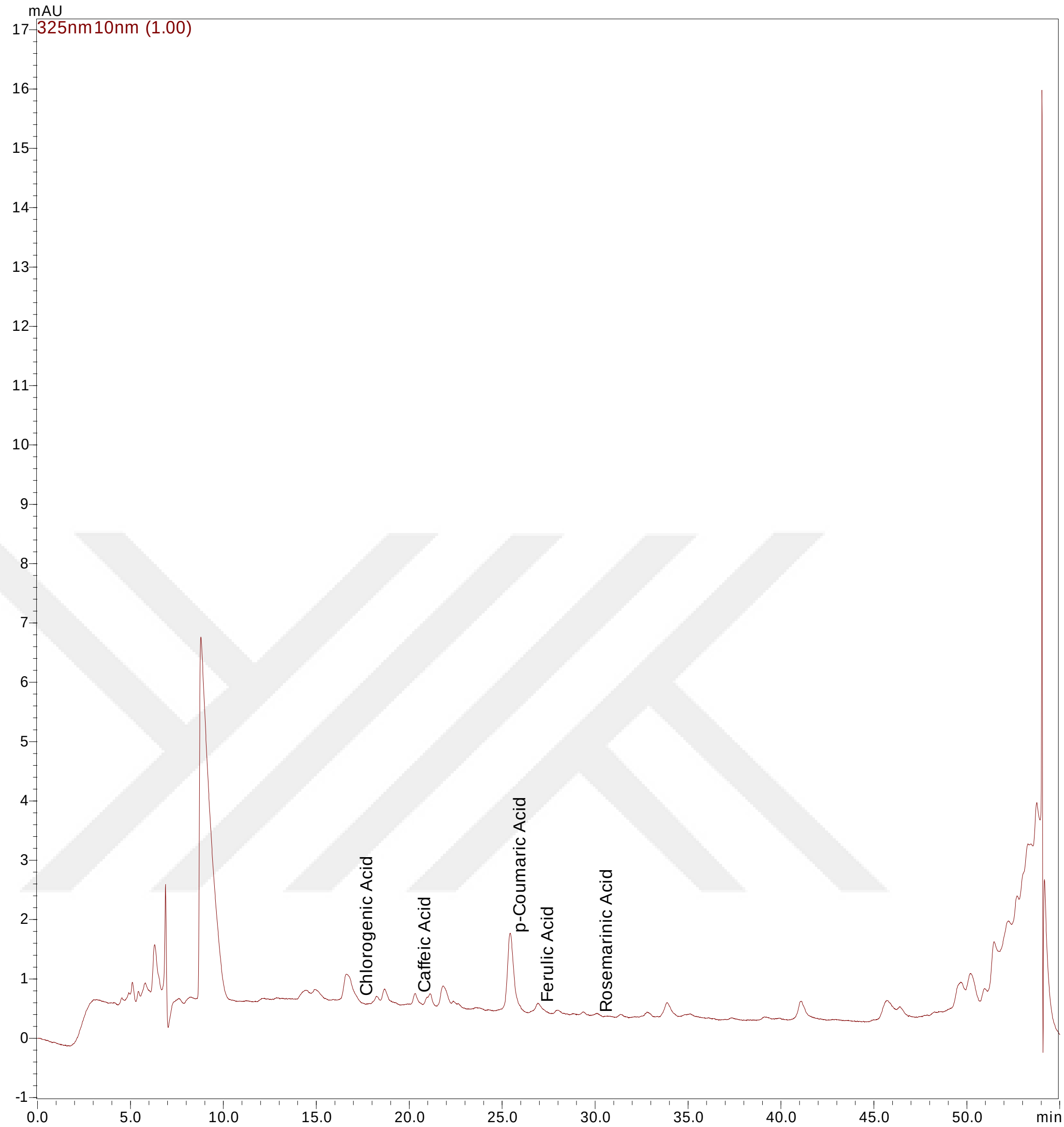

Figure B.10: HPLC chromatogram of the capsule added kefir sample in 21st day.

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