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STUDY ON EXTRACTION TECHNICS OF SWEET POTATO LEAF FLAVONOIDS, SUSTAINED RELEASE OF ITS NANOPARTICLES, AND SWEET POTATO LEAF FORTIFIED BREAD

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Résumé

Jiang LIU (2020). " Etude des techniques d'extraction des flavonoïdes de feuilles de patate douce, de la libération prolongée de ses nanoparticules, et feuilles de patate douce pain enrichi " (Thèse de doctorat en anglais). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège, 157 pages, 11 tableaux, 19 figures.

Résumé :

Les études précédentes portant sur les polyphénols de feuilles de patate douce étaient principalement axées sur les acides phénoliques, peu d'études se focalisent sur la partie flavonoïdes. Chimiquement, les flavonoïdes possèdent un squelette à 15 atomes de carbone, qui se compose de deux cycles phényle (A et B) et d'un cycle hétérocyclique (C), que l'on peut abréger en une structure de type C_6-C_3-C_6.

Dans un premier temps, les flavonoïdes de feuilles de patate douce (SPL) ont été extraits à l'aide d'une technique couplée ultra-son-micro-onde (UMSE) à l'aide de la méthodologie des surfaces de réponse (RSM). Les conditions optimales pour l'extraction des flavonoïdes permettant d'obtenir un rendement de 5,1 % sont : un rapport solide-liquide de 1:40 (g/ mL), 57 ° C comme température d'extraction, 76 s comme temps d'extraction et 72 % (v/v) d'éthanol pour une extraction réalisée deux fois successivement. Après purification, la pureté des flavonoïdes atteint jusqu'à 76,1 % (poids sec). Le résultat de la chromatographie liquide à haute performance (HPLC) réalisée sur l'extrait de feuilles de patate douce a révélé 11 composés, dont astragaline, quercétrine, acide 4,5-chlorogénique, isoquercitrine, tiliroside, quercétine, acide 3,4,5-chlorogénique, acide caféique, kaempférol, myricétrine, rhamnétine. Cet extrait possède, par ailleurs, une bonne activité antioxydante lorsque l'on compare celle-ci aux isoflavones de soja, à l'extrait de *Ginkgo biloba* et à la flavone de propolis.

Dans un deuxième temps, nous avons étudié l'effet du traitement thermique, du traitement à haute pression hydrostatique (HHP), du pH, de la lumière, de la température et de la digestion simulée sur la stabilité du SPLF. Le traitement thermique à 75 °C pendant 90 min ou le traitement HHP à 600 MPa pendant 30 min n'a pas eu d'effet significatif sur le SPLF. Un traitement thermique à 100 ° C

pendant 60 min et 90 min a conduit à une diminution de l'activité antioxydante de 20 % et 25 % respectivement, tandis que le pH 7,0 et 8,0 a diminué de manière significative la quantité de SPLF d'environ 75 %, avec une chute concomitante de l'activité antioxydante de 30% et 47 % respectivement. Les échantillons traités à la lumière ont enregistré une diminution du SPLF de 52% et de l'activité antioxydante de 24 %. Aucun effet significatif sur le SPLF n'a été observé pour les échantillons conservés à -18 °C, 4° C ou à température ambiante (RT) (≈ 20 ° C). A la suite d'un essai de digestion simulée, il a été constaté qu'il restait respectivement 45 % de flavonoïdes par rapport aux conditions initiales et que 56 % de l'activité antioxydante était conservée à l'issue de procédé.

Dans un troisième temps, des nanoparticules de SPLF ont été préparées par encapsulation par lyophilisation à l'aide de maltodextrine à 20 mg/mL. Ces nanoparticules ont montré une grande stabilité avec un potentiel zêta absolu élevé (-41,6 mV), une efficacité d'encapsulation (EE) de 59,0 %, une faible taille moyenne des particules (277,4 nm) et indice de polydispersité (PDI) de 0,417. La microscopie confocale à balayage laser (CLSM) et la spectrométrie infrarouge ont confirmé ces observations. Après une digestion orale, gastrique et intestinale simulée, les nanoparticules ont en outre conservé 16, 31, 28 % des flavonoïdes initiaux et 16, 16, 10 % de l'activité antioxydante de départ. Ces résultats ont été étayés par les observations réalisées en microscopie électronique à balayage (SEM).

Enfin, la SPL a été utilisée pour remplacer 1 ; 2, ; 3,5 et 5% (p/p) de farine de blé lors de la fabrication de pains. La préparation réalisée avec 1 % de SPL change la couleur du pain de manière significative, visible à l'œil nu. La dureté et les propriétés liées à la mastication de la mie augmente également avec le taux d'incorporation de SPL. Lors de l'ajout de 5 % de SPL dans la farine, le volume spécifique du pain a diminué de moitié par rapport au témoin. Les pores de la mie se détériorent et se désintègrent pour des ajouts de SPL supérieurs à 3,5 %. La teneur totale en polyphénols (TPC) et la teneur totale en flavonoïdes (TFC) du pain ont été multipliées par 6 et par 10 respectivement, et l'activité antioxydante a été multipliée par 10 lors de l'ajout de 5 % de SPL dans la farine. L'ajout de SPL n'influence pas de manière importante le profil en composés organiques volatils du pain mais a tendance à altérer de manière significative l'odeur typique du pain . D'une façon générale, le SPL à une concentration ne dépassant pas 1 % pourrait

être ajouté à la farine pour faire du pain présentant un TPC deux fois plus élevé, un TFC multiplié par 2,5 et une excellente activité antioxydante (multipliée par 3), et cela sans affecter significativement les caractéristiques physiques et organoleptiques du produit fini.

Mots clés: Flavonoïdes des feuilles de patate douce; Activité antioxydante; Traitement et préservation de la stabilité; Encapsulation et bioaccessibilité; Pain fortifié aux feuilles de patate douce

Abstract

Jiang LIU (2020). "Study on extraction technics of sweet potato leaf flavonoids, sustained release of its nanoparticles, and sweet potato leaf fortified bread" (PhD Dissertation in English). Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège, 157 pages, 11 tables, 19 figures.

Summary:

The previous studies of sweet potato leaf polyphenols were mainly focus on the non-flavonoids part (phenolic acids), there weren't enough study on the flavonoids part. Chemically, flavonoids have the specific structure of a 15-carbon skeleton, which consists of two phenyl rings (A and B) and a heterocyclic ring (C), and can be abbreviated as C_6-C_3-C_6 structure.

Firstly, ultrasonic-microwave synergistic extraction (UMSE) was used to extract flavonoids from sweet potato leaves (SPL) by response surface methodology (RSM). The optimal conditions for flavonoids extraction were 1:40 (g/mL) of solid-liquid ratio, 57 °C of extraction temperature, 76 s of extraction time and 72 % (v/v) ethanol for 2 times of extraction, and the highest flavonoids yield from SPL was 5.1 %. After purification, the flavonoids purity reached up to 76.1 (%, DW). The result of high performance liquid chromatography (HPLC) revealed 11 compounds including astragalin, quercetrin, 4,5-chlorogenic acid, isoquercitrin, tiliroside, quercetin, 3,4,5-chlorogenic acid, caffeic acid, kaempferol, myricetrin and rhamnetin in sweet potato leaf flavonoids (SPLF), which possessed good antioxidant activity compared to soy isoflavones, *Ginkgo biloba* extract and propolis flavone.

Secondly, the effect of heat treatment, high hydrostatic pressure (HHP) treatment, pH, light, temperature and simulated digestion on the stability of SPLF was studied. Heat treatment at 75 °C for 90 min or HHP treatment at 600 MPa for 30 min didn't cause significant effect on SPLF. Heat treatment at 100 °C for 60 min and 90 min led to a decrease in antioxidant activity by 20 % and 25 % respectively, while pH 7.0 and 8.0 significantly decreased amount of SPLF by approximately 75 %, decreased antioxidant activity by about 30 % and 47 % separately. Light treated samples recorded a decrease in SPLF by 52 % and

antioxidant activity by 24 %. No significant effect on SPLF was observed for samples stored at -18, 4 °C or room temperature (RT) (≈ 20 °C). The retention of flavonoids and antioxidant activity was 45 % and 56 %, individually in SPLF after simulated digestion.

Thirdly, nanoparticles of SPLF were prepared by freeze-drying encapsulation using 20 mg/mL maltodextrin, which showed the highest stability with high absolute zeta potential (-41.6 mV) and encapsulation efficiency (EE) (59.0 %), low mean particle size (277.4 nm) and polydispersity index (PDI) (0.417). Confocal laser scanning microscopy (CLSM) and infrared spectrum provided the evidence. After simulated oral, gastric and intestinal digestion, nanoparticles additionally reserved 16, 31, 28 % of flavonoids and 16, 16, 10 % of antioxidant activity, separately, compared with the sample without encapsulation, which could be intuitively demonstrated by scanning electron microscopy (SEM).

Finally, SPL was used to substitute 1, 2, 3.5 and 5 % (w/w) of wheat flour to make bread. 1 % SPL could change the bread color significantly, which could be clearly distinguished by human eyes. The hardness and chewiness of the crumb increased with increased SPL level. When adding 5 % of SPL to the flour, specific volume of the bread shrank in half, compared with the control. Pores in the crumb became deteriorating and disintegrating when the adding of SPL was higher than 3.5 %. Total polyphenols content (TPC) and total flavonoids content (TFC) of the bread increased 6-fold and 10-fold, and antioxidant activity enhanced 10-fold, separately when adding 5 % SPL to the flour. The addition of SPL won't alter volatile compounds of the bread, but suppressed the generation of typical bread odors significantly. Overall, SPL (no more than 1 %) could be supplemented to the flour to make bread with high TPC (2-fold), TFC (2.5-fold), excellent antioxidant activity (3-fold), and no significant adverse effect on the physical characteristics and flavor.

Keywords: Sweet potato leaf flavonoids; Antioxidant activity; Processing and preserving stability; Encapsulation and bioaccessibility; Sweet potato leaf fortified bread

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May god bless all.

Jiang Liu

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List of Abbreviations

AAE, Ascorbic acid equivalents
ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)
ACE, Angiotensin-converting-enzyme
ANOVA, One-way analysis of variance
CA, Caffeic acid
CAR/PDMS, Carboxen/polydimethysiloxane
CHA, Chlorogenic acid
CLSM, Confocal laser scanning microscopy
CQA, Caffeoylquinic acids
DE, Dextrose equivalents
DPPH, 2,2- diphenyl-1-picrylhydrazyl
DVB/CAR/PDMS, Divinylbenzene/carboxen/polydimethysiloxane
DW, Dry weight
EE, Encapsulation efficiency
EGCG, (-)-Epigallocatechin-3-gallate
FRAP, Ferric reducing antioxidant power
FT-IR, Fourier transform infrared spectroscopy
GAE, Gallic acid equivalents
GC-MS, Gas chromatography-mass spectrum
GIN, *Ginkgo biloba* extract
HHP, High hydrostatic pressure
HPLC, High performance liquid chromatography
HS, Head space
HUVECs, Human umbilical vascular endothelial cells
IC_{50}, Half maximum inhibitor concentration (IC_{50})
MAE, Microwave-assisted extraction
MAL, Maltodextrin
MMP, Matrix metalloproteinase
PDI, Polydispersity index
PDMS, Polydimethylsiloxane
PDMS/DVB, Polydimethysiloxane /divinylbenzene
PRO, Propolis flavone

PSPL, Purple SPL
QE, Quercetin equivalent
RE, Rutin equivalent
RH, Relative humidity
RSM, Response surface methodology
RT, Room temperature
SD, Standard deviation
SEM, Scanning electron microscopy
SGF, Simulated gastric fluid
SIF, Simulated intestinal fluid
SPL, Sweet potato leaves
SPLB, Sweet potato leaf fortified bread
SPLF, Sweet potato leaf flavonoids
SPME, Solid phase microextraction
SSF, Simulated salivary fluid
TAC, Total anthocyanin content
TFC, Total flavonoids content
TPC, Total polyphenols content
TPTZ, 2, 4, 6-Tri (2-pyridyl)-1, 3, 5-triazine
UAE, Ultrasonic-assisted extraction
UMSE, Ultrasonic microwave synergistic extraction
β-Lg, β-lactoglobulin

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Chapter Ⅰ. General Introduction

1.Background

Sweet potato (*Ipomoea batatas* L.) is one of the 8 top crops in the world ranking after maize, wheat, rice, potato, soybean, cassava and barley. China is the largest sweet potato producer in the world, the annual yield of 2018 was 53 million tons, which account for 57 % of the total yield (FAOSTAT, 2020). After the roots been harvested as the starch resources, equal tons of sweet potato leaves (SPL) need to be dealt with.

According to the index of nutritional quality, SPL was a good source of protein, fiber and minerals, especially K, P, Ca, Mg, Fe, Mn and Cu, which represented SPL possessing high nutritive value. More importantly, the correlation coefficient between antioxidant activity and total polyphenols content (TPC) was high (0.8, $p<0.0001$), indicated that polyphenols were key antioxidants in SPL (Sun, Mu, Xi, Zhang, & Chen, 2014).

Polyphenols were classified into two major branches: flavonoids and non-flavonoids (Khan, Zill-E-Huma, & Dangles, 2014). Non-flavonoids part (phenolic acids) of polyphenols in SPL has been comprehensively studied, flavonoids part because of its specific structure (two aromatic rings (A ring and B ring) connected by pyran ring (C ring), often contained hydroxyl, methoxy, methyl, isoamyl group and other substituents), possessing high antioxidant, anti-bacterial, anti-cancer activity etc., began drawing arising attention (Chen et al., 2011; Wang, Cao, Wu, Wang, & Xiao, 2016).

Soxhlet extraction has been widely used to extract flavonoids, however, this method presents the major drawback related with the thermal degradation of target compounds, due to the high temperature maintained for long time, as well as the large use of organic solvents (Chua, 2013). Therefore, emerging extraction technologies become popular, such as ultrasonic-assisted extraction (UAE) (Ameer, Shahbaz, & Kwon, 2017), microwave-assisted extraction (MAE) (Angiolillo, Del Nobile, & Conte, 2015), infrared irradiation-assisted extraction (Duan, Chen, & Chen, 2010), pressurized liquid-assisted extraction (Carro, González, & Lorenzo, 2013), enzyme-assisted extraction (Puri, Sharma, & Barrow, 2012) and supercritical fluid extraction (Liza et al., 2010).

Ultrasonic microwave synergistic extraction (UMSE) united advantages of UAE and MAE, which could increase the extraction yield in a short time. Žlabur et al. (2015) optimized UAE conditions to maximize total flavonoids content (TFC) as well as antioxidant activity in *Stevia rebaudiana* Bertoni leaves extracts, which increased 60 % of the TFC and 10 % of the antioxidant activity, compared with conventional extraction. Wu et al. (2015) took a study on the extraction of antioxidants from *Nitraria tangutorun* Bobr. juice by-products, the results clearly showed that MAE could increase 28 % of the TFC, 19 % of the total anthocyanin content (TAC) and 25 % of the antioxidant activity, compared with Soxhlet

extraction.

High hydrostatic pressure (HHP) is a novel food processing technology of conserving foods as it maintains their sensory quality while stabilizing the concentrations of phytochemical compounds as well as inhibiting microbial growth and enzyme activity (Jiménez-Aguilar et al., 2015). HHP processed strawberry puree at 400 to 600 MPa notably preserved higher TPC and TAC as compared to conventional thermal treatments. Similar results were achieved in case of blackberry purees, in which HHP treated samples showed higher retention of anthocyanins and anti-radical powers as compared to conventional processing techniques (Patras, Brunton, Da Pieve, & Butler, 2009).

Products related to SPL included green beverage powder (Mu, Sun, Xi, & Zhang, 2013), SPL tea (Kim, 2012), pickled SPL (Wei et al., 2015). SPL as a nutritional waste has been studied (Hong, Mu, Sun, Richel, & Blecker, 2020), especially phenolic acids and flavonoids among it, which might make SPL possessing the potential application in staple food-bread.

2.Objective

The first aim of this thesis was to optimize the parameters of UMSE on the yield of SPLF (ethanol concentration, extraction time, extraction temperature), and determine the flavonoids composition of SPLF and its antioxidant activity. The second aim was to evaluate the stability of SPLF under thermal processing and non-thermal (HHP) processing, and the stability during 3 months storage in different environments (light, pH, temperature). The third aim was to study the effect of encapsulation on the stability of SPLF during simulated gastrointestinal digestion. The last aim was to supplement SPL which was rich in flavonoids to wheat flour to make bread, and evaluated its physical-chemical characteristics.

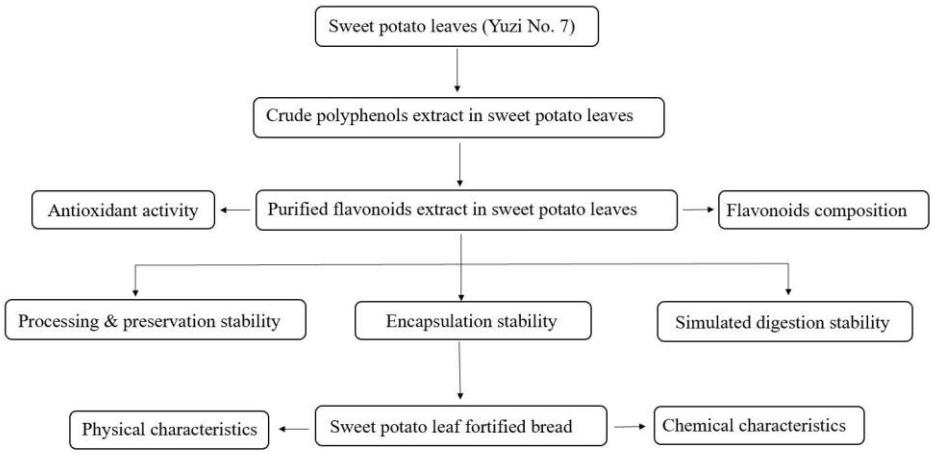

Figure 1-1 Technology roadmap of this thesis

3.Research strategy

The literature review introduces the work undertaken in this thesis by describing the research status of SPLF, and the utilization of plant materials which rich in flavonoids to the baked products.

It was optimized the parameters of UMSE on the extraction yield of SPLF, purified the SPLF and determined the flavonoids composition and its antioxidant activity (Chapter 3), evaluated the stability of SPLF after processing (heat and HHP) and preserving (pH, light, temperature) (Chapter 4). It also determined the stability of SPLF-MAL nanoparticles and possible sustained release during simulated digestion (Chapter 5). Then, flavonoids-rich SPL was supplemented to wheat flour to evaluate the physical and chemical characteristics of SPLB (Chapter 6). Finally the thesis was summarized by a general discussion, conclusion and perspective (Chapter 7).

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Chapter Ⅱ. Literature review on sweet potato leaf flavonoids, bioactivity of plants flavonoids, and the quality of flavonoids-rich bread

Abstract

This review covers recent research advances in sweet potato leaf flavonoids (SPLF), the bioactivity of flavonoids, encapsulation to improve the stability of flavonoids, and the application of flavonoids-rich materials to bakery products. Sweet potato as a nutritious staple food, while its leaves possess high content of flavonoids and strong antioxidant activity, which could be used as a kind of functional additive in food industry. Furthermore, encapsulation could improve the stability of flavonoids in some circumstances (light, pH etc.). In addition, the positive and negative effects of flavonoids-rich materials incorporation to baked foods were discussed. This review focuses on the comprehensive introduction of SPLF, encapsulation technics for protecting flavonoids, and the influence of flavonoids-rich materials on the quality of baked foods.

Keywords

Sweet potato leaf flavonoids; Bioactivity; Encapsulation; Baked food

1.Introduction

1.1. Sweet potato

Sweet potato (*Ipomoea batatas* L.) belongs to the *Convolvulaceae* family, which originates from Central or South America, widespread cultivated throughout warm and high humidity regions, which is the sweet-tasting, starchy, tuberous root vegetable with white, orange or purple flesh (Wang, Nie, & Zhu, 2016). China is the largest sweet potato producer in the world, the annual yield of 2018 was 53 million tons, which account for 57 % of sweet potato yield (FAOSTAT, 2020). Besides simple energy provision, sweet potato is rich in dietary fiber, β-carotene and other nutrients (Wang, Nie, & Zhu, 2016).

1.2. Sweet potato leaves

Sweet potato leaves (SPL) are medium in size and cordate or heart-shaped with pointed tips. After harvesting the roots, there are almost equal amount of leaves, eaten as greens in some places, waiting for discarding (Ishida et al., 2000). The contents of carbohydrate, dietary fiber, protein, ash and fat in leaves from 40 sweet potato cultivars in China ranged of 42.0-61.3, 5.9-14.3, 3.7-31.1, 1.5-14.7 and 0.3-5.3 g/100g in dry weight (DW), individually. Macro-elements K, Ca, P, Mg and Na with ranges of 479.3-4280.6, 229.7-1958.1, 131.1-2639.8, 220.2-910.5 and 8.1-832.3 mg/100 g DW, respectively, while micro-elements Fe, Mn, Zn and Cu with ranges of 1.9-21.8, 1.7-10.9, 1.2-3.2 and 0.7-1.9 mg/100g DW, separately (Sun, Mu, Xi, Zhang, & Chen, 2014). Meanwhile, SPL had higher contents of β-carotene, vitamins B_1, B_2, B_6, niacin, pantothenic acid and biotin with ranges of

273-400, 53-128, 248-254, 120-329, 856-1498, 320-660 and 3-8 μg/mL, separately, and vitamins C and E of 62.7-81 and 1.4-2.8 mg/mL in the varieties cultivated in Japan (Ishida et al., 2000).

1.3. Sweet potato leaves polyphenols

The total phenolic content (TPC) in leaf of 8 sweet potato cultivars in Japan ranged from 6.3-13.5 g/100g gallic acid equivalent (GAE) (DW) (Nagai et al., 2011), meanwhile TPC of SPL in 40 varieties cultivated in China differed from 2.7-12.3 mg chlorogenic acid /100g, DW. The end of stems contained significantly more phenolics than other parts of sweet potato (Jung, Lee, Kozukue, Levin, & Friedman, 2011). Flavonoids as the main branch of polyphenols which possessed typical C_6-C_3-C_6 structure, arousing more and more attention for its bioactivities (Wang, Li, & Bi, 2018).

1.4. The content of flavonoids in sweet potato leaves

Total flavonoids content (TFC) of fresh leaves varied among Taiwan (4 varieties of sweet potato) was 0.2 -0.7 mg quercetin equivalent (QE)/g, DW) (Liao, Lai, Yuan, Hsu, & Chan, 2011). After steaming, boiling and fermenting treatment, TFC in fresh tips (15 cm from the growing end of sweet potato) was 0.5, 0.5 and 0.3 mg rutin equivalent (RE)/ g, DW, individually, higher than the unprocessed sample in which TFC was 0.2 mg RE/ g, DW (Cui, Liu, Li, & Song, 2011). After extracted at 37 °C for 24, 48, 72 h by 80 % methanol, TFC of stems in acidified and non-acidified samples was about 0.3 g quercetin equivalent (QE)/100 g, DW, meanwhile TFC of leaves in acidified and non-acidified samples was about 0.8 g QE/100g, DW (15-38 cm of bottom part of sweet potato vine) (Anastácio & Carvalho, 2013). TFC in methanol (50 %, 70 %, 90 %), ethanol (50 %, 70 %, 90 %), acetone (50 %, 70 %, 90 %) and water extract of SPL was ranging from 0.6-3.4 mg QE/g DW, in which 70 % ethanol possessed the highest extraction ability of flavonoids (3.4 mg QE/g DW) (Fu et al., 2016).

1.5. The composition of sweet potato leaf flavonoids

(+)-Catechin, (-)-epicatechin, quercetin, myricetin and kaempferol were qualitatively and quantitatively analyzed from SPL cultivated in Portugal (Carvalho, Cavaco, Carvalho, & Duque, 2010). Quercetin-3-O-hexosylhexoside, hyperoside, quercetin-3-O-hexoside and ombuin-sulfate were identified in 50 % acetone extract from SPL cultivated in China (Fu et al., 2016). Quercetin-3-O-glucosidase, 7,3-dimethylquercetin and rhamnetin were found in ethyl acetate fraction of 70 % ethanol extract from orange-fleshed SPL (Cultivar: Jishu No. 16) (Zhang et al., 2016). Cyanidin and peonidin had been determined in the leaves of sweet potato from purple-fleshed, orange-fleshed and white-fleshed varieties (Montilla et al., 2010). Rutin, myricetin, luteolin, quercetin, ombuin and kaempferol had been found in 70 % acetonitrile extract from SPL through

microwave-enhanced accelerated solvent extraction, from 10 different regions around China (Lu, Zhou, Ren, & Zhang, 2019).

1.6. In vitro activities of sweet potato leaf flavonoids

1.6.1. Antioxidant activity

Antioxidant activity of water extracts from 4 SPL cultivars in Taiwan, was evaluated by DPPH scavenging ability (Half maximum inhibitor concentration (IC_{50})), 0.1-0.4 mg/mL of sample), reducing power (IC_{50}, 0.3-0.5 mg/mL of sample), superoxide radical scavenging capacity (IC_{50}, 0.1-0.6 mg/mL of sample), and iron-chelating activity (10.0-23.3 %, 0.5 mg/mL), flavonoids contributed the key power for the antioxidant activities (Liao, Lai, Yuan, Hsu, & Chan, 2011). DPPH scavenging ability was positively correlated with TPC in SPL, the correlation coefficient (0.9; $p < 0.0001$) was high (Nagai et al., 2011).

Leaf extracts exhibited variety-dependent antioxidant activities, and ethyl acetate extracts had the highest antioxidant activity, in which contained high amount flavonoids (Xu et al., 2010). TFC accounted for more than 60 % of TPC from European sweet potato vines, which holding a Protected Geographic Indication (PGI), possessing *in vitro* antioxidant activities of 1.2 g ascorbic acid equivalent /100 g DW for total antioxidant activity, 50.8 g gallic acid equivalent /100 g DW for reducing power, 58.6 μM Trolox/g DW for DPPH scavenging capacity and 29.3 μM Trolox/g DW for FRAP, respectively (Anastácio & Carvalho, 2013). The dynamic high-pressure microfluidization-assisted extract exhibited high dose-dependent antioxidant activities, when after purification by LK001 macro-porous absorption resin, purified extract showed similar antioxidant activities to rutin, at the same concentration in the range of 0.1-1.2 mg/mL (Huang et al., 2013).

1.6.2. Hypotensive activity

The angiotensin-converting-enzyme inhibitor (ACE inhibitor) was primarily used for the control of hypertension and congestive heart failure, which found in freeze-dried powders of Chinese Cuilv sweet potato tips (leaves and stem, 15 cm from the growing end). Boiling, steaming and fermenting increased the activity of ACE inhibitor by 46.9 %, 41.1 % and 44.1 %, respectively in which high content of flavonoids exited (Cui, Liu, Li, & Song, 2011). The increase might due to thermal processing inducing phenolic polymerization or oxidation, which modified the reactive hydroxyl groups in the aromatic ring structure. The increase in the activity of ACE inhibitor of fermented samples might due to the ACE inhibitory peptides synthesizing by bacterial cells.

1.6.3. Anti-angiogenesis activity

Angiogenesis is critical to the regeneration of injured body parts. However, this process also contributes to the development and progression of several neoplastic

and non-neoplastic diseases, such as cancer and atherosclerosis. SPL inhibited proliferation, migration and formation of human umbilical vascular endothelial cells (HUVECs) at 0.2-0.6 mM gallic acid equivalents. Moreover, the activity of secreted matrix metalloproteinase-2 (MMP-2) decreased by 13.8 %. However, 5 % SPL serum increased migration and tube formation of HUVECs by 110 % and 56.9 %, separately, compared with serum getting from low flavonoids diet. In the meantime, the activity of MMP-9 was increased by 128 % in SPL serum (Chen et al., 2011).

1.6.4. Anti-cancer activity

SPL extract had high content of anthocyanins (11 mg monomeric anthocyanin equivalent /L), which could significantly inhibit cellular proliferation of prostate cancer cell, with IC_{50} values in the range of 145-315 μg/mL. Especially, the IC_{50} value of SPL extract for normal prostate epithelial cells was 1000-1250 μg/mL, about 5-fold higher than the cancer cells, indicated that SPL extract preferred targeting cancer cells compared with normal cells (Karna et al., 2011).

1.7. In vivo activity of sweet potato leaf flavonoids

1.7.1. Prevention of damage by exercise-induced oxidative stress

200 g cooked purple SPL (PSPL) which had high amount of flavonoids (426.8 μg/g DW), was consumed as the diet by 15 healthy male humans, in which the average age was 24, the average height was 174 cm, the average weight was 67 kg and individual maximal oxygen uptake ($V_{O2max)}$ was 40.5 $mL \cdot min^{-1} \cdot kg^{-1}$. After 70 % V_{O2max} exercise for 1 h during 3 h recovery period, consumption of a flavonoids-rich PSPL diet for 1 week decreased plasma lipid peroxidation by ≈63 %, compared with the control group 3 h after exercise, decreased protein oxidation by ≈50 % compared with the control group 1 h after exercise. In the meantime, exercise-induced oxidative stress could cause an acute inflammatory response, an increase concentration of plasma interleukin after exercise was a significant index, which could decrease by ≈25 %, compared with the control group 1 h after exercise (Chang, Hu, Huang, Yeh, & Liu, 2010).

1.7.2. Anti-hyperglycemic effects

SPL extract (at the dose of 100, 200 and 400 mg/kg/day of rat weight) was fed orally to healthy rats and diabetic rats for 14 days, flavonoids was the main contributor to decrease the fasting blood glucose level for both groups. In this range (100-400 mg/kg/day), SPL extract showed the dose-dependent anti-hyperglycemic effect, in which the maximum effect of SPL extract was a dose of 400 mg/kg/day in both groups, which was equal to the glibenclamide (an antidiabetic drug) at a dose of 1 mg/kg/day, meanwhile there was no evidence that SPL extract had cytotoxicity, even up to 1000 mg/kg/day (Adeyemi, Olowu, & Adeneye, 2011).

1.7.3. Inhibition of carcinogenesis

Mice in the treatment group were fed 400 mg/kg/day SPL extract, in which high content of anthocyanins existed, by oral gavage for 6 weeks. The study was monitored by bioluminescent imaging, and mice without feeding SPL extract was taken as the control group. For this group, tumors exhibited unrestricted growth, on the contrary, SPL extract feeding group showed a time-dependent tumor inhibition during 6 weeks, quantification analysis of relative photon counts demonstrated a ≈69 % reduction in tumor volume after 6 weeks, compared with the control group. Body weight of the mice was recorded twice a week to evaluate overall health state, SPL extract treatment group maintained normal weight with no sign of discomfort during the whole process. In the meantime, mice fed with SPL extract maintained 87.5 % of their surviving longevity after 10 weeks, significantly prolonged the survival time compared with the control group, where was only 6 weeks (Karna et al., 2011).

1.8. Simulated gastrointestinal digestion of plants flavonoids

"Bioactivity" is usually defined as the fraction of a given compound or its metabolite that reaches the systemic circulation, which includes gastrointestinal digestion, absorption, metabolism, and tissue distribution. Digestion is the first step, and because of the ethical constraint for experiment *in vivo*, simulated digestion *in vitro* (relatively inexpensive and technically simple) becomes a good substitute (Carbonell-Capella, Buniowska, Barba, Esteve, & Frígola, 2014).

In-vitro gastrointestinal digestion is useful to estimate the stability of phytonutrients experiencing the pre-absorptive process. *In vitro* study of grape showed that the content of flavonoids and anthocyanins decreased when the environment changed from acidic gastric phase to alkaline intestinal phase. At the end of digestion phase, the retention of flavonoids and anthocyanin was 56.1 % and 7.6 %, respectively, catechin and quercetin did not significantly degrade under pancreatic conditions (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Flavonoids of 12 potato clones extracts after simulated digestion was studied, the retention of anthocyanins in purple-fleshed potato clones ranged from 40 % to 81 %, meanwhile the retention of rutin and kaempferol-3-rutinoside was ranging from 32 % to 84 % and 71 % to 92 %, individually (Andre et al., 2015). Simulated gastric digestion had no substantial effect on anthocyanins, flavan-3-ols and flavonols in chokeberry juice, which however significantly decreased each flavonoid by approximately 43, 26 and 19 %, respectively. Moreover, interactions between the digestive enzymes were not responsible for the observed loss, chemical conditions (pH) during intestinal digestion influenced a lot (Bermúdez-Soto, Tomás-Barberán, & García-Conesa,

2007). The retention of cyanidin-3-glucoside, cyanidin-3-rutinoside and rutin in fresh figs (*Ficus carica* L.) reduced to 19, 35 and 40 %, respectively after simulated gastric digestion, and further reduced to 3 %, not detected and 12 %, separately after simulated intestinal digestion. Meanwhile a significant decrease of antioxidant activity in fresh figs after simulated intestinal digestion was observed, and only 22 % of the initial value could be preserved (Kamiloglu & Capanoglu, 2013).

Effect of dehydration was studied on the retention of flavonoids in green *amaranthus* mixture, which was 38 % in fresh mixture and 33 % in dehydrated mixture. Similar results were obtained in chickpea *amranthus* mixture, where the retention of flavonoids reduced from 40 % (fresh) to 36 % (dehydrated). The reason might be explained that although dehydration was able to destroy the heat sensitive antioxidants, however concentration of heat stable ones remained the same (Oghbaei & Prakash, 2015). *In vitro* digestion of hand-squeezed and pasteurized commercial orange juices was studied by Gil-Izquierdo, Gil, Ferreres, & Tomás-Barberán (2001), that the retention of total flavanones in orange juices was 13 % and 23 %, respectively, and pasteurization increased the retention of flavonoids in orange juice.

Cooking process exerted a positive effect on the retention of flavonoids due to cell wall softening (Palermo, Pellegrini, & Fogliano, 2014). The retention of anthocyanins depended on the stability of molecular structure, which was highly unstable and degraded to chalcone in mild alkaline environment of the small intestine (Pérez-Vicente, Gil-Izquierdo, & García-Viguera, 2002).

Freezing is a useful way to preserve food, which cause structural changes resulting in softening of the tissue. The retention of flavonoids in frozen strawberries was ranging from 72 % to 89 %, higher than in fresh strawberries. In other words, freezing not only prolonged the shelf life of strawberries, but also increased the retention of anthocyanins (Kamiloglu, 2019).

1.9. Encapsulation of flavonoids for improving stability

Since main contributors to the poor stability of flavonoids are environmental (oxygen, light, heat, pH etc.), the effective approach to improve stability is to form a wall between the flavonoids and the external environment. Encapsulation encase the compound within a wall material, increasing the ability to isolate and protect the compounds from the surroundings, in which wall materials should not react with active ingredients, be inexpensive, be consistent during storage, and have food-grade status (Labuschagne, 2018).

Spray-drying and freeze-drying are the most popular encapsulation methods, accounting for ≈84 % of the publications, the other novel encapsulation technologies (electrospinning, vibrating nozzle technique, inclusion complexation) only appear in the last ≈10 years (Ozkan, Franco, De Marco, Xiao, & Capanoglu,

2019).

Spray-drying is the most common encapsulation technology relating atomization of a liquid into a dry powder, by means of an injector with a hot gas stream. Firstly, the feed liquid (a solution, an emulsion or a suspension including core and wall material) is injected into the drying vessel through a nozzle in order to obtain small droplets followed by evaporation of the solvent, secondly, these dried particles are removed from the drying gas into the collector by a cyclone or filter. Spray-drying is used commercially in food industry due to the availability of equipment, low production costs, ability to process a wide range of materials, excellent stability of the final product, and continuous production in the large-scale (Ozkan, Franco, De Marco, Xiao, & Capanoglu, 2019).

Freeze-drying is the process of removing ice from the material through sublimation and desorption. Compared with spray-drying, freeze-drying could prevent or limit the microbial reaction, oxidation and other chemical reaction, due to the absence of liquid water, air and low temperature, which is an excellent method for a wide variety of heat-sensitive compounds. However, the disadvantage of freeze-drying is the cost, usually 30 to 50 times higher than the spray-drying (Massounga Bora, Ma, Li, & Liu, 2018).

Freeze-drying using maltodextrin as the coating material, was more appropriate for preserving the flavonoids extracted from spent coffee grounds, which providing the highest retention of flavonoids (73 %) within the matrix, meanwhile preserving 86 % of the antioxidant activity. On the other hand, although Arabic gum was more thermal stable compared with maltodextrin, when taken as the wall material encapsulated by spray-drying showed mediocre performance, in which 45 % of flavonoids and 35 % of antioxidant activity could be preserved (Ballesteros, Ramirez, Orrego, Teixeira, & Mussatto, 2017).

Gelatin and gum Arabic as the wall materials for encapsulation of black raspberry anthocyanins, showed the improved thermal and storage stability of core anthocyanins. The formula (ratio of 1: 1: 1 of gelatin, gum Arabic and black raspberry anthocyanins with the concentration of 0.075 g/mL) was more efficient taken as the as the wall material, which capsules with high loading capacity (≈40 %) and good morphological feature were obtained, compared with isolate black raspberry anthocyanins, which could significantly increase the stability of anthocyanins up to 24 % after 2 months of storage at 37±2 °C (Shaddel et al., 2018).

The flavonoids in citrus peel extracts was sensitive to pH, which could be protected by whey protein concentrate nanoparticles generally. The nanoparticles significantly slowed down the release of flavonoids under gastrointestinal condition, while the antioxidant activity exhibited the same tendency, and after 4 h, the nanoparticles could extra preserved ≈32 % of its $DPPH^{\cdot+}$ scavenging activity, compared with naked flavonoids extracts (Hu, Kou, Chen, Li, & Zhou, 2019).

Sour cherry was rich in flavonoids, which was highly sensitive towards temperature, pH, oxygen and light, leading to a low stability, could be stabilized by encapsulation through uniaxial or coaxial electrospinning with gelatin and lactalbumin. Encapsulation efficiency was found as 70 % and 78 % in terms of flavonoids for the uniaxially electrospun samples with gelatin and gelatin-lactalbumin, respectively. The content of flavonoids in sour cherry decreased after intestinal digestion, whereas encapsulated samples showed improved stability, which provided 8-fold higher protection ability of cyanidin-3-glucoside, compared with non-encapsulated sour cherry flavonoids (Isik, Altay, & Capanoglu, 2018).

Blueberry juice with high content of anthocyanins, was encapsulated by spray-drying and freeze-drying, using hydroxypropyl-β-cyclodextrin and β-cyclodextrin as the wall materials. The loss of anthocyanins during spray-drying reached to 57 % on average, while freeze-dried powder showed better retention of anthocyanins, which was ≈1.5-fold higher than spray-dried powder. The result indicated that high inlet gas temperature and low final product yield had a negative effect on anthocyanins preservation of blueberry juice (Wilkowska, Ambroziak, Czyzowska, & Adamiec, 2016).

(-)-Epigallocatechin-3-gallate (EGCG) was an oxidation-sensitive, water-soluble flavonoids, which need to be protected by β-lactoglobulin (β-Lg). The result showed that although particle size increased with the rising of EGCG concentration, zeta potential stayed around -40 mV, showing particles were quite stable in the solution. Good loading efficiency (60-70 %) of EGCG within β-Lg was obtained by freeze-drying, and these nanoparticles dramatically suppressed the astringency and bitterness of EGCG. Simulated gastric digestion of β-Lg-EGCG nanoparticle exhibited limited release of EGCG (≈25 %), indicated nanoparticles could be potentially used for protecting EGCG in the stomach, then sustained release in the intestine (Shpigelman, Cohen, & Livney, 2012).

Argentine propolis extracts with high content of flavonoids (289 mg/g of galangin, 180 mg/g of pinocembrin and 96 mg/g of chrysin), had the potential to be utilized in functional foods. Spray-drying propolis powder encapsulated by maltodextrin possessed high degree of encapsulation rate (89 %) and antioxidant activity retention (86 %), which indicated to be a promising method for protecting flavonoids (Busch et al., 2017).

1.10. Effect of flavonoids-rich materials on the qualities of bread

Flavonoids-rich materials have been widely used in bakery products because of their nutritional properties. The positive effects of flavonoids incorporation include increasing antioxidant activities, scavenging food-borne toxins to prolong the shelf life, meanwhile negatively influencing the color, texture and flavor of

baked foods (Xu, Wang, & Li, 2019).

1.10.1. Effect of baking on flavonoids

Anthocyanins are quite sensitive to baking, most of them was lost by up to 50 %, while quercetin-, delphinidin- and myricetin-glucoside exhibited excellent thermal stability, could still be preserved more than 90 % of their content, which could be clearly proved by the study of muffins (fortified with strawberry, raspberry and black currant pomace) (Górnaś et al., 2016). Tartary buckwheat flour was taken to prepare breads, in which the content of rutin and quercetin in the crust decreased by 53 % and only 6 %, respectively (Vogrinčič, Timoracka, Melichacova, Vollmannova, & Kreft, 2010).

1.10.2. Effect of flavonoids on flavor of bakery products

The incorporation of flavonoids-rich materials negatively influenced the aroma of baked foods. The addition of fruit pomace (20 %) (blackcurrant, rowan, rosehip and elderberry) decreased the aroma score of shortbread cookies (Tańska et al., 2016). The prepared bread showed low acceptability, when adding 10 % grape pomace powder into the flour (Hayta, Özuğur, Etgü, & Şeker, 2014). Adding 5 % of ground green coffee bean powder significantly decreased the bread aroma (Zain, Baba, & Shori, 2018). Catechin and genistein effectively suppressed the generation of 2-acetyl-1-pyrroline, methional and 2-acetyl-2-thiazoline, during processing and storage, methional reduced by 80 % and 75 %, respectively, which could be the evidence that flavonoids inhibited the baked aroma (Kokkinidou & Peterson, 2014). However, no difference was found in overall acceptability between the control and the fortified sponge cakes with *Clitoria ternatea* extract (5, 10, 15 and 20 %, w/w) (Pasukamonset et al., 2018).

1.10.3. Effect of flavonoids on the color and texture of baked foods

When added 0.25 % (w/w) epicatechin, quercetin, naringenin into cookies, the chromatic value and hardness of the cookies could significantly increase compared with the control (Zhang, Chen, & Wang, 2014). The addition of 0.2 % quercetin in the bread decreased the specific volume by 9.7 %, but increased the hardness and chewiness by 23.8 % and 30.5 %, individually, in comparison with the control (Lin & Zhou, 2018). Adding of *Clitoria ternatea* extract (5, 10, 15 and 20 %, w/w) to the sponge cakes increased the hardness, adhesiveness, gumminess and resilience of the texture, meanwhile decreased the lightness, redness and yellowness of the color (Pasukamonset et al., 2018).

1.10.4. Effect of flavonoids on the antioxidant activity of baked foods

0.25 % (w/w) of quercetin, epicatechin, naringenin were individually added in the dough to prepare cookies, resulting in the increase of antioxidant activity, however, was much lower than that carried by the quantity of flavonoids originally added to the cookie recipe, especially for quercetin and epicatechin fortification, which might be explained by that they were relatively sensitive to thermal

processing, which suffered serious thermal loss of their antioxidant activity (66 % for quercetin and 52 % for epicatechin, respectively) (Zhang, Chen, & Wang, 2014).

2.Conclusion and future trends

SPLF and the stability of plant flavonoids, encapsulation of flavonoids for improving their stability, and the effect of flavonoids-rich materials on the baked foods were discussed in this review, demonstrated that SPL is a cheap resource of flavonoids, utilization of SPL as a flavonoids-rich additive to bakery food increase its antioxidant activity significantly.

3

Chapter Ⅲ. Optimization of ultrasonic-microwave synergistic extraction of flavonoids from sweet potato leaves by response surface methodology

China is the largest sweet potato producer in the world, after the roots been harvested as the starch resources, equal tons of leaves left to be dealt with. Most previous studies were focus on non-flavonoids part（such as phenolic acids）of polyphenols, and their bioactivities. However, few studies discussed the flavonoids part, which possessed the specific structure of C_6-C_3-C_6. The aim of this chapter was to study the high-tech extraction of flavonoids from sweet potato leaves, flavonoids composition and their antioxidant activities.

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Abstract

Ultrasonic-microwave synergistic extraction was used to extract flavonoids from sweet potato leaves by response surface methodology. The optimal conditions for flavonoids extraction were 1:40 (g/mL) of solid-liquid ratio, 57 °C of extraction temperature, 76 s of extraction time and 72 % (v/v) ethanol for 2 times, the highest yield of flavonoids from sweet potato leaves was 5.13 % (DW). After purification, the flavonoids purity reached to 76.1 (%, DW). The result of high performance liquid chromatography revealed 11 compounds including astragalin (473.8 mg/g, DW), quercetrin (86.5 mg/g, DW), 4,5-chlorogenic acid (76.4 mg/g, DW), isoquercitrin (62.4 mg/g, DW), tiliroside (18.8 mg/g, DW), quercetin (12.5 mg/g, DW), 3,4,5-chlorogenic acid (6.5 mg/g, DW), caffeic acid (6.1 mg/g, DW), kaempferol (6.0 mg/g, DW), myricetrin (5.9 mg/g, DW) and rhamnetin (4.3 mg/g, DW) in sweet potato leaf flavonoids, which possessed good antioxidant activity compared to soy isoflavones, *Ginkgo biloba* extract and propolis flavone. The IC_{50} value of sweet potato leaf flavonoids were 13.26 μg/mL in ferric reducing antioxidant power and 5.41 μg/mL in 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) scavenging capacity, respectively.

Keywords

Ultrasonic-microwave synergistic extraction; Sweet potato leaves; Flavonoids; Response surface methodology; Antioxidant activity

1. Introduction

Flavonoids are a large class of secondary metabolites widely existing in plants. Its basic structure is flavane nuclear parent (2- benzyl ketone), which means two benzene rings (A ring and B ring) are connected by pyran ring (C ring), often containing hydroxyl, methoxy, methyl, isoamyl group and other substituents, widely exists in the fruit, leaf, wood and bark of plant. Flavonoids possess biological activities such as antioxidant activity, improving blood circulation, reducing cholesterol, preventing ultraviolet, inhibiting angiogenesis, anti-bacterial and anti-inflammatory (Chen et al., 2011).

Some studies found that sweet potato leaves (SPL) are rich in flavonoids and the content ranges from 18-73 mg quercetin equivalent/100 g (DW) in 4 cultivars in Taiwan (Liao, Lai, Yuan, Hsu, & Chan, 2011). At present, the flavonoids including quercetin, myricetin, luteolin and apigenin have been found in SPL (Ojong et al., 2008). Peonidin has been found in purple sweet potato cultivar Eshu No. 8 (Zhang, Luo, Zhou, & Zhang, 2018). Meanwhile, it has been reported that SPL flavonoids possess antioxidant activity (Huang et al., 2013), anti-LDL oxidation activity (Taira, Taira, Ohmine, & Nagata, 2013), anti-cytotoxic activity (Liao, Lai, Yuan, Hsu, & Chan, 2011), anti-proliferation activity (Taira, Taira,

Ohmine, & Nagata, 2013).

It is well known that flavonoids are soluble in polar solvents and are usually extracted by aqueous mixtures of organic solvent such as methanol, ethanol and acetone and 70 % ethanol extract had the highest yield of total flavonoid (3.4 mg QE/g DW) (Fu et al., 2016), meanwhile methanol and acetone are toxic and ethanol and its aqueous mixture are the safest solutions for extraction. The extraction of flavonoids from SPL included conventional stirring extraction (Miu et al., 2011), reflux extraction (Liao, Lai, Yuan, Hsu, & Chan, 2011), sonication extraction (Isabelle et al., 2010) and dynamic high pressure microfluidization-assisted extraction (Huang et al., 2013), which were time-consuming, activity-destroying, solvent-wasting and not eco-friendly.

Ultrasonic-microwave synergistic extraction (UMSE) is the extraction method combined with ultrasonic and microwave which is economic and time-saving comparing with other new extraction technologies such as pressurized liquid extraction, supercritical fluid extraction, enzyme assisted extraction and pulsed electric field extraction (Vivekananda, Yogesh, & Hemalatha, 2007). Ultrasound-assisted extraction (UAE) uses ultrasound to produce "cavitation" in liquid, destroying plant cell and cell membrane structure, thus enhancing the penetration of cell contents through cell membrane, which is beneficial to the release and dissolution of flavonoids (Chemat, Zill-E-Huma, & Khan, 2011). The biggest advantage of UAE is to increase the yield significantly such as the yield of chlorogenic acid from artichoke leaves extracted by 80 % methanol for 15 min increased 50 % towards to maceration at ambient temperature, which was close to the yield by boiling extraction (Saleh et al., 2016). Microwave-assisted extraction (MAE) is based on the principle of its penetrating heating, in the microwave field, the absorption difference of various materials makes certain components of the matrix material or some components in the extraction system be selectively heated and get enough energy to escape (Mustapa, Martin, Gallego, Mato, & Cocero, 2015). The biggest advantage of MAE is time-saving, compared to 2 h of conventional reflux extraction of total polyphenols from the leaves of *Pistacia lentiscus*, the MAE could only take 60 s to attain the highest yield of total polyphenols, saving more than 99 % of the time (Dahmoune et al., 2014). Meanwhile, the highest antioxidant activity could be obtained in 70 % ethanol extracts from elecampane (*Inula helenium*) roots collected by UAE which was 86.0 mM TE/g DW in ABTS and 67.0 mM TE/g DW in FRAP (Petkova, Ivanov, Vrancheva, Denev, & Pavlov, 2017).

Response surface methodology (RSM) is an experimental design which utilize minimum trials to optimize the multiple variables experiments, at the same time detecting the interrelationship between the variables comparing to the traditional orthogonal design (Ferreira et al., 2007). Especially for the bioactive compound extraction optimization experiments, RSM shows its superiority which could

flexibly optimize the dependent variables more than one according to the same variables (Derrien, Badr, Gosselin, Desjardins, & Angers, 2017).

In the present study, flavonoids were extracted from SPL by UMSE. The optimal extraction parameters were assessed with RSM. The individual flavone composition was analyzed by high performance liquid chromatography (HPLC). In addition, the antioxidant activity was investigated and compared with some common commercial flavonoids (soy isoflavones, *Gingko biloba* extract and propolis flavone). The aim of this study was to exploit the potentialities of UMSE application in the industrial extraction of flavonoids and the application of SPL as a good source in commercial flavonoids production.

2. Materials and methods

2.1. Materials

According to the previous study in our lab (Sun, Mu, Xi, Zhang, & Chen, 2014), the SPL (leaf and petiole) from sweet potato cultivar Yuzi No. 7 was selected in the present study, which was heart type leaf, contained the highest polyphenols content and highest antioxidant activity among 40 cultivars around China. Fresh SPL were provided by Chongqing Sweet Potato Engineering and Technology Research Center, Chongqing, China. Tubers were sowed in the late period of June and SPL were harvested in the early period of September, 2017, approximate 70 days of growth period at Baiyun Village, Muer Town, Yubei District, Chongqing. Air transported to the lab immediately, washed gently and dried by vacuum freezing and then grounded by ultra-micro pulverizer, sieved through 100 mesh screens and then sealed in aluminum foil bags and stored in the refrigerator at 4 °C for further use.

Quercetin, kaempferol, myricetrin, astragalin, tiliroside, quercitrin, isoquercitrin, rhamnetin, caffeic acid (CA), 4, 5-caffeoylquinic acids (4, 5-CQA) and 3, 4, 5- caffeoylquinic acids (3, 4, 5-CQA) were purchased from An Apoptosis and Epigenetics Company (Houston, USA), the purity of the standards was more than 98 %. Soy isoflavones, *Ginkgo biloba* extract and propolis flavone were purchased from Shanghai Yuanye Biotech Co., Ltd. (Shanghai, China). 2, 4, 6-Tri (2-pyridyl)-1, 3, 5-triazine (TPTZ) and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Solarbio Life Sciences (Beijing, China). Other analytical grade chemicals were obtained from Sinopharm Chemical Reagent (Beijing, China). HPLC grade solvents were purchased from Fisher Chemical (Beijing, China). HPLC water was prepared by the Molgene water purification system from Molecular (Shanghai, China).

2.2. Basic components of sweet potato leaves

The SPL powder was analyzed for moisture, ash, crude protein, crude fat, crude

fiber, carbohydrate and flavonoids content. The moisture content was determined by AOAC method 930.15, ash content was determined by AOAC method 923.03, crude protein was analyzed according to Kjeldahl procedure by AOAC method 955.04, crude fat was determined by AOAC method 960.39 and crude fiber was determined by AOAC method 991.43. Carbohydrate content was calculated by subtracting the sum of ash, crude protein, crude fat and crude fiber from 100.

2.3. Determination of total flavonoids content

Total flavonoids content (TFC) in extraction solution was measured by colorimetric aluminum method described by Shi et al. (2016) with some modifications: 0.1 mL of crude extract was absorbed and added into 10 mL volumetric flask, 4.9 mL of 30 % ethanol was complemented, shook the solution for even and 0.3 mL of 5 % $NaNO_2$ was added later, mixing uniformity and stood still for another 5 min, then 0.3 mL of 10 % $Al(NO_3)_3$ was added to the mixture, mixing evenly and stood still for another 6 min, finally 4.0 mL of 4 % NaOH was added and constant volume to 10 mL with 30 % ethanol, lasting for 10 min and measured the absorbance by spectrophotometer at 320 nm (maximum absorbance), quercetin was taken as the standard.

2.4. Single factor for extraction yield of sweet potato leaf flavonoids

5.00 g SPL powder was extracted by Ultrasonic-Microwave Assisted Extractor CW-2000 (Ultrasonic frequency was 40 kHz, ultrasonic power was 50 W; microwave frequency was 2450 MHz, microwave power was 100 W, microwave wavelength was 0.12 m) (Shanghai Xintuo Analytical Instruments Co., LTD, China) for 1, 2, 3 times, immersed in ethanol solution of 10, 30, 50, 70, 90 and 100 % by solid-liquid ratio (g/mL) of 1:10, 1:20, 1:30, 1:40 and 1:50 at the temperature of 35, 45, 55, 65 and 75 °C continued for 25, 50, 75, 100, 200, 600 and 999 s, centrifuged at 7000 rpm for 30 min, the supernatant was combined and constant the volume to 500 mL with 30 % ethanol. The extraction yield of SPL flavonoids was expressed as gram quercetin equivalent per dry weight of SPL (g QE/100 g DW).

2.5. Response surface methodology optimization for extraction yield of sweet potato leaf flavonoids

Since 2 times and 3 times of extraction had no significant difference and 1:40 (g/mL) of solid-liquid ratio had the highest extraction yield of SPL flavonoids, according to result of single factor experiments, meanwhile took solvent saving into consideration, extraction times and solid-liquid ratio were settled down as 2 and 1:40. Three factors and three levels model consisted of 17 experiments with 5 replicates at central point were shown in Table 3-1. Three experimental factors included extraction temperature (X_1: 45, 55, 65 °C), extraction time (X_2: 50, 75,

100 s) and ethanol concentration (X_3: 50, 70, 90 %), which were optimized by Box-Behnken design, extraction yield of SPL flavonoids (Y) was chosen as the dependent variable. The experimental data were fitted into the equation:

$$Y = Ao + \sum_{i=1}^{3} AiXi + \sum_{i=1}^{3} AiiXi2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} AijXiXj \quad (1),$$

where Y was the response variable, A_0 was the intercept constant, A_i, A_{ii}, A_{ij} were the regression coefficients for linear, quadratic, cross effect of X_1, X_2, X_3, X_i, X_j were coded values of independent variables.

The optimal extraction parameters were calculated by the ‘‘desirability” algorithm (Wong, Li, Li, Razmovski-naumovski, & Chan, 2017). To determine the optimization of extraction, the experimental variables in the Design Expert 8.0 were set as ‘‘in the range”, meanwhile the response variable was set as ‘‘maximize”. The combination of independent variables which made the highest desirability was chosen as the optimal extraction parameters.

2.6. Purification of sweet potato leaf flavonoids

Liquid-liquid extraction is the method to separate materials with different polarity using different organic solvents. Phenolic acids in SPL were mainly chlorogenic acid with different position substituted (Xi, Mu, & Sun, 2015), which hardly dissolved in ethyl acetate while flavonoids could dissolve easily (Lu, Wang, Xie, & Ding, 2013). Therefore, crude ethanol extract obtained by optimum process was vacuum concentrated to half volume and extracted by the same volume of petroleum ether in order to remove chlorophyll and other lipid soluble impurities. Water phase was extracted by ethyl acetate with the same volume, the mixture was shaken thoroughly and stood for 5 min to make the ester phase and water phase separate completely, ethyl acetate phase was vacuum concentrated and evaporated to dryness, which was SPL flavonoids.

2.7. Qualitative and quantitative analysis of sweet potato leaf flavonoids

The SPL flavonoids standards (quercetin, myricetrin, astragalin, tiliroside, quercitrin, isoquercitrin, kaempferol, rhamnetin, CA, 4,5-CQA, 3,4,5-CQA) were qualified and quantified by HPLC (Shimadzu), which was equipped by the system control unit (CBM-20A), the UV detector (SPD-20A), the degaser (DGU-$20A_3$), the liquid infusion unit (LC-20AB), the automatic sampler (SIL-20AC) and the column oven (CTO-20AC), C_{18} was the column (4.6×150mm, 5μm, Inertsil ODS-SP, Shimadzu). The mobile phase was consisted of A and B, A: ultrapure water with 0.5 % (v/v) phosphoric acid, B: 100 % acetonitrile, the elution procedure was performed as follows: 0-15.0 min, 20-65 % B; 15.0-15.1 min, 65-80 % B; 15.1-20.0 min, static 80 % B; 20.0-20.1 min, 80-20 % B; 20.1-25.0 min, static 20 % B, the elution rate was 1.0 mL/min and the injection volume was 20 μL, the oven temperature was kept at 30 °C and detection wavelength was set at 326 nm

according to the method of Xi, Mu, & Sun (2015) with some modification.

The SPL flavonoids and standards were precisely weighed and dissolved in the HPLC grade methanol to prepare the stock solution at the concentration of 1 mg/mL, kept at 4 °C until use. Standard stock solutions were diluted to 50 μg/mL with methanol and 1 mg/mL of SPL flavonoids were filtered through 0.22 μm membrane and injected into HPLC to detect and compare the retention time and response of each peak with standards. The concentration of standards was adjusted to 25, 50, 100, 150, 200 μg/mL for quercetin, myricetrin, tiliroside, quercitrin, isoquercitrin, kaempferol, rhamnetin, CA, 4, 5-CQA, 3, 4, 5-CQA and 100, 200, 300, 400, 500 μg/mL for astragalin according to the response of the SPL flavonoids.

2.8. Antioxidant activity of sweet potato leaf flavonoids

2.8.1. Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) was carried out according to the method of Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne (2006) with some modification, the specific procedure was as follows: 10 mmol/L TPTZ (40 mmol/L HCl was the solvent), 20 mmol/L $FeCl_3$ (0.3 mol/L, pH 3.6 phosphate buffer solution (PBS) was the solvent). 1 portion of TPTZ, 1 portion of $FeCl_3$ and 10 portions of PBS were mixed together and incubated in the water bath at 37 °C away from light, that was how to prepare the FRAP working solution.

The sample solutions with different flavonoid concentration (5, 10, 20 μg/mL) were prepared by diluting with distilled water. 0.15 mL flavonoid sample solution was mixed with 2.85 mL FRAP working solution, incubated for 30 min from light at room temperature and measured the absorbance by spectrophotometer at 593 nm immediately, the blank control was the mixture while flavonoid sample solution was replaced by distilled water. The scavenging rate was calculated according to the equation:

$$\text{Scavenging rate } (\%) = \frac{A0-A}{A0} \times 100\% \quad (2)$$

A_0 was the absorbance of the mixture using distilled water to substitute the flavonoids sample solution at 593 nm and A was the absorbance of the mixture with flavonoids sample solution at 593 nm.

2.8.2. 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) scavenging activity

According to the method of Li, Lin, Gao, Han, & Chen (2012) with some modification: 2.5 mL of ABTS (7.4 mM) was mixed with 2.5 mL of $K_2S_2O_8$ (2.6 mM), the mixture was kept quite away from light at 4 °C for 24 h to produce $ABTS^{\cdot+}$, then diluted with ethanol for about 50 times to make the absorbance reach to 0.70±0.02 at 734 nm which was $ABTS^{\cdot+}$ working solution.

2.0 mL $ABTS^{\cdot+}$ working solution reacted with 1.0 mL flavonoids sample

solution of different flavonoids concentration (5, 10, 20 μg/mL), incubated for 6 min and detected their absorbance at 734 nm, the scavenging rate was calculated according to the equation:

$$\text{Scavenging rate } (\%) = \frac{A0-A}{A0} \times 100\% \quad (3)$$

A_0 was the absorbance of the mixture using ethanol to substitute the flavonoids sample solution at 734 nm and A was the absorbance of the mixture with flavonoids sample solution at 734 nm.

2.9. Data analysis

The data of RSM was processed by Design Expert 8.0, other were analyzed by SPSS (Version 24). Experiments were carried out in triplicate and data was expressed as mean ± standard deviation (SD), $p<0.05$ was considered as there was no significant difference.

3. Results and discussion

3.1. Basic components of sweet potato leaves

The ash, crude protein, crude fat, crude fiber content of SPL was 10.47±0.12, 28.79±0.04, 3.28±0.23, 18.49±0.20 g/100g DW, respectively, and the carbohydrate content was 38.97±0.28 g/100g DW.

3.2. Analysis of single factor experiments

The effects of UMSE variables, including extraction times, solid-liquid ratio, ethanol concentration, extraction temperature and extraction time on the yield (%) of SPL flavonoids were evaluated by single factor experiments [Figure 3-1]. For the effect of extraction times on the yield of SPL flavonoids, there was no significant difference between 2 and 3 times. Considering the solvent saving and follow-up concentration operation simplifying, extracted 2 times would be better. For the effect of varying solid-liquid ratio on the yield of SPL flavonoids, there was a significant increase when solid-liquid ratio increased from 1:10 to 1:40 and then decreased. This might due to the reason that huge solution volume made complete stirring difficult. In addition, the large volume of solution needed to absorb more energy to heat itself up, thus led to inadequate energy diffusion in ultrasonic and microwave field, which would slow down the cell wall breaking and flavonoids leaching (Alara, Abdurahman, & Olalere, 2018). For the effect of ethanol concentration on the yield of SPL flavonoids, the maximum yield was obtained at the concentration of 70 %, following with the flavonoids decreasing when the ethanol concentration kept increasing. Relative high ethanol concentration made the alcohol-soluble substances dissolved into the solvent easily. For the effect of extraction temperature on the yield of SPL flavonoids,

there was a significant rising when the temperature reached to 55 °C compared with 45 °C, which increased 23.1 % then slightly decreased 2.5 % when the temperature kept increasing to 65 °C. Relatively high temperature could lower the viscosity of solvent and made bioactive components transferring through cell membrane more easily from plant matrix (Chew et al., 2011), but high temperature environment could accelerate the bioactive compounds degradation (Alara, Abdurahman, & Olalere, 2018). The yield of SPL flavonoids achieved to maximum when the extraction time lasting to 75 s compared with 50 s and 100 s, which were 9.1 % and 2.8 % higher, respectively, ultrasonic and microwave power needed time to transfer the energy to the matrix.

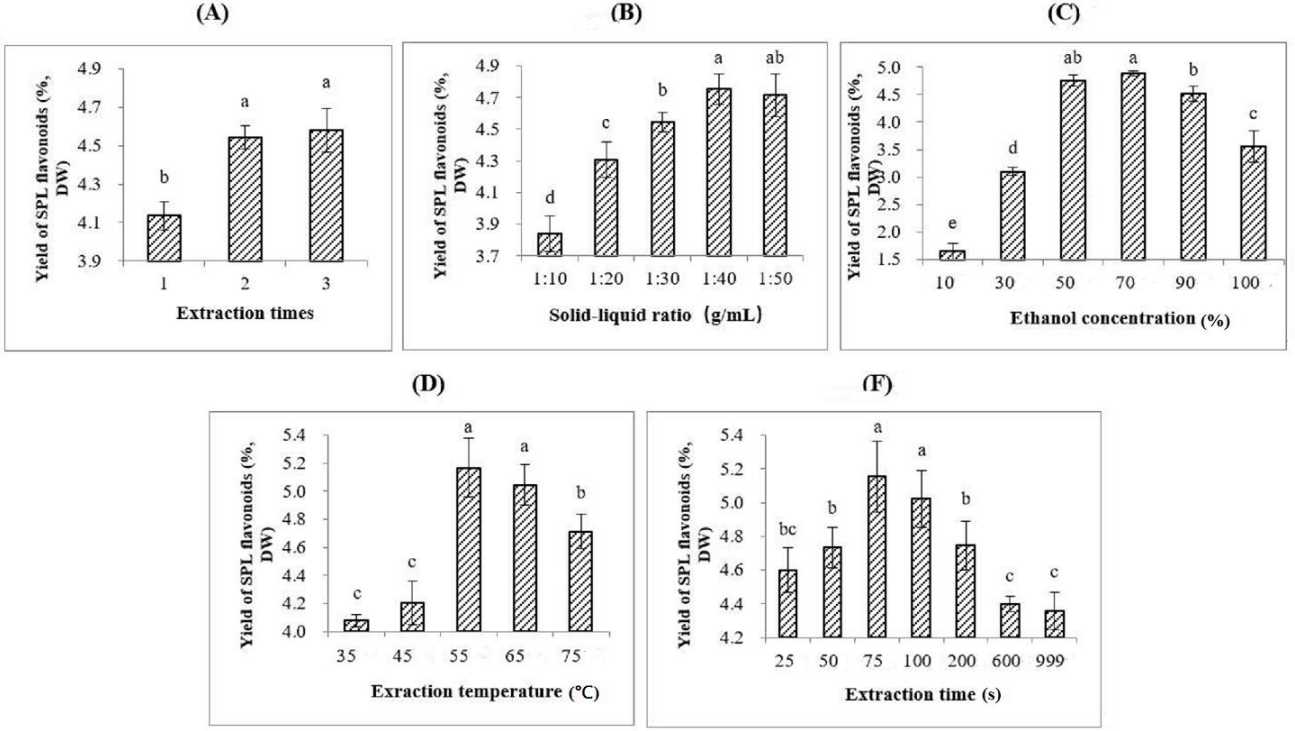

Figure 3-1 (A) Extraction times on the yield of SPL flavonoids. (B) Solid-liquid ratio (g/mL) on the yield of SPL flavonoids. (C) Ethanol concentration on the yield of SPL flavonoids. (D) Extraction temperature on the yield of SPL flavonoids. (E) Extraction time on the yield of SPL flavonoids.

Columns and bars represent mean ± SD (n = 3). Different letters (a-e) mean values are significantly different ($p < 0.05$).

3.3. Statistical analysis and model fitting of response surface methodology

The advantage of Box-Behnken design was trying minimum times to obtain the optimal model of the experiment. The experimental and predicted values were shown in Table 3-1. The analysis of variance (ANOVA) was summarized in Table 3-2. *P*-value of the model was below 0.0001, indicated the model was approximate to the reality ($p < 0.05$). X_1, X_2, X_3, X_1X_2, X_1X_3, X_1^2 and X_3^2 were below 0.05, which showed these factors were quite significant to the yield of SPL flavonoids.

The lack of fit was insignificant ($p = 0.67 > 0.05$), represented the model was significant compared with the pure error, which might due to the noise, the model could predict the extraction process quite well. The $R^2 = 0.99$ indicated the model could explain 99 % of the real extraction process, there was only 1 % of the total variation could not be explained (Wai, Alkarkhi, & Easa, 2010). Moreover, the adjusted $R^2 = 0.97$ showed the high significance of the model, after deleting the insignificant parameters, there was still 97 % of the data could be explained by this model.

Table 3-1 Experimental and predicted values of extraction efficiency in Box-Behnken design

Factor	Coded symbol	Level		
		-1	0	1
Extraction temperature	X_1	45	55	65
Extraction time	X_2	50	75	100
Ethanol concentration	X_3	50	70	90

Run	Independent variables			Measured Y	Predicted Y
	X_1	X_2	X_3		
1	-1	-1	0	4.00±0.16	3.91
2	1	-1	0	4.67±0.11	4.62
3	-1	1	0	4.28±0.17	4.33
4	1	1	0	4.17±0.11	4.25
5	-1	0	-1	4.04±0.16	3.92
6	1	0	-1	4.74±0.09	4.67
7	-1	0	1	4.50±0.07	4.35
8	1	0	1	4.84±0.16	4.94
9	0	-1	-1	4.48±0.12	4.38
10	0	1	-1	4.15±0.19	4.30

11	0	-1	1	4.65±0.17	4.77
12	0	1	1	3.75±0.08	3.73
13	0	0	0	5.06±0.17	5.02
14	0	0	0	5.16±0.19	5.02
15	0	0	0	4.93±0.12	5.02
16	0	0	0	4.99±0.13	5.02
17	0	0	0	4.96±0.22	5.02

X_1: Extraction temperature (°C); X_2: Extraction time (s); X_3: Ethanol concentration (%);
Y: Yield (%) of SPL flavonoids

Table 3-2 Analysis of variance (ANOVA) for response surface model

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	3.35	9	0.37	54.31	< 0.0001	significant
A-Temperature	1.24	1	1.24	180.64	< 0.0001	
B-Time	0.14	1	0.14	19.74	0.0030	
C-Concentration	0.041	1	0.041	6.05	0.0435	
AB	0.28	1	0.28	40.26	0.0004	
AC	0.32	1	0.32	46.63	0.0002	
BC	0.022	1	0.022	3.29	0.1127	
A^2	1.49	1	1.49	217.72	< 0.0001	
B^2	0.029	1	0.029	4.19	0.0800	
C^2	0.55	1	0.55	80.81	< 0.0001	
Residual	0.048	7	6.846E-003			
Lack of Fit	0.014	3	4.708E-003	0.56	0.6707	not significant
Pure Error	0.034	4	8.450E-003			
Cor Total	3.39	16				
R-Squared			0.99			
Adj R-Squared			0.97			
Pred R-Squared			0.92			

Three-dimensional and contour plots were chosen to represent the predicted model and the interaction between different parameters. The plots showed the interaction between two factors while another was kept at medium level, which were exhibited in Figure 3-2.

3.4. Interaction of variables on yield of sweet potato leaf flavonoids

The yield of SPL flavonoids, which ranged from 3.75 to 5.16 % (Table 3-1), depending on the extraction temperature, extraction time and ethanol concentration and their interaction (Table 3-2).

Extraction temperature was the most important factor (p-value < 0.0001) for the extraction, which affected the yield of SPL flavonoids effectively. When the extraction temperature rising from 45 °C to 65 °C, the yield of SPL flavonoids showed first rising then falling tendency, which clearly exhibited in Figure 3-2(A,C,D,F). The increase of extraction temperature could significantly enhance the flavonoids dissolving, but high temperature would also affect the stability of flavonoids and induced them degradating rapidly.

Extraction time was the second critical factor for the extraction. We could see from Figure 3-2(B,C,E,F) that increased time from 50 s to 100 s could enhance the microwave and ultrasonic reacting with the sample, and accelarate the solvent and eneregy penetrating into the matrix, but too much time of severe reaction would definitely destroy the chemical bonds of flavonoids and induced them dissociating (Wong, Li, Li, Razmovski-naumovski, & Chan, 2017).

Ethanol concentration was also vital for the extraction because it decided the polarity of the whole system. When the ethanol concentration changed from 50 % to 90 %, the yield showed first rising then falling tendency as we saw from Figure 3-2(A,B,D,E). Flavonoids were a class of weak polarity organic compounds, which could easily dissolved in high percentage of ethanol. If the ethanol concentration was not high enough, there were a large number of water-soluble impurities escaped into the solution, however, if the ethanol concentration was too high, which meant the polatiry of the solution was not high enough to dissolve the flavonoids completely (Fu et al., 2016).

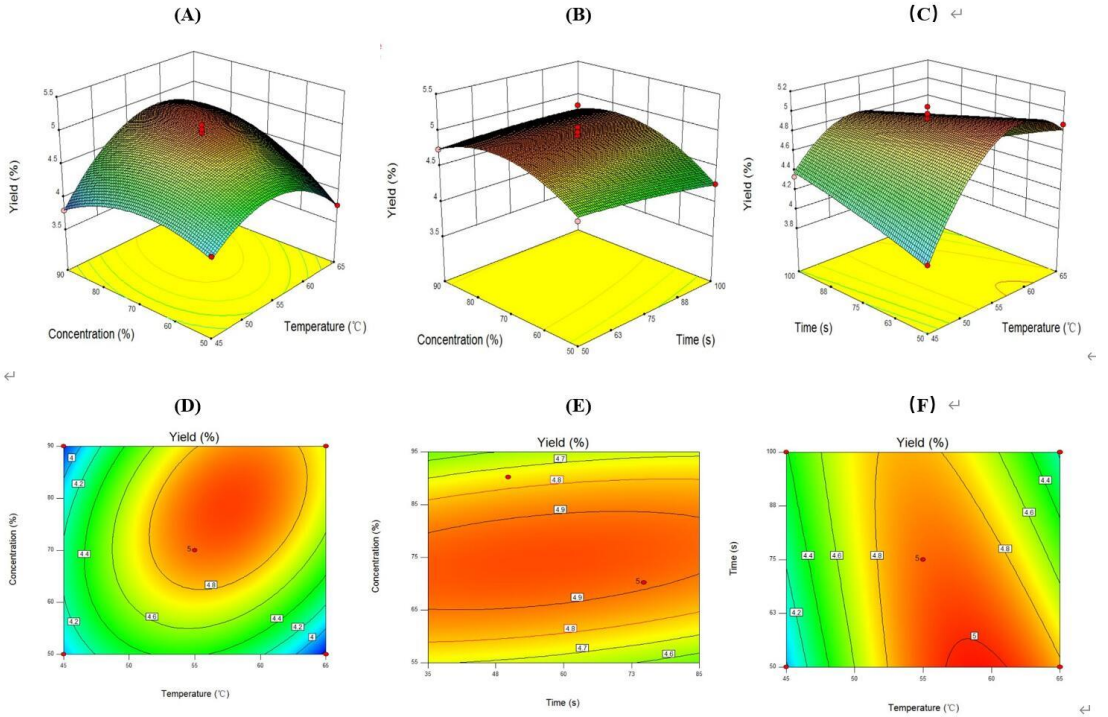

Figure 3-2 Three dimensional and contour plots of extraction temperature, extraction time and ethanol concentration on the yield of SPL flavonoids (A, D: Interaction of extraction temperature and ethanol concentration on the yield of SPL flavonoids; B, E: Interaction of extraction time and ethanol concentration on the yield of SPL flavonoids; C, F: Interaction of extraction temperature and extraction time on the yield of SPL flavonoids).

3.5. Optimization of extraction condition and method validation

The quadratic regression model for SPL flavonoids resulted in the R-Squared 0.99, indicating that only 1 % of the variation couldn't be explained by this model. The adjusted R-Squared was 0.97, and the predicted R-Squared was 0.92, confirmed that the model was highly significant. The lack of fit associated with *P* value of 0.67, indicated a non-significance, supporting the model fitted with the data. A *P*-value lower than 0.0001 was found, demonstrated high significance of the regression model. Neglected the insignificant terms, the model was given by the following second order polynomial equation:

$$Y = 5.02 + 0.65 \times X_1 + 0.065 \times X_2 + 0.047 \times X_3 - 0.00105 \times X_1X_2 + 0.001412 \times X_1X_3 - 0.00595 \times X_1^2 - 0.0009062X_3^2 \quad (4)$$

The small difference between the yield predicted by the model and the experimental results, due to the ANOVA of the model and the insignificant lack of fit, all indicated that the accuracy and the fitness of the model was highly satisfactory.

According to the results and discussion, the optimum extraction was required to find the desire condition for maximizing the yield of SPL flavonoids, which was extraction temperature of 57 °C, extraction time of 76 s, ethanol concentration of 72 %, solid-liquid ratio of 1:40 and extracted 2 times according to the above condition. The maximum yield of SPL flavonoids was 5.13 %, the predicted maximum yield fitted by the software was 5.10 %, which correlated quite well with the actual data, demonstrated the model could simulate the reality, and the optimum condition was quite valid for this experiment.

3.6. Purification of sweet potato leaf flavonoids

The crude solution (extracted by optimum parameters) went through liquid-liquid extraction to obtain petroleum ether phase, ethyl acetate phase and water phase. The purity of SPL flavonoids in crude ethanol extract was 16.8 (%, DW), and in ethyl acetate phase it rose up to 76.1 (%, DW).

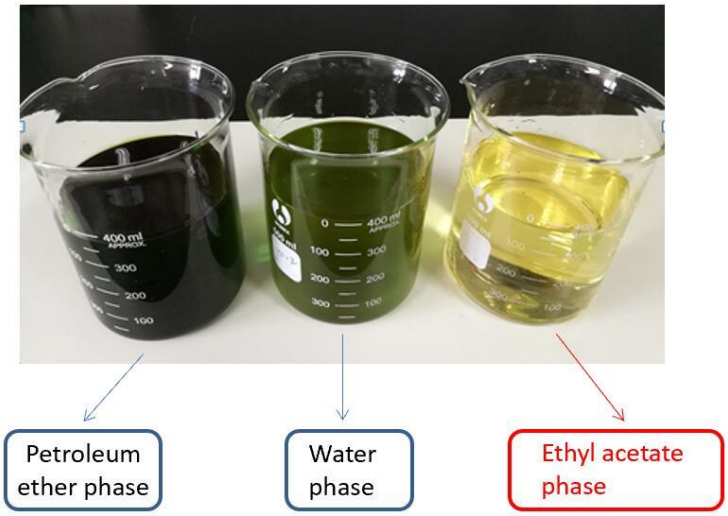

Figure 3-3 Petroleum ether phase, ethyl acetate phase and water phase of SPL crude ethanol extract

3.7. Qualitative and quantitative analysis of sweet potato leaf flavonoids

Qualitative and quantitative analysis were shown in Figure 3-4, 3-5 and Table 3-3. There were 8 flavonoids, 2 phenolic acids and caffeic acid detected in SPL flavonoids, which were CA, 4,5-CQA, myricetrin, 3,4,5-CQA, isoquercitrin, astragalin, quercitrin, tiliroside, quercetin, kaempferol and rhamnetin, according to the references and comparing with the retention time of standards (Ojong et al., 2008; Anastácio & Carvalho, 2013; Xi, Mu, & Sun, 2015).

Astragalin was the highest amount of flavonoids in SPL flavonoids, which was 473.8 mg/g DW, followed by quercitrin (86.5 mg/g DW), 4,5-CQA (76.4 mg/g DW), isoquercitrin (62.4 mg/g DW), tiliroside (18.8 mg/g DW), quercetin (12.5 mg/g DW), 3,4,5-CQA (6.5 mg/g DW), CA (6.1 mg/g DW), kaempferol (6.0 mg/g), myricetrin (5.9 mg/g DW) and rhamnetin (4.3 mg/g DW). Ojong et al. (2008) found out that apigenin, kaempferol, luteolin, quercetin and myricetin existed in SPL, which usually grown in southern United States. Anastácio & Carvalho (2013) also took a research on purple SPL flavonoids, luteolin, myricetin and quercetin were detected. There was some difference in our results, which might due to the cultivar and physiological stage differences, compared with other studies.

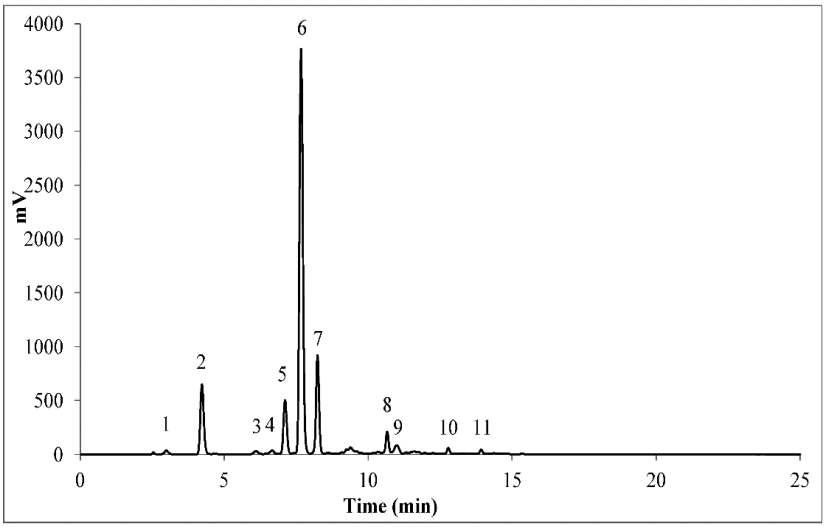

Figure 3-4 The HPLC of SPL flavonoids. Peak 1: CA, peak 2: 4, 5-CQA, peak 3: myricetrin, peak 4: 3, 4, 5-CQA, peak 5: isoquercitrin, peak 6: astragalin, peak 7: quercitrin, peak 8: tiliroside, peak 9: quercetin; peak 10: kaempferol, peak 11: rhamnetin.

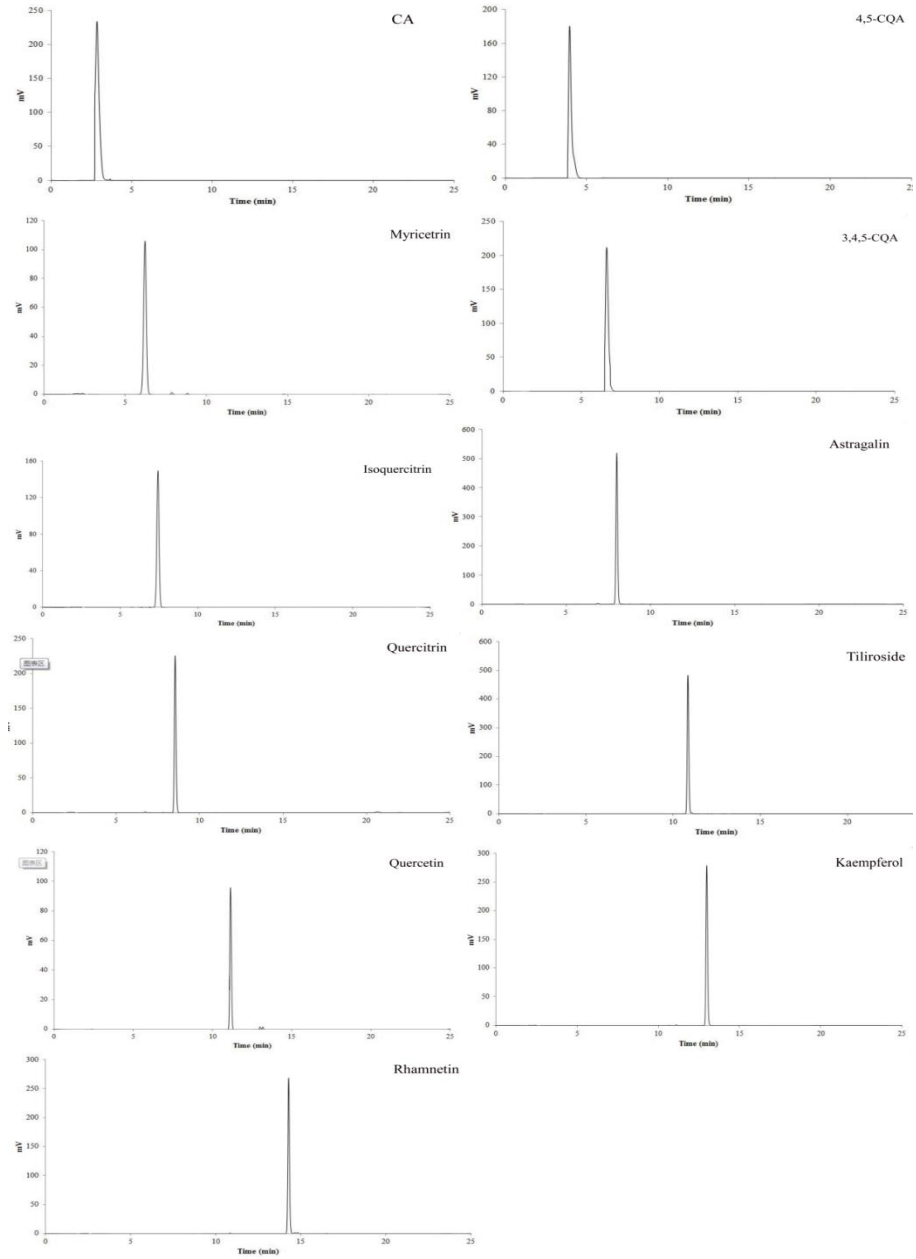

Figure 3-5 Chromatography of standards (CA, retention time (RT) 2.98 min; 4,5-CQA, RT 4.20 min; Myricetrin, RT 6.10 min; 3,4,5-CQA, RT 6.67 min; Isoquercitrin, RT 7.10 min; Astragalin, RT 7.65 min; Quercitrin, RT 8.21 min; Tiliroside, RT 10.62 min; Quercetin, RT 10.97 min; Kaempferol, RT 12.75 min; Rhamnetin, RT 13.88 min; All samples' concentration was adjusted to 50 μg/mL except astragalin, the latter concentration was 100 μg/mL)

Table 3-3 Qualitative and quantitative analysis of SPL flavonoids by HPLC

No.	RT (min)	Identification	Standard curve	R^2	Peak area	Content (mg/g DW)
1	2.98	CA	y=114133x-292197	0.9993	323630±10611	6.1±0.2[g]
2	4.198	4,5-CQA	y=72434x-293019	0.9984	3611795±23637	76.4±0.5[c]
3	6.099	myricetrin	y=29502x-120219	0.9979	347722±5894	5.9±0.1[g]
4	6.662	3,4,5-CQA	y=74873x-468134	0.9958	321973±9907	6.5±0.2[g]
5	7.095	isoquercitrin	y=34321x-101233	0.9996	3293152±21110	62.4±0.4[d]
6	7.642	astragalin	y=40295x-219384	0.9995	25006624±385286	473.8±7.3[a]
7	8.207	quercitrin	y=35135x-132682	0.9991	5064650±40986	86.5±0.7[b]
8	10.613	tiliroside	y=61425x-236252	0.9923	992268±15834	18.8±0.3[e]
9	10.965	quercetin	y=33083x-92209	0.9983	664719±10636	12.5±0.2[f]
10	12.735	kaempferol	y=44840x-122699	0.9990	316534±10551	6.0±0.2[g]
11	13.879	rhamnetin	y=42177x-71175	0.9979	229180±5330	4.3±0.1[h]
Sum						759.2±7.3

[a] Different letter (a-h) mean values are significantly different ($p < 0.05$).

[b] SPL, sweet potato leaves; HPLC, high performance liquid chromatography; RT, retention time; DW, dry weight; CA, caffeic acid; CQA, caffeoylquinic acids.

3.8. Antioxidant activity of sweet potato leaf flavonoids

Antioxidant activity of SPL flavonoids was evaluated by FRAP and ABTS method. The results were shown in Figure 3-6. The flavonoids purity of SPL flavonoids, soy isoflavones, *Ginkgo biloba* extract and propolis flavone were 76.1^{a}, 59.3^{b}, 45.8^{c} and 56.9^{b} (%, DW), respectively.

Figure 3-6(A) showed the Fe^{3+} scavenging capacities of SPL flavonoids and soy isoflavones, *Ginkgo biloba* extract and propolis flavone at the concentration of 5, 10 and 20 μg/mL, SPL flavonoids had the significant advantage than the other three positive controls. The Fe^{3+} scavenging capacities of SPL flavonoids was 17.7 %, 37.4 % and 76.2 % at the concentration of 5, 10 and 20 μg/mL, which was approximately 0.3 times and 3.1 times higher than *Ginkgo biloba* extract and propolis flavone at the concentration of 5 μg/mL, 0.2 times and 2.3 times higher than *Ginkgo biloba* extract and propolis flavone at the concentration of 10 μg/mL and 0.2 times and 1.8 times higher than *Ginkgo biloba* extract and propolis flavone at the concentration of 20 μg/mL. Soy isoflavones showed poor activity in this experiment. IC_{50} values of SPL flavonoids, soy isoflavones, *Ginkgo biloba* extract and propolis flavone were 13.3^{a}, 143.7^{d}, 16.0^{b}, 34.0^{c} μg/mL, respectively.

Figure 3-6(B) showed $ABTS^{\cdot+}$ scavenging capacity at the flavonoid concentration of 5, 10 and 20 μg/mL. The $ABTS^{\cdot+}$ scavenging rate of SPL flavonoids at the concentration of 5 μg/mL was 38.2 %, which was 61.3 %, 59.9 % and 132.4 % of scavenging capacity, compared with the corresponding concentration of soy isoflavones, *Ginkgo biloba* extract and propolis flavone. When the concentration reached to 10 μg/mL, the $ABTS^{\cdot+}$ scavenging rate of SPL flavonoids achieved to 85.6 %, which was 17.1 % and 24.0 % higher than soy isoflavones and propolis flavone and 5 % a little lower than *Ginkgo biloba* extract. When the concentration reached up to 20 μg/mL, the $ABTS^{\cdot+}$ scavenging rate of SPL flavonoids achieved to 91.5 %, which was 14 % higher than soy isoflavones, and had no significant difference between *Ginkgo biloba* extract and propolis flavone. IC_{50} values of SPL flavonoids, soy isoflavones, *Ginkgo biloba* extract and propolis flavone were 5.4^{c}, 2.1^{a}, 3.2^{b} and 7.0^{d} μg/mL, respectively.

The antioxidant activity was related to many factors, including the structure of the flavonoids from different sources, other ingredients in the flavonoids samples (Chen et al., 2017). For example, it has been reported that daidzin and genistin were the dominant flavonoids in soy isoflavones (Szymczak et al., 2017), quercetin, kaempferol and isorhamnetin were the dominant flavonoids in *Ginkgo biloba* extract (Sati, Dhyani, Bhatt, & Pandey, 2019), rutin, isorhamnetin, kaempferol, luteolin, naringenin and quercetin-3-glucoside were the main flavonoids detected in propolis flavone (Andrade et al., 2018).

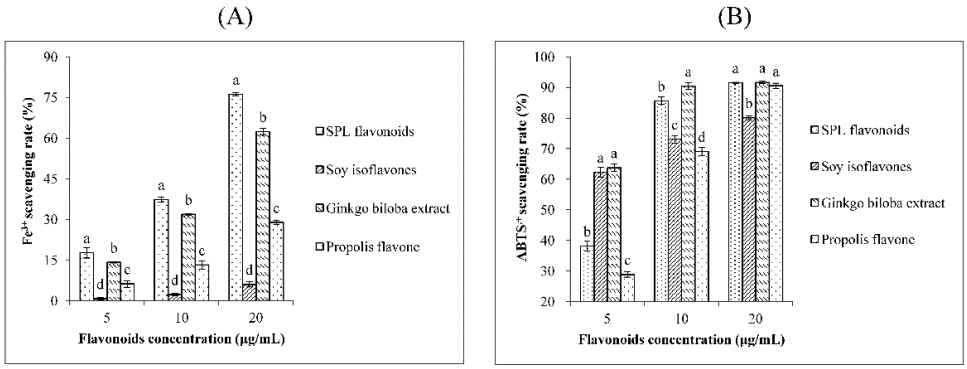

Figure 3-6 Antioxidant activities of SPL flavonoids. (A) Fe^{3+} scavenging capacity. (B) $ABTS^{\cdot+}$ scavenging capacity

Different letters (a-d) meant values were significantly different ($p < 0.05$)

SPL, sweet potato leaves; ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

4. Conclusion

The results showed that the highest yield of SPL flavonoids at the optimum condition (extraction temperature was 57 °C, extraction time was 76 s, ethanol concentration was 72 %, solid-liquid ratio (w/v) was 1:40 and extracted 2 times) was 5.13 %, which was confirmed through the validation experiment. The crude extract was selected by petroleum ether and ethyl acetate, the latter phase was collected to obtain the SPL flavonoids with the purity of 76.1 (%, DW). HPLC analysis showed that the SPL flavonoids mainly consisted of astragalin, quercetrin, 4, 5-CQA, isoquercitrin, tiliroside, quercetin, 3, 4, 5-CQA, CA, kaempferol, myricetrin and rhamnetin, possessing high antioxidant capacity. Meanwhile, UMSE was an economic and efficient method to obtain SPL flavonoids, which was time-saving, easy to scale up at the pilot test and industrial scale, providing a potential possibility for industrial extraction of flavonoids from SPL, enriching the flavonoids health products market.

4

Chapter Ⅳ. Effects of processing and storage conditions on the stability of sweet potato (*Ipomoea batatas* L.) leaf flavonoids

The content, composition and antioxidant activity of sweet potato leaf flavonoids were determined in chapter 3. The application into food industry depending on their stability during processing and preservation, which haven't been studied yet. Therefore, the aim of this chapter was to figure out the influence of heat, pressure, light, pH, temperature on the stability of sweet potato leaf flavonoids.

Liu, J., Mu, T., Sun, H., & Fauconnier, M. L. (2020). Effects of processing and storage conditions on the stability of sweet potato (*Ipomoea batatas* L.) leaf flavonoids. *International Journal of Food Science and Technology*, *55*(5), 2251–2260

Abstract

The effect of heat treatment, high hydrostatic pressure (HHP) treatment, pH, light, temperature and simulated digestion on the stability of sweet potato leaf flavonoids (SPLF) was studied. Results showed that heat treatment at 75 °C for 90 min or HHP treatment at 600 MPa for 30 min didn't cause significant effect on SPLF. Heat treatment at 100 °C for 60 min and 90 min led to a decrease in antioxidant activity by 20 % and 25 % respectively, while pH 7.0 and 8.0 significantly decreased amount of SPLF by approximately 75 %, decreased antioxidant activity by about 30 % and 47 % separately. Light treated samples recorded a decrease in SPLF by 52 % and antioxidant activity by 24 %. No significant effect on SPLF was observed for samples stored at -18, 4 °C or room temperature (RT). The retention of flavonoids and antioxidant activity was 45 % and 56 %, individually in SPLF after simulated digestion.

Keywords

Sweet potato leaf flavonoids; Ultrasonic-microwave synergistic extraction; Processing storage stability; Simulated digestion; Individual flavonoid; Antioxidant activity

1. Introduction

The world sweet potato production is about 112.8 million tons, China accounts for 64 % of the total production (FAOSTAT, 2017). After harvesting the roots, there is almost equal amount of sweet potato leaves (SPL) waiting to be discarded. SPL is rich in flavonoids, possessing high antioxidant activity which could slow down chronic diseases, such as rheumatism, cardiovascular diseases and cancers (Chen, Fan, Wu, Li, & Guo, 2019).

The stability of flavonoids during processing and storage was critical to factories and consumers. In order to attain the desired nutritional and functional effects, heat treatment and HHP treatment were usually employed to attain processing and bactericidal effect (Li, Chen, Zhang, & Fu, 2017). Compared to traditional heat treatment, HHP treatment had less adverse effects on flavonoids during processing or preservation (Khan et al., 2018).

Zeng et al. (2019) studied the influence of hydrothermal treatment at 60, 80 and 100 °C on total flavonoid content (TFC), found out that TFC decreased by 3.5-5.8 % after 30 min, with a slight decrease in the antioxidant activity (<10 %). Fernández-Jalao, Sánchez-Moreno, & De Ancos (2019) recorded a 30 % of increase in TFC for Spain apples after HHP treatment (400 MPa, for 5 min). Echeverry, Medina, Costa, & Aragón (2018) revealed a decrease of TFC in *Passiflora quadrangularis* leaf extracts by 28.2 % under alkali hydrolysis, and

by 7.8 % under acid hydrolysis. A decrease (>54 %) in total anthocyanin content (TAC) was reported when been exposed to sunlight after 3 months (Bakowska, Kucharska, & Oszmiański, 2003). Furthermore, TFC in pomegranate juice, pulp and extract showed a decrease by 13.8 %, 16.4 % and 26.7 %, respectively, after simulated gastric digestion (Mosele, Macià, Romero, Motilva, & Rubió, 2015). Industrial processed tomato sauce with higher amounts of flavonoids showed a decrease in antioxidant activity by 60.3 %, 60.5 % and 75.3 %, respectively, after digestion in oral, stomach and intestinal phase (Tomas et al., 2017). Based on the literatures, flavonoids were sensitive to digestion, environmental and processing conditions, with the rate of degradation varying with the severity of the process.

The overall stability of SPLF during processing and storage is not well understood, thus, the purpose of this study was to elucidate the effects of different treatments (heat, HHP, pH, light, temperature and simulated digestion) on the stability of SPLF. The information obtained from this research will be useful in providing the stability profile of SPLF with potential application as a food additive.

2. Materials and methods

2.1. Materials

The SPL (Cultivar: Yuzi No.7), which exhibited the highest amount of polyphenols content and strongest antioxidant activity, when assessed in the leaves among 40 sweet potato cultivars harvested in China, was chosen for this study, based on previous work (Sun, Mu, Xi, Zhang, & Chen, 2014). The leaves were provided by Chongqing Sweet Potato Engineering & Technology Research Center (Chongqing, China), transported to the lab by plane, washed, dried by vacuum freezer and grounded by ultra-micro pulverizer. The grounded material was sieved through the 100 meshes screen, sealed in aluminum foil bags and stored at 4 °C in the refrigerator.

Myricetrin, isoquercitrin, astragalin, quercitrin, tiliroside, quercetin, kaempferol, rhamnetin were obtained from An Apoptosis and Epigenetics Company (Houston, USA). The purity of the individual flavonoid was more than 98 %. α-amylase with the activity ≥ 16 units/mg, pepsin with the activity ≥ 250 units/mg, trypsin with the activity ≥ 1500 units/mg and bile salt with the purity of choleic acid ≥ 60 % were purchased from Sigma Chemical Co (St. Louis, MO, USA). 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was obtained from Solarbio Life Sciences (Beijing, China). High performance liquid chromatography (HPLC) grade solvents were obtained from Fisher Chemical (Beijing, China). HPLC grade water was prepared by the Molgene water purification system from Molecular

(Shanghai, China). All other analytical grade reagents were purchased from Sinopharm Chemical Reagent (Beijing, China).

2.2. Preparation of sweet potato leaf flavonoids

Briefly, 50 g of SPL was extracted 2 times at 57 °C for 76 s using ultrasonic-microwave assisted extractor CW-2000 (Ultrasonic frequency and power was 40 kHz and 50 W, microwave frequency and power was 2450 MHz and 100 W, respectively, and microwave wavelength was 0.12 m) (Shanghai Xintuo Analytical Instruments Co., LTD, China). Thereafter, the crude ethanol extract attained above was extracted by petroleum ether and ethyl acetate to get 1.55 g SPLF with the purity of 76.1 (%, DW) (Liu, Mu, Sun, & Fauconnier, 2019). Based on the solubility of flavonoids and antibacterial capacity of ethanol, 1.4 g SPLF was dissolved into 70 mL ethanol (approximately 5 % of the total volume), and divided into 28 portions (2.5 mL for each portion) for processing and storage experiment. In addition, 0.15 g SPLF was dispersed into 15 mL of distilled water, and used for simulated digestion study.

2.3. Preparation of sample solutions for processing and storage stability evaluation

SPLF was diluted by distilled water to obtain the control sample, heat treated samples and HHP treated samples. Heat treated samples were placed in plug test tubes, and HHP treated samples were placed in high-pressure bags. SPLF was prepared at different pH solutions using phosphate buffer solution (PBS) (pH 3.0, 5.0, 7.0, 8.0). The SPLF used to study the storage stability at -18 °C was diluted by 40 % ethanol, in which the freezing point was -23 °C. For other storage stability studies, the SPLF was prepared using distilled water. The final concentration of all the SPLF samples prepared was adjusted to 1 mg/mL.

2.4. Processing and storage treatment on the sample solutions

Processing treatment on SPLF was performed as explained by Cao, Cai, Wang, & Zheng (2018), storage treatment on SPLF was carried out according to the method of Struck, Plaza, Turner, & Rohm (2016). Heat treatment on SPLF was done at 50, 75, 100 °C for 30, 60, 90 min in the oven to simulate enzyme inactivation, heating and boiling process. HHP treatment on SPLF was done at 200, 400 and 600 MPa for 10, 20 and 30 min at RT, using high pressure equipment (HPP. L3, Huataisenmiao Bioengineering Technology Co., Ltd., Tianjin, China) to evaluate the stability of SPLF after HHP treatment comprehensively. However, industrial level conditions would be much milder, usually no more than 10 min, as such long time was not need to inactivate microorganism (main application of HHP), which might have less effect on SPLF.

SPLF was prepared at different pH (3.0, 5.0, 7.0, 8.0) which simulated acid, weak acid, neutral and weak alkaline environment. The samples were placed at RT away from light, following with the method as described by Bradwell, Hurd, Pangloli, McClure, & Dia (2018). SPLF stored under light was placed at laboratory in the presence of natural light, while SPLF stored without light was kept away from light under the same environment (Holzwarth, Wittig, Carle, & Kammerer, 2013). Also, SPLF was kept at -18 °C and 4 °C in the refrigerator, while other samples were kept at RT (≈20 °C) in the laboratory away from light (Touati, Tarazona-Díaz, Aguayo, & Louaileche, 2014).

2.5. Simulated digestion in vitro of sweet potato leaf flavonoids

Simulated digestion was carried out according to the standard static *in vitro* digestion method described by Minekus et al. (2014) with some modification. The SPLF, 15 mL, at the concentration of 10 mg/mL, was mixed with 10.5 mL simulated salivary fluid (SSF) (pH 7.0) and 1.5 mL α-amylase (1500 U/mL). Then, 75 μL $CaCl_2$ and 2.9 mL distilled water was added, and the pH adjusted to 7.0. The contents were agitated for 2 min at 37 °C to simulated oral digestion. After that, 25 mL oral digestive juice was mixed with 15 mL simulated gastric fluid (SGF) (pH 3.0) and 3.8 mL pepsin (25000 U/mL). Following, 11.8 μL $CaCl_2$ and 1.6 mL distilled water was added. Next, the pH was adjusted to 3.0 and agitated continuously for 2 h at 37 °C to simulate gastric digestion. The gastric digested juice (36.5 mL) was mixed with 20 mL simulated intestinal fluid (SIF) (pH 7.0), 7.5 mL trypsin (500 U/mL), 5.5 mL bile salts (10 mg/mL), 85 μL $CaCl_2$ and 2.8 mL distilled water. Subsequently, the pH was adjusted to 7.0 and agitated continuously for another 2 h at 37 °C to simulated intestinal digestion.

2.6. Determination of total flavonoids, antioxidant activity and individual flavonoid

TFC was determined by the method of Lim, Huang, Zhao, Zou, & Yan (2016) with some modifications. The result was calculated by the standard curve of quercetin in the linear range of 0-0.05 mg/mL. TFC was expressed in milligrams of quercetin equivalent per g of dry weight (mg QUE/g DW). The retention of TFC was calculated by the equation as follows:

$$R_1\ (\%) = C_1/C_0 \times 100\ \%\ (1)$$

where R_1 was the retention of TFC in the sample after processing, storage or digestion, C_1 was the TFC in the sample after processing, storage or digestion, C_0 was the TFC in the initial sample.

The antioxidant activity of SPLF was evaluated by the method of Li, Lin, Gao, Han, & Chen (2012) with some modifications. Equal volume of ABTS (7.4 mM) and $K_2S_2O_8$ (2.6 mM) were mixed thoroughly, and kept in the dark for 24 h at 4 °C

to produce $ABTS^{\cdot+}$, then adjusted the absorbance of $ABTS^{\cdot+}$ to 0.70±0.02 at 734 nm by distilled water, the working solution was obtained. Then, 2.0 mL $ABTS^{\cdot+}$ working solution reacted with 1.0 mL sample solution in which flavonoids concentration was adjusted to 5 μg/mL, incubated for 6 min and detected the absorbance at 734 nm, the retention of antioxidant activity was calculated by the equation as follows:

$$R_2\ (\%) = A_1/A_0 \times 100\ \%\ (2)$$

Where R_2 was the retention of $ABTS^{\cdot+}$ scavenging rate in the sample after processing, storage or digestion, A_1 was the $ABTS^{\cdot+}$ scavenging rate in the sample after processing, storage or digestion, A_0 was the $ABTS^{\cdot+}$ scavenging rate in the initial sample.

Quantitative analysis of individual flavonoid was performed by HPLC (Shimadzu LC-20A) using the C_{18} column (4.6×150 mm, 5 μm, Inertsil ODS-SP, Shimadzu), according to the method of Xi, Mu, & Sun (2015) with some modification. Standard curves obtained in our previous studies (Liu, Mu, Sun, & Fauconnier, 2019), were used to calculate the content of individual flavonoid.

2.7. Data analysis

All the experiments were carried out in triplicate, and data was expressed as mean ± SD. Statistic difference among diversely treated samples was calculated through one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. The IBM SPSS 24.0 (Chicago, USA) software was used, and statistical difference considered significant when $P < 0.05$.

3.3. Results and discussion

3.1. Stability of sweet potato leaf flavonoids after heat treatment

As shown in Figure 4-1(A, B), no significant difference was reported for the flavonoids content and antioxidant activity in SPLF for heat treated samples at 50 and 75 °C for 30, 60 even 90 min. However, at the temperature of 100 °C for 60 and 90 min, a significant decrease in the flavonoids content and antioxidant activity was observed. The result showed that the heat treatment stability of SPLF was excellent at temperature not higher than 75 °C.

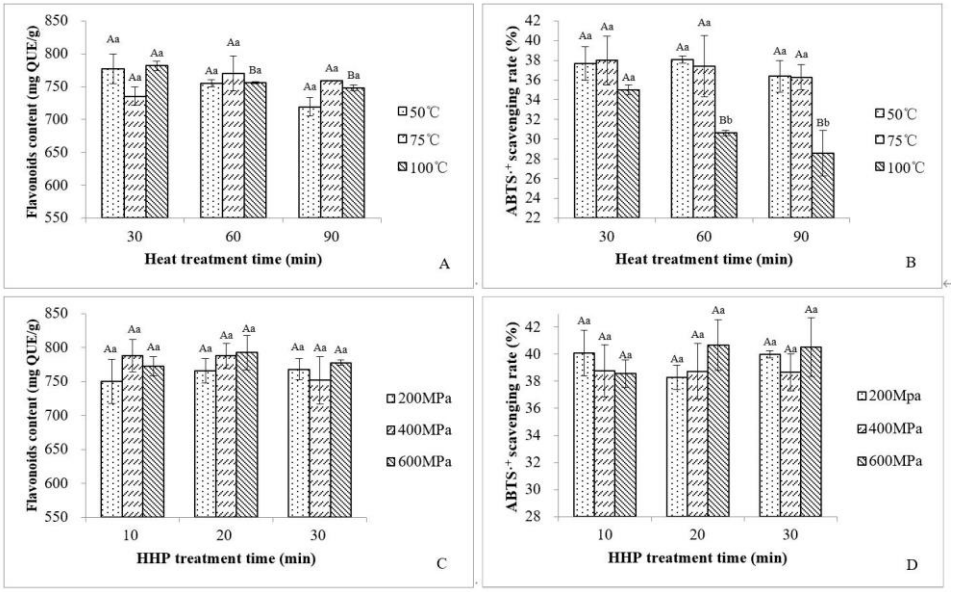

Figure 4-1 Processing stability of SPLF (A: Flavonoids content (mg QUE/g) after heat treatment; B: ABTS$^{\cdot+}$ scavenging rate (%) after heat treatment; C: Flavonoids content (mg QUE/g) after HHP treatment; D: ABTS$^{\cdot+}$ scavenging rate (%) after HHP treatment).

SPLF, sweet potato leaf flavonoids; QUE, quercetin equivalent; HHP, high hydrostatic pressure; ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).

Different letters (A, B) represented values were significant difference ($P < 0.05$) at the same temperature or pressure, (a, b) represented values were significant difference ($P < 0.05$) at the same treatment time

The stability of individual flavonoid under heat treatment was shown in Table 4-1. It was observed that most of individual flavonoid was stable except tiliroside and quercetin. However, in another study, the content of myricetin and quercetin in bilberry spreads increased after vacuum-heated at 77 °C or home-made boiling. This could be ascribed to the difference in food structure or food state, such as jams possessed higher solid content and lower water activity, which might resist the heat shock to a certain extent, when compared with juices (Može Bornšek et al., 2015).

Table 4-1 The content of individual flavonoid (mg/g, DW) after heat treatment

Individual flavonoid	Untreated	50 °C			75 °C			100 °C		
		30 min	60 min	90 min	30 min	60 min	90 min	30 min	60 min	90 min
Myricetrin	6.51±0.02[a]	6.73±0.02[a]	6.64±0.01[a]	6.67±0.03[a]	6.62±0.01[a]	6.65±0.02[a]	6.53±0.03[a]	6.69±0.01[a]	6.68±0.02[a]	6.84±0.02[a]
Isoquercitrin	62.44±0.13[a]	62.85±0.07[a]	62.37±0.14[a]	62.84±0.11[a]	62.90±0.09[a]	62.51±0.22[a]	62.91±0.16[a]	62.90±0.08[a]	62.71±0.12[a]	62.88±0.15[a]
Astragalin	480.40±1.71[a]	478.31±1.93[a]	478.11±2.34[a]	476.72±3.72[a]	477.10±2.55[a]	476.77±3.22[a]	475.25±3.93[a]	474.40±2.75[a]	472.39±2.61[a]	471.35±2.30[a]
Quercitrin	96.07±0.29[a]	95.34±0.27[a]	95.11±0.21[a]	95.64±0.25[a]	95.67±0.20[a]	95.04±0.23[a]	95.19±0.17[a]	94.91±0.22[a]	95.22±0.31[a]	95.30±0.32[a]
Tiliroside	18.83±0.04[a]	18.05±0.03[ab]	17.88±0.05[ab]	16.31±0.06[c]	16.77±0.02[c]	15.75±0.08[cd]	16.07±0.02[cd]	15.21±0.07[d]	15.24±0.03[d]	14.66±0.04[e]
Quercetin	12.64±0.04[a]	12.42±0.04[a]	12.55±0.03[a]	11.71±0.06[ab]	11.56±0.03[b]	11.44±0.06[b]	11.28±0.05[bc]	11.46±0.05[b]	11.28±0.03[bc]	11.15±0.03[c]
Kaempferol	6.03±0.02[a]	6.05±0.03[a]	5.91±0.01[a]	5.93±0.02[a]	5.93±0.02[a]	5.97±0.02[a]	6.06±0.03[a]	5.95±0.02[a]	5.98±0.02[a]	5.95±0.03[a]
Rhamnetin	4.31±0.02[a]	4.35±0.02[a]	4.27±0.01[a]	4.33±0.02[a]	4.36±0.02[a]	4.39±0.01[a]	4.33±0.01[a]	4.28±0.02[a]	4.31±0.02[a]	4.35±0.01[a]

[a] Different letter (a-e) meant values were significantly different ($p < 0.05$).

3.2. Stability of sweet potato leaf flavonoids after high hydrostatic pressure treatment

Stability of SPLF at different HHP treatment was shown in Figure 4-1 (C, D). There was no significant difference in flavonoids content or antioxidant activity as affected by HHP treatment. Our results were in good agreement with Patras, Brunton, Da Pieve, & Butler (2009), that the anthocyanin content in strawberry and blackberry purees had no significant difference after treated at 400, 500, 600 MPa for 15 min at RT (≈20 °C). In the meantime, no significant effect was reported for their antioxidant activity. Not to mention that the intensity of industrial level conditions, which would be much milder (usually less than 10 min), might have less effect on SPLF.

The stability of individual flavonoid under HHP treatment was shown in Table 4-2. Results had no significant difference after HHP treatment, indicating that the small molecule compounds could endure HHP and their antioxidant activities could be well kept. This result was similar to Jiménez-Aguilar et al (2015), who observed the content of quercetin in prickly pears beverage under 400 MPa processing for 16 min, had no statistical difference compared with non-treated beverage. In the same way, no statistical difference was reported in their study for isorhamnetin.

Table 4-2 The content of individual flavonoid (mg/g, DW) after HHP treatment

Individual flavonoid	Untreated	200 MPa			400 MPa			600 MPa		
		10 min	20 min	30 min	10 min	20 min	30 min	10 min	20 min	30 min
Myricetrin	6.53±0.02[a]	6.60±0.03[a]	6.51±0.03[a]	6.55±0.01[a]	6.64±0.04[a]	6.57±0.03[a]	6.53±0.02[a]	6.59±0.03[a]	6.56±0.03[a]	6.48±0.02[a]
Isoquercitrin	62.41±0.13[a]	62.55±0.17[a]	62.30±0.13[a]	62.53±0.12[a]	61.61±0.13[a]	61.09±0.11[a]	61.72±0.15[a]	61.77±0.18[a]	61.50±0.14[a]	61.47±0.13[a]
Astragalin	480.42±1.75[a]	474.20±3.51[a]	477.72±2.99[a]	479.03±2.44[a]	479.11±2.10[a]	472.30±3.13[a]	479.41±2.57[a]	470.33±2.20[a]	470.56±1.96[a]	475.12±3.37[a]
Quercitrin	96.04±0.29[a]	95.88±0.19[a]	95.65±0.20[a]	96.08±0.16[a]	96.05±0.24[a]	95.57±0.26[a]	95.64±0.22[a]	95.48±0.17[a]	95.63±0.24[a]	95.80±0.21[a]
Tiliroside	18.88±0.04[a]	18.50±0.05[a]	18.04±0.04[a]	18.75±0.03[a]	18.13±0.03[a]	18.26±0.04[a]	18.38±0.06 [a]	18.51±0.05[a]	18.73±0.03[a]	18.26±0.04[a]
Quercetin	12.64±0.04[a]	12.43±0.03[a]	12.47±0.01[a]	12.70±0.02[a]	12.66±0.03[a]	12.44±0.02[a]	12.03±0.02[ab]	12.56±0.03[a]	12.35±0.03[a]	12.12±0.01[ab]
Kaempferol	6.03±0.02[a]	6.01±0.02[a]	5.95±0.01[a]	5.97±0.03[a]	5.92±0.02[a]	5.92±0.03[a]	6.00±0.02[a]	5.99±0.03[a]	5.97±0.01[a]	5.94±0.02[a]
Rhamnetin	4.31±0.02[a]	4.33±0.02[a]	4.25±0.01[a]	4.28±0.01[a]	4.20±0.02[a]	4.22±0.02[a]	4.36±0.01[a]	4.29±0.02[a]	4.20±0.01[a]	4.24±0.02[a]

[a] HHP, high hydrostatic pressure.

[b] Letters (a, ab) meant values were not significantly different ($p < 0.05$).

3.3. Influence of pH on the stability of sweet potato leaf flavonoids

Storage of SPLF for 16 weeks resulted in decrease tendency at pH 3.0, 5.0, 7.0, 8.0, especially for samples at pH 7.0 and 8.0. Figure 4-2(A) showed that the TPC didn't decrease significantly at pH 3.0 or 5.0 after 4 weeks of storage. The retention of flavonoids, after 16 weeks of storage, was 89.1 % (pH 3.0) and 82.0 % (pH 5.0). In the meantime, the retention of flavonoids was 26.6 % for pH 7.0 and 20.9 % for pH 8.0. The results clearly showed that SPLF was quite sensitive to pH. Storage of SPLF in acid environment is encouraged.

The antioxidant activity of SPLF gradually declined in neutral and alkaline environment. As depicted in Figure 4-2(B), after 16 weeks of storage, retention of antioxidant activity in SPLF fell to 73.8 % and 56.1 % at pH 7.0 and 8.0, respectively, but increased by 2.5 % and 9.7 % at pH 3.0 and 5.0, revealing SPLF was quite stable in acid environment.

As observed in Figure 4-2(C), isoquercitrin and quercitrin were more stable at pH 5.0, and tiliroside and quercetin were completely destroyed at pH 3.0 after 16 weeks of storage. It should be pointed out that all individual flavonoids were almost completely destroyed within a week in pH 7.0 or 8.0 according to the result of HPLC, especially in pH 8.0, because the structure of flavonoids in alkaline environment underwent the opening of ring C, resulting in two fragments of ring A and ring B being produced, which kept consistent with Echeverry, Medina, Costa, & Aragón (2018).

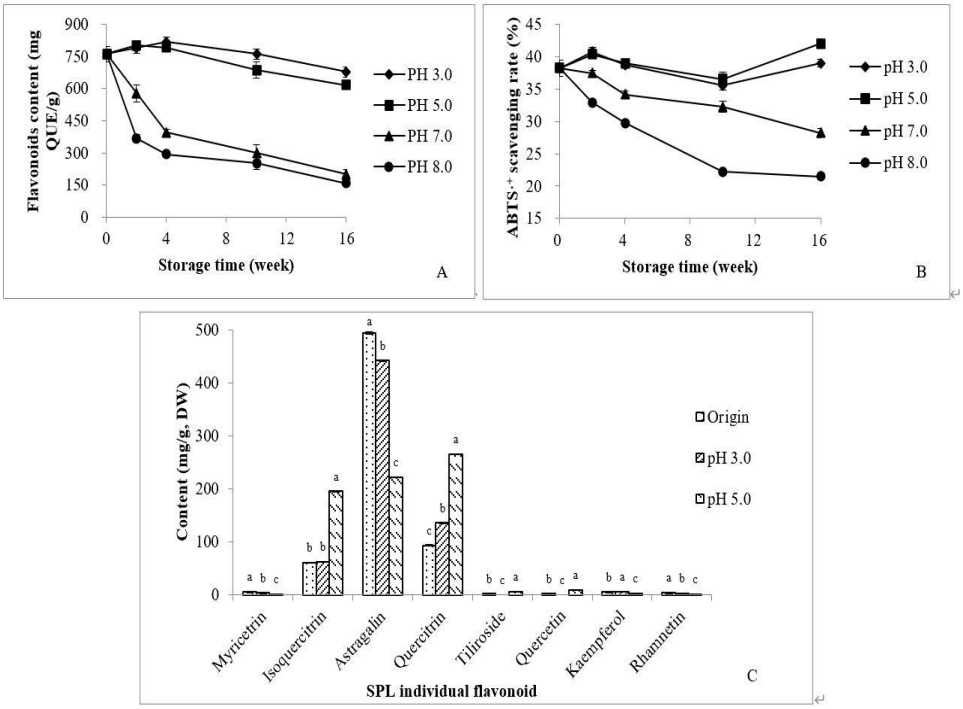

Figure 4-2 Influence of pH on the storage stability of SPLF (A: Flavonoids content during 16 weeks; B: ABTS·$^{+}$ scavenging rate during 16 weeks; C: The content of individual flavonoid after 16 weeks). SPLF, sweet potato leaf flavonoids; ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).

Different letters (a-c) represented values were significant difference for the same individual flavonoid stored at different pH ($P < 0.05$).

3.4. Influence of light on the stability of sweet potato leaf flavonoids

The retention of flavonoids was stable in first 4 weeks, and there was no significant difference between samples exposed to light or not, then showed a descending tendency as was observed in Figure 4-3(A). The difference in flavonoids retention became more pronounced as storage time increased. The retention of flavonoids after 16 weeks of storage was 80.5 % for samples kept away from light. However, a significant lower value was reported for samples exposed to light (47.9 %). Our findings clearly showed that SPLF was quite sensitive to light, with detrimental effects after long time exposure. In the same way, Rabadán, Álvarez-Ortí, Pardo, & Alvarruiz (2018) reported that polyphenols content of almond, walnut and pistachio was significantly lower under light storage at RT, compared with dark storage.

The antioxidant activity of SPLF showed a continuous decreasing tendency. The retention of antioxidant activity after 16 weeks of storage was 75.6 % in SPLF under light storage, and 85.2 % under dark storage [Figure 4-3(B)].

Myricetrin, isoquercitrin, astragalin, quercitrin and kaempferol were sensitive to light resulting in extra loss of 36 %, 66 %, 65 %, 72 % and 40 %, respectively, while tiliroside, quercetin, rhamnetin were completely destroyed, as observed in Figure 4-3(C). Similar findings were reported by Bakowska, Kucharska, & Oszmiański (2003) on the loss of anthocyanin-3-glucoside stored in the dark for 3 months.

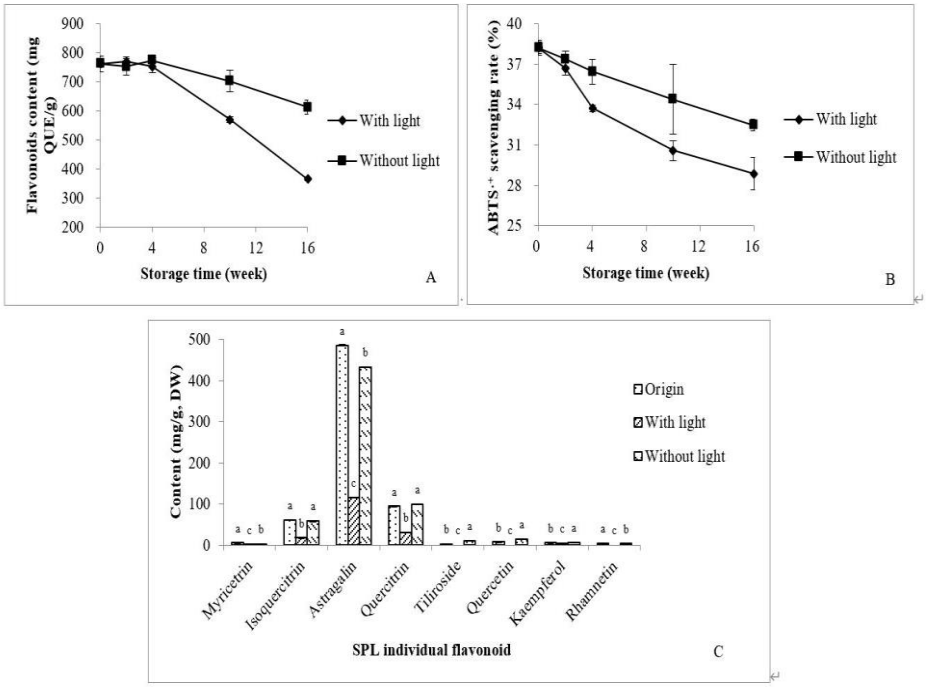

Figure 4-3 Influence of light on the storage stability of SPLF (A: Flavonoids content during 16 weeks; B: ABTS·$^{+}$ scavenging rate during 16 weeks; C: The content of individual flavonoid after 16 weeks).

SPLF, sweet potato leaf flavonoids; ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).

Different letters (a-c) represented values were significant difference for the same individual flavonoid stored at different light environments ($P < 0.05$).

3.5. Influence of storage temperature on the stability of sweet potato leaf flavonoids

As shown in Figure 4-4(A), the retention of flavonoids was stable in first 4

weeks, then decreased tendency was observed for the remaining period of storage. The retention of flavonoids dropped to 84.9 %, 83.2 % and 81.3 % for samples stored at -18, 4 °C and RT away from light, respectively. It can be deduced that SPLF was not temperature sensitive with more than 80 % TFC preserved as revealed in this study, which was similar to the findings of Bradwell, Hurd, Pangloli, McClure, & Dia (2018), who reported that TFC in sorghum phenolic extracts had no significant difference at 4 and 22 °C, during the first 6 weeks of storage. However, a decrease in the flavonoid content was reported after weeks of storage.

The antioxidant activity of SPLF was shown in Figure 4-4(B). A sharp decrease in the antioxidant activity was observed in the first 4 weeks, before slowing down for the rest of the time. The decrease in the antioxidant activity could be attributed to the increase in temperature with storage time, achieving final retentions of 84.2 %, 79.9 % and 74.0 % for -18, 4 °C and RT, respectively. Even so, nearly 75 % of antioxidant activity could still be preserved, after 16 weeks of storage at RT without any refrigeration equipment. Our results were consistent with Tsai, Huang, & Huang (2004) who observed that the antioxidant activity in new mulberry wine decreased to 80.9 % after a year's storage at 20 °C.

As observed in Figure 4-4(C), the individual flavonoid showed different stabilities after 16 weeks. Isoquercitrin and quercitrin had no significant difference at 4 °C or RT, and quite unstable when compared with at -18 °C. The stability of astragalin, tiliroside and rhamnetin decreased significantly with increase of temperature. Our findings were similar to Teleszko, Nowicka, & Wojdyło (2016) who illustrated that lower temperature could limit the degradation process generally. On the other hand, Häkkinen, Kärenlampi, Heinonen, Mykkänen, & Törronen (1999) observed that storage at -18 °C for 3 to 9 months decreased myricetin and kaempferol more than quercetin.

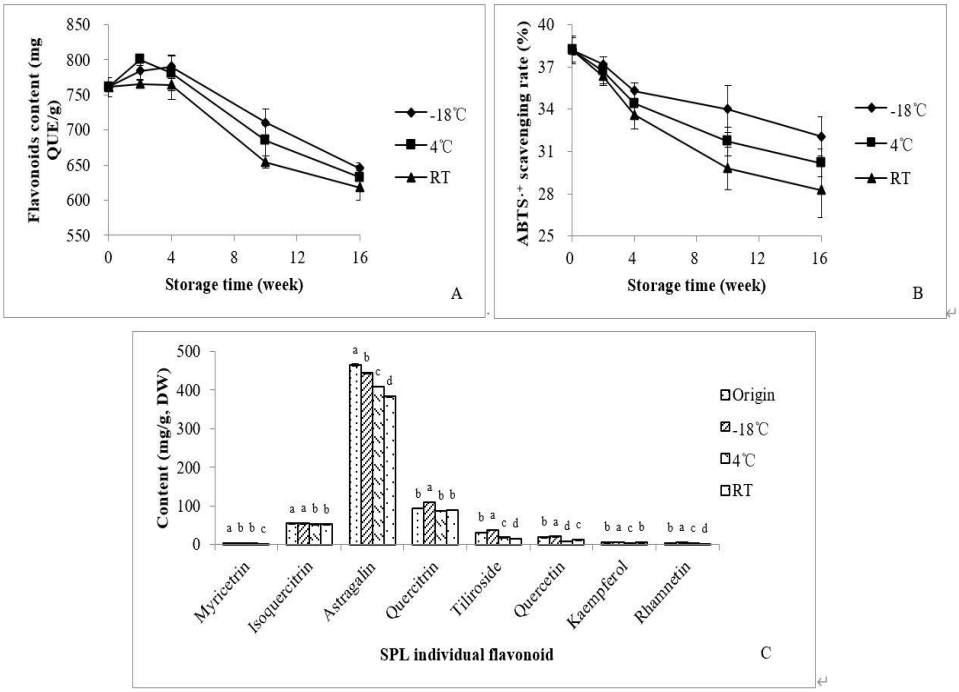

Figure 4-4 Influence of temperature on the storage stability of SPLF (A: Flavonoids content during 16 weeks; B: ABTS·+ scavenging rate during 16 weeks; C: The content of individual flavonoid after 16 weeks).
SPLF, sweet potato leaf flavonoids; ABTS, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid).
Different letters (a-d) represent values were significant difference for the same individual flavonoid stored at different temperature ($P < 0.05$)

3.6. Retention of sweet potato leaf flavonoids after simulated digestion

Retention of SPLF after simulated digestion was shown in Table 4-3. *Ginkgo biloba* extract (GIN) and propolis flavone (PRO) were taken as the control. TFC in SPLF, GIN and PRO was 761.0, 457.7 and 568.5 mg/g, respectively. After simulated digestion, the retention of flavonoids decreased to 45.9 %, 27.5 % and 8.4 % in SPLF, GIN and PRO, respectively. Our results were similar to Gunathilake, Ranaweera, & Rupasinghe (2018) who found that the retention of flavonoids in *Centella asiatica*, *Gymnema lactiferum*, *Sesbania grandiflora* and *Passiflora edulis* after gastrointestinal digestion as 55 %, 48 %, 42 % and 40 %, respectively. Meanwhile the retention of antioxidant activity after simulated

digestion in SPLF, GIN and PRO was 56.2 %, 34.5 % and 28.2 %, respectively. Our results were supported by Spínola, Llorent-Martínez, & Castilho (2018) who reported the retention of antioxidant activity in *Rumex maderensis* as 51.8 %, 60.0 % and 50.7 %, in leaves, flowers and stems, respectively, after *in vitro* simulated gastrointestinal digestion. Compared with the control samples (GIN and PRO), SPLF had higher stability during simulated digestion.

Table 4-3 The stability of SPLF after simulated digestion

	Oral digestion			Gastric digestion			Intestinal digestion		
	SPLF	GIN	PRO	SPLF	GIN	PRO	SPLF	GIN	PRO
Retention of flavonoids (%)	75.4±2.6[b]	78.0±1.6[b]	88.6±2.6[a]	55.0±2.6[b]	59.1±3.5[b]	73.7±4.8[a]	46.0±3.6[a]	27.5±0.3[b]	8.4±2.3[c]
Retention of antioxidant activity (%)	75.9±5.2[a]	81.6±1.0[a]	76.0±1.9[a]	65.0±4.4[a]	53.6±2.2[b]	46.4±2.4[c]	56.2±2.6[a]	34.5±0.7[b]	28.2±1.5[c]

[a] SPLF, sweet potato leaf flavonoids; GIN, *Ginkgo biloba* extract; PRO, propolis flavone.

[b] Different letters (a-c) meant values were significantly different ($p < 0.05$).

4.Conclusion

The results of our study showed that the stability of SPLF after heat and HHP treatment was high. More specifically, heat treatment at 75 °C for 90 min or HHP treatment at 600 MPa for 30 min did not cause any significant destruction. The stability of SPLF under acid environment was good (able to preserve 50 % of TFC and 35 % of antioxidant activity after 16 weeks of storage), compared with neutral or alkaline environment. Likewise, SPLF kept away from light exhibited better retention of TFC (30 %) and antioxidant activity (10 %) after 16 weeks of storage. No significant difference in SPLF was observed for the samples stored at -18, 4 °C and RT. Interestingly, more than 80 % of TFC and 75 % of antioxidant activity was preserved under these conditions (-18, 4 °C and RT) after 16 weeks. Overall, the retention of SPLF was high compared to GIN and PRO, after simulated digestion, retaining 46 % of TFC and 56 % of antioxidant activity.

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Chapter Ⅴ. *In-vitro* digestion of sweet potato leaf flavonoids and its sustained release from maltodextrin nanoparticles

The stability of sweet potato leaf flavonoids when processing and storing was high, except towards neutral or alkaline environment, which was exactly the digestion pH in vivo. Thus, in this chapter we need to evaluate the stability of sweet potato leaf flavonoids during in vitro digestion, and whether nanoparticles encapsulated by maltodextrin could protect it to some certain degree and postpone its release.

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Abstract

The nanoparticles of sweet potato leaf flavonoids, produced by freeze-drying using 20 mg/mL maltodextrin, showed the highest stability with zeta potential of -41.6 mV, mean particle size of 277.4 nm, polydispersity index of 0.417, encapsulation efficiency of 59.0 %, confocal laser scanning microscopy and infrared spectrum also provided the proofs. After simulated oral, gastric and intestinal digestion, the retention of total flavonoids content (TFC) was 75.4 %, 55.0 % and 45.9 % respectively, and the retention of antioxidant activity was 75.9 %, 65.0 % and 56.2 % separately. Nanoparticles could additionally reserve 16 %, 31 %, 28 % of TFC and 17 %, 17 %, 11 % of antioxidant activity in each digestion phase, compared with the sample without encapsulation, which could be intuitively observed by scanning electron microscopy. Individual flavonoid been protected better in oral and gastric phase, which realized partial sustained release to some certain extent.

Keywords

Sweet potato leaf flavonoids; Nanoparticles; Simulated digestion; Antioxidant activity; Microscopic observation

1. Introduction

Sweet potato (*Ipomoea batatas* L.) is one of the 8 top crops in the world ranking after maize, wheat, rice, potato, soybean, cassava, barley and China is the leading producer in the world, the annual yield of 2018 was 53 million tons, accounting for 57 % of the total yield (FAOSTAT, 2020). Compare with roots, sweet potato leaves (SPL) have been basically neglected, directly discarded or limitedly used as the feed, not only polluting the environment, but also wasting the resources, after all, there are plenty of nutrients and bioactive compounds in SPL. Sun, Mu, Xi, Zhang, & Chen (2014) conducted a comprehensive study on SPL in 40 cultivars around China, which included fresh-eaten type, starch-processing type and leafy-vege type, and there was a positive correlation between polyphenol content (2.7-12.5 g chlorogenic acid (CHA) /100g DW) and antioxidant activity (0.08-0.8 mg ascorbic acid equivalent (ACE) / mg DW), with the correlation coefficient of 0.76 ($P < 0.0001$).

Flavonoids contained the typical molecular structure of two aromatic rings conjugated by a three-carbon bridge (C_6-C_3-C_6), which was the major branch of polyphenols, arousing huge interest because of its excellent antioxidative, anticancer, antihypertensive, antimicrobic activities etc. (Sathishkumar, Gu, Zhan, Palvannan, & Mohd Yusoff, 2018).

The study of sweet potato leaf flavonoids (SPLF) was mainly focused on the processing methods (boiling, steaming, fermenting) (Liao, Lai, Yuan, Hsu, &

Chan, 2011; Cui, Liu, Li, & Song, 2011), extraction parts (tips, leaves, stems) (Cui, Liu, Li, & Song, 2011; Anastácio & Carvalho, 2013), and high-tech extraction methods (dynamic ultra-high pressure micro-jet extraction, ultrasonic microwave synergestic extraction) (Huang et al., 2013; Liu, Mu, Sun, & Fauconnier, 2019). But these studies were almost carried out in the form of crude extract with low purity of flavonoids, could not excluded the interference of other substances. In addition, the premise of absorption for these bioactive substances was digestion, however conducting *in vivo* trial was often costly, resource intensive, and ethically disputable, while simulated gastrointestinal digestion was a better alternative, which widely employed in many fields of food and nutritional sciences. Therefore, the stability of SPLF during simulated digestion will be investigated.

As we know from literatures, the flavonoids in pomegranate juice, pulp and extract decreased 13.8 %, 16.4 % and 26.7 %, respectively after gastric digestion, and further decreased 60.9 %, 76.3 % and 59.1 %, separately after intestinal digestion. These differences might due to different chemical structures of flavonoids existed and the food matrix characteristics (Mosele, Macià, Romero, Motilva, & Rubió, 2015). Industrial processed tomato sauce which had plenty of flavonoids, decreased 60.3 %, 60.5 % and 75.3 % of its antioxidant activity, respectively after oral, stomach and intestinal digestion phase, which might be related to the correlation of digestive enzymes and pH (Tomas et al., 2017).

The destruction of flavonoids during digestion could be delayed after encapsulation, the operation of freeze-drying was gentle, compared with spray-drying, often applied to the encapsulation of heat-sensitive polyphenols. When maltodextrin was taken as the wall material, especially with relatively low value of dextrose equivalents (DE), which DE was 10 rather than 20, could obtain microcapsule from anthocyanin-rich extracts with high anthocyanin retention by freeze-drying (Yamashita et al., 2017). TFC in spent coffee grounds was 12.1 and 7.9 mg quercetin equivalents (QE) /100 mL, respectively by freeze-drying and spray-drying encapsulation, meanwhile the antioxidant activity of microcapsules was 506.3 and 380.2 mg α-tocopherol equivalent /100mL, individually (Ballesteros, Ramirez, Orrego, Teixeira, & Mussatto, 2017). The cumulative release of flavonoids from microparticles was about 41 % and 33 % lower than the pure extracts in citrus peel after gastric and intestinal digestion, separately, meanwhile, antioxidant activity of citrus peel extracts was about 26 % and 20 % lower than the microparticles, after corresponding simulated digestion (Hu, Kou, Chen, Li, & Zhou, 2019). We could find out that damage of flavonoids and decrease of antioxidant activity in the sample after simulated digestion could be alleviated to a certain extent by maltodextrin encapsulation.

In this study, encapsulation effect of SPLF by maltodextrin was observed through confocal laser scanning microscopy (CLSM) and fourier transform infrared spectroscopy (FT-IR). The stability of their nanoparticles after simulated

digestion was observed by scanning electron microscopy (SEM). TFC, individual flavonoid and antioxidant activity were evaluated during the process. The obtained data might provide preliminary supports for applications of plant extracts in food and pharmaceutical fields.

2. Material and methods

2.1. Material and reagents

SPL was provided by Chongqing Sweet Potato Engineering & Technology Research Center (Chongqing, China). Yuzi No. 7 was the chosen cultivar, which possessed the highest polyphenols content and the strongest antioxidant activity in the leaves from 40 cultivars around China, according to the previously study done in our laboratory (Sun, Mu, Xi, Zhang, & Chen, 2014). The leaves were harvested and transported to the lab after approximate 70 days of growing period (June to September, 2017), washed, freeze-dried and grounded to fine powder.

2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), maltodextrin (MAL) with the dextrose equivalents (DE) ranging from 11-14, $K_2S_2O_8$, KCl, KH_2PO_4, $NaHCO_3$, NaCl, $MgCl_2$, $(NH_4)_2CO_3$, NaOH, HCl, $CaCl_2$ were purchased from Sinopharm Chemical Reagent (Beijing, China). α-amylase extracted from porcine pancreas with the activity ≥ 16 units/mg, pepsin extracted from porcine gastric mucosa powder with the activity ≥ 250 units/mg, trypsin extracted from porcine pancreas with the activity ≥ 1500 units/mg, and bile salt with the purity of choleic acid more than 60 % were purchased from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography (HPLC) grade solvents (methanol, acetonitrile) were obtained from Fisher Chemical (Beijing, China). H_2O was prepared by Molgene Water Purification System from Molecular (Shanghai, China).

2.2. Preparation of sweet potato leaf flavonoids

The extraction process was shown as follows: ethanol concentration of 72 %, extraction temperature of 57 °C, extraction time of 76 s, solid-liquid ratio of 1:40 (g/mL) and extracted 2 times by Ultrasonic-Microwave Synergistic Extractor CW-2000 (Shanghai Xintuo Analytical Instruments Co., LTD, China) (Ultrasonic power was 50 W, ultrasonic frequency was 40 kHz, microwave power was 100 W, microwave frequency was 2450 MHz and microwave wavelength was 0.12 m) (Liu, Mu, Sun, & Fauconnier, 2019). The ethanol extract was further separated by equal volume of petroleum ether and ethyl acetate according to the polarity of flavonoids (Fu et al., 2016), to obtained 1.02 g SPLF freeze dried powder from 40 g of SPL, with the purity of 76.1 (%, DW).

2.3. Preparation of nanoparticles

According to the method of Hamid, Thakur, & Thakur (2020) to prepare SPLF-MAL nanoparticles. The specific method was as follows: SPLF solution (30 mg/mL, pH 2.64) and MAL solution (5, 10, 20, 50 and 100, mg/mL) were prepared by distilled water. 10 mL SPLF solution was dropwise added into 50 mL MAL solution and continuously stirred for 2 h using a magnetic stirring (500 rpm). The mixture was pre-freezing at -18 °C in the refrigerator then shifted into a freeze-dryer (FreeZone, LABCNCO, USA), slowly freezing to -46 °C. Drying process was performed by keeping the sample at a pressure of 0.31 mbar, maintaining for 48 h at -46 °C to get the floc.

2.4. Particle size, zeta potential, polydispersity index

Particle size, zeta potential and polydispersity index (PDI) of the nanoparticles were detected by dynamic light scattering equipment ZEN 3600 (Malvern, UK). The size measurement was carried out at a 173° scattering angle at room temperature (RT) (≈20 °C). The zeta potential was calculated based on Smoluchowski's equation. The nanoparticles were dispersed in distilled water and ajusted to 1 mg/mL according to the response.

2.5. Encapsulation efficiency of sweet potato leaf flavonoids

Encapsulation efficiency (EE) of SPLF in the nanoparticles was calculated indirectly by measuring free flavonoids content (FFC) in the nanoparticles suspension (Hu, Li, Zhang, Kou, & Zhou, 2018). The specific treatment was as follows: the suspension was centrifuged at 7000 rpm for 20 min at 4 °C to separate the encapsulated and free SPLF. SPLF in the supernatant was considered as free SPLF, which was determined by UV-visible spectrophotometer (Persee, TU-1810, Beijing, China) using the method mentioned at 2.10. EE was calculated by the equation:

$$EE\ (\%) = (C_T - C_F)/C_T \times 100\ \%\ (1)$$

Where C_T was the TFC in the SPLF, C_F was the FFC in the supernatant.

2.6. Confocal laser scanning microscopy

Freeze-dried samples were dispersed in H_2O uniformly and ajusted to 7.5 mg/mL according to the pre-test, then dropped onto the glass slide and observed under the CLSM (LSM 880, Zeiss, Germany). The objective lens was 20 × and 40 ×, the excitation spectrum was chosen at 488 nm according to the method of (Milea et al., 2019). The experimental operation was performed by Leica ZEN 2.1 SP2 Application Suite, and images were processed by Leica ZEN 2.1 lite Graph Processing Software.

2.7. Fourier transform infrared spectroscopy

The infrared spectrum of SPLF, MAL and nanoparticles was detected by FT-IR spectrometer (TENSOR 27, Bruker, Germany). The freeze-dried powder of all samples (2 mg) was ground with KBr in the agate mortar then pressed into hydraulic pressure tablet machine. The FT-IR spectrum of the sample sheet was collected in transmission mode within the wavelength range of 4000-600 cm^{-1} at a resolution of 2 cm^{-1}.

2.8. Preparation of simulated digestion fluids

Simulated salivary fluid (SSF) (pH 7.0), simulated gastric fluid (SGF) (pH 3.0) and simulated intestinal fluid (SIF) (pH 7.0) were prepared by corresponding proportion of electrolyte stock solutions according to Minekus et al. (2014). KCl (0.5 mol/L), KH_2PO_4 (0.5 mol/L), $NaHCO_3$ (1 mol/L), NaCl (2 mol/L), $MgCl_2$ (0.15 mol/L), $(NH_4)_2CO_3$ (0.5 mol/L) and $CaCl_2$ (0.3 mol/L) were prepared by distilled water, HCl (6 mol/L) and NaOH (1 mol/L) for pH adjustment were diluted by distilled water. Specific steps were given in the previous chapter.

2.9. Simulated digestion in vitro

Simulated digestion of SPLF, *Ginkgo biloba* extract (GIN), propolis flavone (PRO) and SPLF-MAL was carried out according to the method of Minekus et al. (2014) with some modification, detailed description was given in the previous chapter. 5 mL digestive juice after each step was collected, adjusted to pH 2.0 and kept at 4 °C, in order to maintain the stability of flavonoids, and finished analysis as soon as possible (Bernardes et al., 2019).

2.10. Determination of total flavonoids content

TFC was determined by spectrophotometer (Persee, TU-1810, Beijing, China), according to the method of Lim, Huang, Zhao, Zou, & Yan (2016) with some modification. TFC was expressed in milligrams of quercetin equivalent per g of dry weight (mg QUE/g DW). The retention of TFC was calculated by the equation as follows:

$$R_1\ (\%) = C_1/C_0 \times 100\ \%\ (2)$$

where R_1 was the retention of TFC in the sample after encapsulation or digestion, C_1 was the TFC in the sample after encapsulation or digestion, C_0 was the TFC in the initial sample.

2.11. Quantification of individual flavonoid

The content of individual flavonoid was quantified by HPLC which equipped the system control unit (CBM-20A, Shimadzu), the liquid infusion unit (LC-20AB, Shimadzu), the automatic sampler (SIL-20AC, Shimadzu), the degaser (DGU-$20A_3$, Shimadzu), the UV detector (SPD-20A, Shimadzu) and the column oven (CTO-20AC, Shimadzu), C_{18} was the column (4.6×150mm, 5μm, Inertsil ODS-

SP, Shimadzu). The parameters of HPLC were set up according to the method of Liu, Mu, Sun, & Fauconnier (2019), which detailed description in the previous chapter.

2.12. Determination of antioxidant activity

Antioxidant activity of SPLF was performed by $ABTS^{\cdot+}$ scavenging capacity based on the method of Li, Lin, Gao, Han, & Chen (2012) with some modification. The retention of $ABTS^{\cdot+}$ scavenging capacity in the sample was calculated by the equation:

$$R_2\ (\%) = A_1/A_0 \times 100\ \%\ (3)$$

Where R_2 was the retention of $ABTS^{\cdot+}$ scavenging rate of the sample after encapsulation or digestion, A_1 was the $ABTS^{\cdot+}$ scavenging rate of the sample after encapsulation or digestion, A_0 was the initial $ABTS^{\cdot+}$ scavenging rate of the sample.

2.13. Scanning electron microscopy

The surface microsructure of the samples after oral, gastric and intestinal simulated digestion was observed by scanning electron microscope (SEM) (SU8010, Hitachi, Japan), in low vaccum operating mode with the accelerating voltage of 10 kV, and SEM images with a magnification of 200 × were acquired. Before observation, the freeze-dried samples were manually dispersed on the double-side conducting adhesive bands which sticked on the circular aluminum table, and sputtering coated with a thin gold-palladium layer (5-10 nm, 10 mA, 30s).

2.14. Statistical analysis

Statistic difference among diversely treated samples was calculated through one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, IBM SPSS 24.0 (Chicago, USA) was taken as the software, difference was considered significant when $P < 0.05$. The data were the average of three replicates.

3. Results and discussion

3.1. Nanoparticles characterization

Mean particle size, zeta potential, PDI and EE of the prepared nanoparticles dispersing in distilled water, based on the different formularies, which were showed in Table 5-1. Zeta potential was the stability index of nanoparticles, which the higher of the absolute value, the more stable of the nanoparticles dispersing in the solvent. Nanoparticles with absolute zeta potential value which greater than 30 mV could be dispersed stably in the suspension (Hao et al., 2017). There was no significant different between F1-F3 for the zeta potential. In the meantime, the

mean particle size of nanoparticles changed with the formularies, ranging from 207.4 nm to 386.5 nm. PDI was the index of molecular weight dispersion, the value was smaller the better, when the nanoparticles were prepared by formulary 3, the PDI was down to 0.417, which was the smallest among these formularies, showing the highest uniformity of the nanoparticles (Yan et al., 2018). However, there was no significant difference in EE between F3, F4 and F5, which was 59.0 %, 65.4 % and 61.7 %, respectively. On the other hand, according to the result of Hamid, Thakur, & Thakur (2020) that TFC in microencapsulated pomegranate peel powder decreased from 6.2 to 0.6 mg QUE/g with the increase in the concentration of maltodextrin (from 1:1 to 1:10), which might due to the reason that increased amount of coating material around the phenolic extract reduced the concentration of the active ingredient, demonstrated the smaller amount of maltodextrin was preferred. To sum up, F3 was the optimal formulary which had the highest absolute zeta potential value, lesser mean particle size, smallest PDI and relatively highest EE.

Table 5-1 Mean particle size, zeta potential, PDI and EE of the nanoparticles

Formulary	Maltodextrin (mg/mL)	Zeta potential (mV)	Particle size (nm)	PDI	EE (%)
F1	5	-39.3±2.1[a]	207.4±8.9[d]	0.524±0.006[bc]	27.3±1.3[c]
F2	10	-40.6±3.2[a]	216.8±14.5[cd]	0.500±0.004[bc]	41.7±2.4[b]
F3	20	-41.6±3.3[a]	277.4±12.9[bc]	0.417±0.004[c]	59.0±1.4[a]
F4	50	-29.3±0.2[b]	294.6±17.4[b]	0.548±0.089[ab]	65.4±4.2[a]
F5	100	-19.8±1.0[c]	386.5±22.2[a]	0.632±0.056[a]	61.7±3.4[a]

[a] PDI, polydispersity index; EE, encapsulation efficiency.

[b] Different letters (a-d) in the same column represented values (mean ± SD, n = 3) were significantly different ($P < 0.05$).

3.2. Confocal laser scanning microscopy

The autofluorescence characteristics of SPLF made it possible to observe the degree of its sheltered by MAL, because MAL did not fluoresce (Pravinata & Murray, 2019). As it shown in Figure 5-1(A, B), SPLF granules were visible, with bright red fluorescence, distributed in the H_2O. With the adding of MAL, it gathered around SPLF, and the red fluorescence gradually faded following with the increase of MAL concentration [Figure5-1(C-F)]. When the concentration of MAL reached up to 20 mg/mL, the red fluorescence of SPLF had been well sheltered, exhibited the dark object of the nanoparticles in the H_2O, which meant MAL occupied the peripheral layer of the SPLF granule [Figure 5-1(E, F)]. Our result kept consistent with Zhao et al. (2019) that green tea polyphenols contained strong autofluorescence at 488 nm, which could be remarkably weakened even disappeared when been sheltered or ruined.

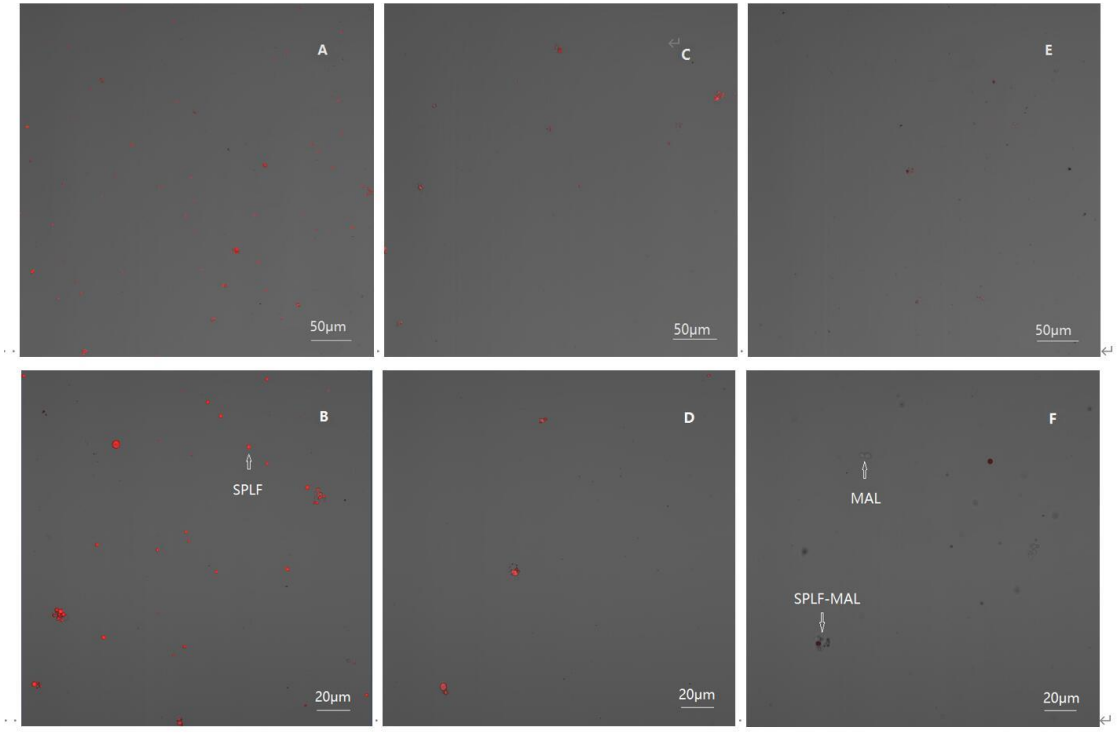

Figure 5-1 CLSM of nanoparticle. A: SPLF (7.5 mg/mL) (Objective lens was 20 ×). B: SPLF (7.5 mg/mL) (Objective lens was 40 ×). C: SPLF (7.5 mg/mL)-MAL (5 mg/mL) (Objective lens was 20 ×). D: SPLF (7.5 mg/mL)-MAL (5 mg/mL) (Objective lens was 40 ×). E: SPLF (7.5 mg/mL) -MAL (20 mg/mL) (Objective lens was 20 ×). F: SPLF (7.5 mg/mL)-MAL (20 mg/mL) (Objective lens was 40 ×).
CLSM, confocal laser scanning microscopy; SPLF, sweet potato leaf flavonoids; MAL, maltodextrin.

3.3. Morphology of sweet potato leaf flavonoids and their nanoparticles encapsulated by 20 mg/mL maltodextrin

The morphology of SPLF and their nanoparticles were exhibited in Figure 5-2. We could see clearly that freeze-dried SPLF was shiny yellow-green powder, and nanoparticles encapsulated by 20 mg/mL maltodextrin (with optimum stability demonstrated previously) was canary yellow flocculent.

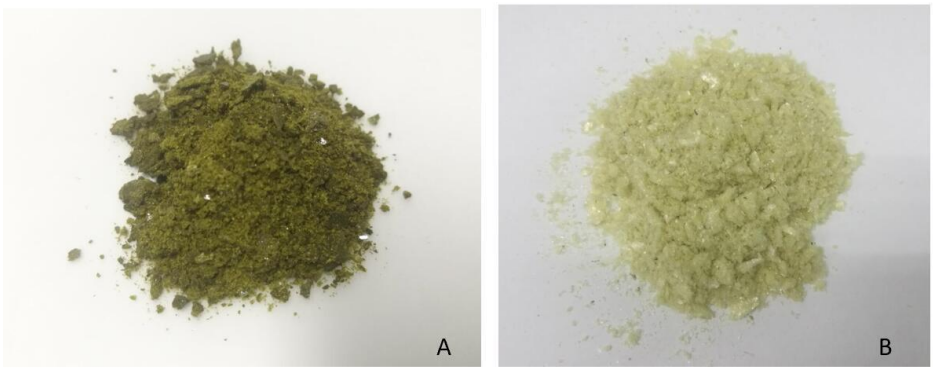

Figure 5-2 Morphology of SPLF and their nanoparticles encapsulated by 20 mg/mL MAL. SPLF, sweet potato leaf flavonoids; MAL, maltodextrin.

3.4. Fourier transform infrared spectroscopy

FT-IR spectroscopy was the important tool to analyze the interaction of chemical bond and spectra of naked SPLF, MAL and their nanoparticles, the result was shown in Figure 5-3. The strong and broad band detected at 3384 cm^{-1} originated from stretching vibration of phenolic O-H stretching in MAL. The stretching vibration band of SPLF was 3367 cm^{-1} which kept consistent with Zhang et al. (2018) that the band at 3413 cm^{-1} indicated phenolic O-H stretching vibration in propolis, while the corresponding band in nanoparticles returned to 3383 cm^{-1}. The band at 2927 cm^{-1} in MAL and nanoparticles was due to the asymmetric stretching vibration of $-CH_2$, while there was two bands at 2927 and 2854 cm^{-1} in SPLF, indicated the asymmetric and symmetric stretching vibration of $-CH_2$, which was similar to Chinese propolis (Wu et al., 2008). The bands at 1691 and 1689 cm^{-1} was attributed to C=C and C-C vibration in SPLF and nanoparticles, while the band at 1649 cm^{-1} indicated the vibration of C=O in MAL. Bands appearing at 1602 cm^{-1} (H-O-H), 1518 cm^{-1} (N-H), 1446 cm^{-1} (-CH2, -CH, =C-H), 1280 and 1114 cm^{-1} (C-O) in SPLF were closely related to aromatic compounds, which was proved to be flavonoids (Kuzniar, Heneczkowski, Kopacz,

& Nowak , 2014; Schulz, Baranska, & Baranski, 2005). Another band for MAL was located at 1022 cm^{-1} which represented C-O stretching vibration (Santiago-Adame et al., 2015), meanwhile the same band at 1026 cm^{-1} in nanoparticles could be observed. Some peaks around 978 cm^{-1}, 854 cm^{-1} and 813 cm^{-1} showed the association of =C-H bending, implying the presence of alkenes. We could see clearly from Figure 5-2 that most of the aromatic functional groups in SPLF be well masked by MAL.

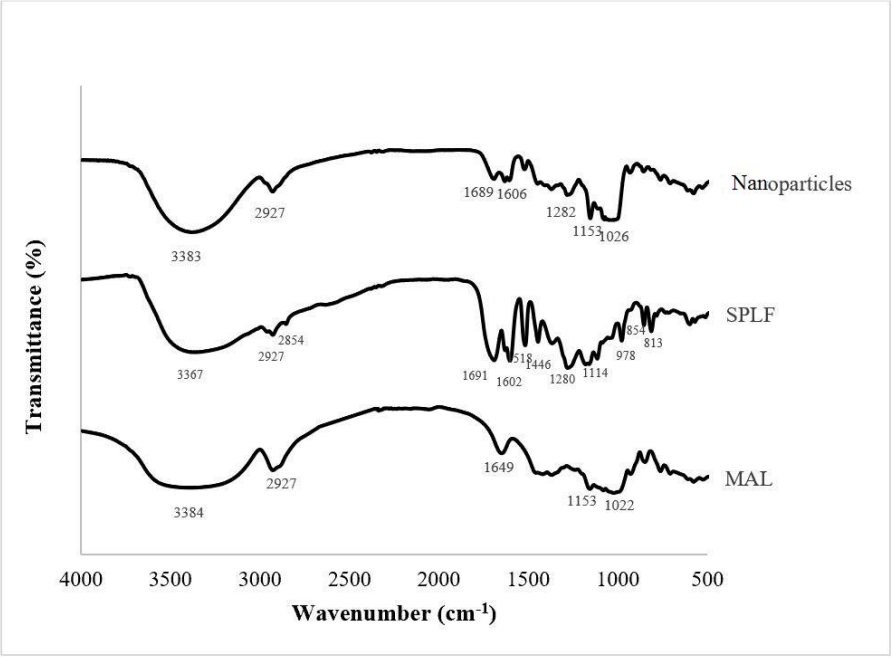

Figure 5-3 FT-IR spectra of SPLF, MAL and SPLF capsulated by 20 mg/mL MAL.
FT-IR, Fourier transform infrared spectroscopy; SPLF, sweet potato leaf flavonoids; MAL, maltodextrin.

3.5. Retention of total flavonoids content after simulated digestion

Bioaccessibility is usually evaluated by digestion procedure *in vitro*, generally simulated gastrointestinal digestion is the alternative, which easy to operate and without ethical dispute. Retention of TFC in SPLF and its nanoparticles after simulated digestion was shown in Figure 5-4(A), GIN and PRO were the flavonoids controls. TFC in SPLF and nanoparticles were 761.0, 175.6 mg/g, TFC in GIN and PRO was 457.7, 568.5 mg/g, correspondingly.

The retention of TFC after oral digestion was 75.4 %, 78.0 % and 88.5 % in SPLF, GIN and PRO, which was similar to Qin et al. (2018) that 21 % and 30 % of TFC in raspberry dried fruit and seeds were released. However, there was only a small amount of flavonoids released from blackthorn fruit, *Arbutus unedo* and pomegranate during simulated oral digestion (Pinacho, Cavero, Astiasarán, Ansorena, & Calvo, 2015; Mosele, Macià, Romero, & Motilva, 2016; Mosele, Macià, Romero, Motilva, & Rubió, 2015). The differences in the decrease of flavonoids during simulated oral digestion, might due to the difference in flavonoids composition and properties among varied plants, which some might more sensitive to pH, and some might not.

After gastric digestion, the retention of TFC was 54.9 %, 59.1 % and 73.6 %, respectively in SPLF, GIN and PRO, which was comparable with *Centella asiatica*, *Gymnema lactiferum*, *Sesbania grandiflora* and *Passiflora edulis* (45 %, 40 %, 42 % and 40 %) (Gunathilake, Ranaweera, & Rupasinghe, 2018). The presence of low pH down to around 2.0 could affect the stability of flavonoids, which kept consistent with the result of Mosele, Macià, Romero, & Motilva (2016).

After intestinal digestion, the retention of TFC was down to 45.9 %, 27.5 % and only 8.4 % in SPLF, GIN and PRO, separately, PRO showed the poorest stability. Significant decrease in TFC was found in four apple cultivars (2 cultivars of red skin and 2 cultivars of green skin) grown in Luxembourg, that TFC retention after simulated digestion was about 60 %, meanwhile, no anthocyanins could be detected after intestinal digestion. The degradation of flavonoids during transition from the acidic gastric environment to the mild alkaline intestinal environment, especially under the influence of bile salts and trypsin might be the reason (Bouayed, Hoffmann, & Bohn, 2011).

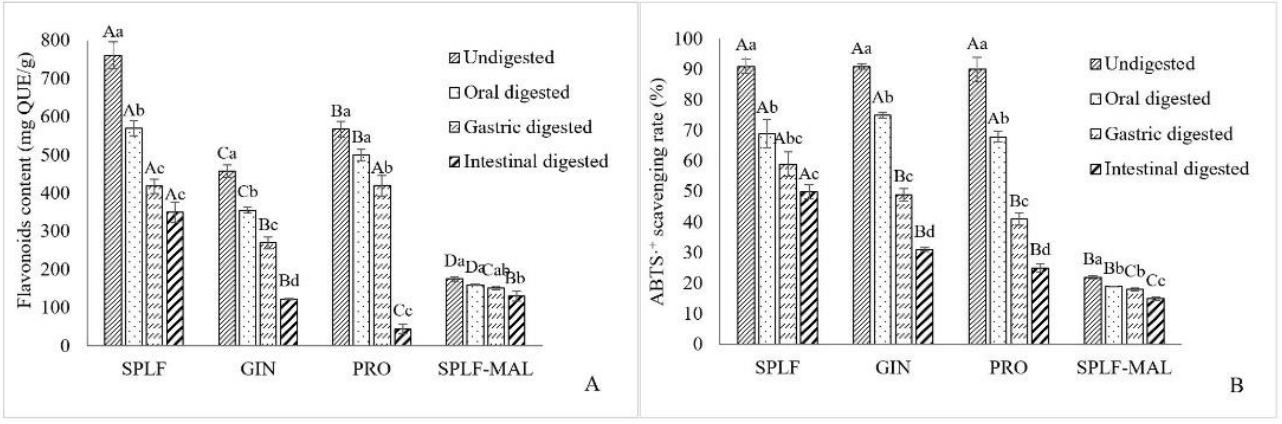

Figure 5-4 TFC and antioxidant activity after simulated digestion. A. TFC in SPLF, GIN, PRO and nanoparticles after simulated digestion. B. ABTS·+ scavenging capacity of SPLF, GIN, PRO and nanoparticles (the concentration of which was adjusted to 20 μg/mL) after simulated digestion.

TFC, total flavonoids content; SPLF, sweet potato leaf flavonoids; GIN, *Ginkgo biloba* extract; PRO, propolis flavone; SPLF-MAL, sweet potato leaf flavonoids and maltodextrin nanoparticles.

Different letters (A-D) represented values (mean ± SD, n = 3) were significant difference, for the same stage of simulated digestion between different samples ($P < 0.05$); Different letters (a-d) represented values (mean ± SD, n = 3) were significant difference, for the same samples at different stage of simulated digestion.

3.6. Retention of antioxidant activity after simulated digestion

Figure 5-4(B) showed that after oral digestion, the retention of antioxidant activity in SPLF, GIN and PRO was 75.9 %, 81.5 % and 75.9 %, severally, which was comparable with raspberry dried fruits, and after oral digestion, the retention of $ABTS^{\cdot+}$ scavenging capacity slightly decreased, for even short time exposure in medium pH environment could affect the stability of flavonoids in the negative way, which in turn affected antioxidant activity (Qin et al., 2018).

After gastric digestion, the retention of antioxidant activity in SPLF, GIN and PRO was 65.1 %, 53.6 % and 46.3 %, respectively, and green tea polyphenols showed the similar behavior when been undergoing the same digestion phase, which could retain 67 % of its Fe^{3+} reducing ability and 74 % of its $ABTS^{\cdot+}$ scavenging ability (López de Lacey et al., 2012). The significant decrease in the antioxidant activity might due to the release of flavonoids from the matrix. This transformation during simulated digestion could influence their antioxidant activity, for TPC in fruits and vegetables were highly associated with their antioxidant activity (Morales et al., 2014).

After intestinal digestion, the retention of antioxidant activity in SPLF, GIN and PRO was 56.2 %, 34.5 % and 28.2 % respectively, which was similar to *Rumex maderensis* that the retention of antioxidant activity evaluated by $ABTS^{\cdot+}$ scavenging capacity in leaves, flowers, stems was 51.8 %, 60.0 %, 50.7 %, separately (Spínola, Llorent-Martínez, & Castilho, 2018). The same behavior was also found from the study done by Dong et al. (2015) that the reduction of antioxidant activity after simulated digestion might depend on the food matrix and the types of flavonoids. Meanwhile, during the simulated digestion, the binding of flavonoids with the bile salts and digestive enzymes could lead to precipitation, then decreased their native bioactivity (Jakobek, 2015).

We could clearly see that TFC in nanoparticles were significantly lower than in SPLF, GIN and PRO before simulated digestion, from Figure 5-4 (A, B). Regarding TFC, SPLF presented the highest content compared with nanoparticles, it should be noted that when SPLF was encapsulated by 20 mg/mL MAL, equaled to 1 part of extract to 3.3 part of wall material, this was the explanation that nanoparticles showed the lowest TFC, which kept consistent with the study done by Bernardes et al. (2019). However, after encapsulated by 20 mg/mL MAL, SPLF was protected better after oral, gastric and intestinal digestion, which could extra retain 16 %, 31 %, 28 % of TFC and 17 %, 17 %, 11 % of $ABTS^{\cdot+}$ scavenging capacity, respectively. Our result was similar to citrus extract encapsulated by whey protein concentrate, that flavonoids in nanoparticles could be extra retained around 35 % and 30 % after gastric and intestinal digestion, meanwhile, $DPPH^{\cdot+}$ scavenging capacity could be extra

retained around 21 % and 20 %, separately (Hu, Kou, Chen, Li, & Zhou, 2019), for the association between antioxidant activity and flavonoids content was highly positive (Schulz et al., 2015).

3.7. Retention of individual flavonoid after simulated digestion

We could see from Table 5-2 that individual flavonoid in SPLF showed decreasing tendency after oral, gastric and intestinal digestion, illustrating flavonoids were sensitive to gastrointestinal environment. Myricetrin, isoquercitrin, quercetin were the individual flavonoids which could endure digestion to some certain extent, the retention of content after digestion was 61.2 %, 65.9 %, 31.4 % respectively. On the other hand, astragalin, quercitrin, tiliroside, kaempferol and rhamnetin were the individual flavonoids which could not especially endure intestinal digestion, retention of the content decreased to only 11.6 %, 12.9 %, 3.5 %, 15.7 % and 11.1 % respectively. Other studies showed the similar results, such as rutin in methanol extract of *Sesbania grandiflor* had already been damaged 22 % after oral digestion (Gunathilake, Ranaweera, & Rupasinghe, 2018), hyperoside and isoquercitrin in Chinese hawthorn decreased 61.6 % and 67.3 % after gastric digestion, and there was no hyperoside and isoquercitrin detected after intestinal digestion (Zheng et al., 2018). Apigenin, isoorientin, orientin, vitexin and acavetin in *Rumex maderensis* leaves decreased 41.3 %, 51.7 %, 53.0 %, 61.3 % and 38.2 %, respectively after simulated digestion, and luteolin even been completely destroyed. Isorhamnetin, quercetin and kaempferol in *Rumex maderensis* flowers decreased 81.1 %, 33.0 % and 70.1 % after the same process. The digestion-sensitive difference of flavonoids depended on the specific structure, such as flavonoids in Madeira sorrel (*Rumex maderensis*) flower, which flavanols were the most stable (reduction of 30 %), followed by flavones (reduction of 57 %) and flavonols (67 %), anthocyanins were completely degraded (Spínola, Llorent-Martínez, & Castilho, 2018). The significant reduction of flavonoids in the digesta after simulated digestion could be due to the instability in the mild alkaline environment (SSF and SIF), the basic aromatic structure of flavonoids in this pH environment underwent the opening of ring C, resulting in producing two fragments of ring A and ring B (Echeverry, Medina, Costa, & Aragón, 2018).

During digestion process, SPLF after encapsulation been protected better in oral and gastric phase, the content of individual flavonoid after encapsulation significantly higher than not encapsulated one, but had no significant difference after intestinal digestion. As MAL was a kind of polysaccharide, which could be digested preferentially by α-amylase, resisted to mild alkaline environment of SSF, and protected SPLF from damage during simulated oral digestion.

Meanwhile, SPLF could be released from nanoparticles and was relatively stable in SGF (pH 3.0). But we need to know whether SPLF been encapsulated by MAL or not, won't change the fact that intestinal digestion would seriously digest SPLF.

Table 5-2 The content of individual flavonoid in SPLF after simulated digestion (mg/g, DW)

	Before digestion	Oral digestion		Gastric digestion		Intestinal digestion	
		Not encapsulated	Encapsulated	Not encapsulated	Encapsulated	Not encapsulated	Encapsulated
Myricetrin	8.5±0.07[a]	6.1±0.01[d]	8.4±0.02[a]	5.3±0.26[e]	7.5±0.01[b]	5.2±0.09[e]	6.8±0.01[c]
Isoquercitrin	118.2±0.9[a]	101.6±0.07[d]	112.8±0.11[b]	99.4±0.08[e]	108.6±0.04[c]	77.9±0.13[g]	84.1±0.35[f]
Astragalin	639.5±12.8[a]	572.6±4.6[b]	581.2±2.3[b]	528.4±2.5[d]	548.5±1.8[c]	74.3±0.7[e]	79.7±0.3[e]
Quercitrin	206.9±3.7[a]	147.6±0.1[d]	176.7±0.1[b]	140.1±0.2[e]	157.2±0.6[c]	26.7±0.3[f]	28.5±0.08[f]
Tiliroside	54.6±0.5[a]	25.3±0.17[d]	42.8±0.71[b]	22.5±0.02[e]	30.1±0.11[c]	1.9±0.23[f]	2.1±0.07[f]
Quercetin	41.7±0.2[a]	17.9±0.03[d]	36.2±0.53[b]	16.8±0.06[e]	25.3±0.07[c]	13.1±0.13[g]	14.4±0.29[f]
Kaempferol	6.0±0.03[a]	3.6±0.05[e]	5.2±0.01[b]	4.2±0.01[d]	4.7±0.01[c]	0.94±0.00[f]	0.96±0.00[f]
Rhamnetin	4.5±0.02[a]	2.7±0.03[e]	4.1±0.01[b]	3.3±0.01[d]	3.6±0.01[c]	0.50±0.01[f]	0.49±0.01[f]

[a] SPLF, sweet potato leaf flavonoids.

[b] Different letters (a-g) in the same individual flavonoid represented values (mean ± SD, n = 3) were significantly different during simulated digestion ($P < 0.05$).

3.8. Microstructure of sweet potato leaf flavonoids and their nanoparticles after simulated digestion

The microstructure of SPLF and SPLF-MAL granules after simulated digestion was exhibited in Figure 5-5. We could see clearly that the freeze-dried powder of SPLF was lamellar [Figure 5-5(A)], while the microstructure of SPLF-MAL granule demonstrated that MAL particles inserted into SPLF granule and scattered around [Figure 5-5(E)]. After oral digestion, lamellar SPLF granule was digested obviously and the shape became blunt, losing its sharp edge [Figure 5-5(B)]. On the other hand, because of the MAL existence, which could be digested by α-amylase preferentially, could slowed down the digestion of SPLF granule to some certain extent [Figure 5-5(F)]. After gastric digestion, SPLF granule was digested further and "melted" gradually [Figure 5-5(C)], while the SPLF-MAL granule lost its form to a considerable degree, but still better than SPLF granule [Figure 5-5(G)]. After intestinal digestion, SPLF granule was digested to a loose minced appearance [Figure 5-5(D)], while SPLF-MAL granule was also digested seriously and even disintegrated [Figure 5-5(H)]. In general, simulated digestion process was intuitively observed by SEM, and MAL could make SPLF granule more tolerant towards oral and gastric digestion to some extent, making the latter been digested in the main digestion site (small intestine), achieving delayed release effect. Similarly, high concentration of flavonoids could reach through the stomach and to the upper part of the intestine. The metabolism of undestroyed flavonoids by microbiota producing smaller molecules which could be absorbed, entered into the blood circulation (Oteiza, Fraga, Mills, & Taft, 2018).

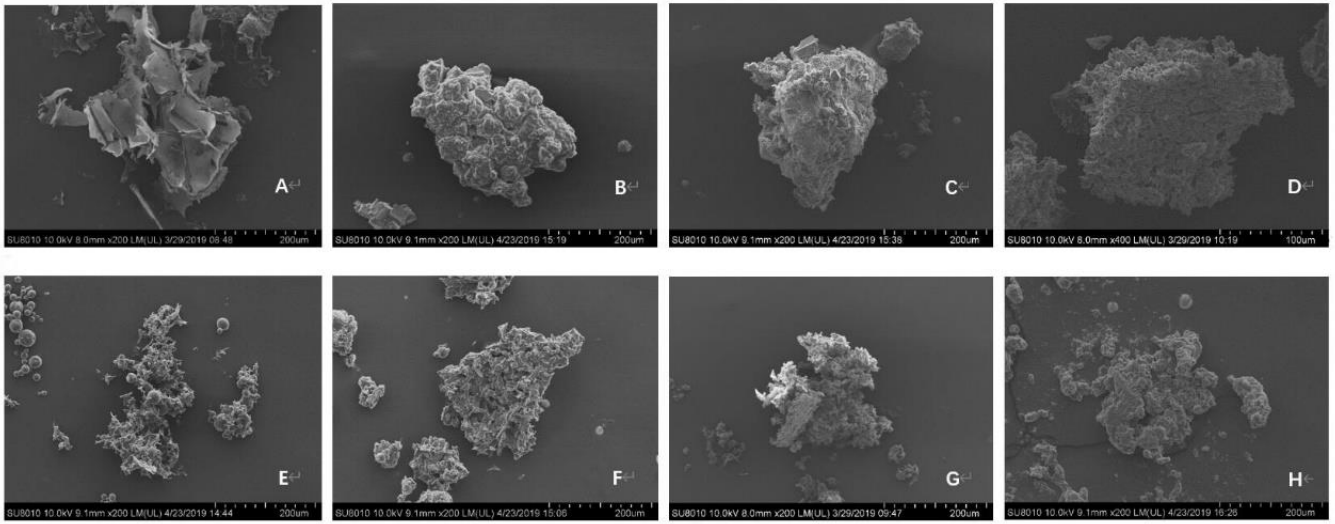

Figure 5-5 SEM of SPLF granule and its nanoparticles granule after simulated digestion (200 ×).

A: SPLF granule before digestion; B: SPLF granule after oral digestion; C: SPLF granule after gastric digestion; D: SPLF granule after intestinal digestion; E: Nanoparticles granule before digestion; F: Nanoparticles granule after oral digestion; G: Nanoparticles granule after gastric digestion; H: Nanoparticles granule after intestinal digestion.

SEM, scanning electron microscopy; SPLF, sweet potato leaf flavonoids.

4. Conclusions

In this study, SPLF was extracted by UMSE and SPLF-MAL nanoparticles were prepared by freeze-drying encapsulation. When the concentration of MAL was adjusted to 20 mg/mL, zeta potential and PDI were the lowest, EE was the highest, illustrating the stability of these nanoparticles was excellent, which could also be proved by CLSM and FT-IR that most of SPLF was sheltered. After simulated digestion, MAL as the polysaccharide inserted into SPLF granule, which was digested preferentially by α-amylase, could protect SPLF during oral and gastric digestion to some extent, postponed the digestion process of SPLF to the main digestion site (small intestine).

6

Chapter Ⅵ. Physical-chemical properties and volatile compounds of flavonoids-rich sweet potato leaf fortified bread

Sweet potato leaves possessing high content of flavonoids and excellent antioxidant activity, which might become a promising food additive. Commercial product of SPL green beverage powder has been developing, the possibility applied in bakery products also waiting to be discussed. In this chapter 1, 2, 3.5 and 5 % (w/w) of wheat flour was replaced by sweet potato leaves to make flavonoids enriched breads, and physical and chemical qualities of these fortified breads were evaluated.

This article has not been published yet.

Abstract

Sweet potato leaves (SPL) (cultivar: Yuzi No. 7) was used to substitute 1, 2, 3.5 and 5 % (w/w) of wheat flour to make bread. 1 % SPL changed the bread color significantly with ⊿E of 15.10, which could be clearly distinguished through human eyes. The hardness and chewiness of the crumb increased with increased SPL level, while specific volume of the bread shrank 53 %, compared with the control. Pores in the crumb became deterioration and disintegration when adding of SPL was higher than 3.5 % of the wheat flour. Total polyphenols content (TPC) and total flavonoids content (TFC) of the bread increased 6-fold and 10-fold, and antioxidant activity enhanced 10-fold, when adding 5 % SPL to the wheat flour. The addition of SPL (up to 5 %) won't alter volatile compounds of the bread, but suppressed the generation of typical flavor in bread significantly. Overall, SPL (no more than 1 %) could be supplemented to wheat flour to make breads with high TPC (2-fold), high TFC (2.5-fold) and excellent antioxidant activity (3-fold), without significantly adverse effect on the physical characteristics and the flavor.

Keywords

Sweet potato leaf fortified Bread; Physical characteristics; Polyphenols and flavonoids; Antioxidant activity; Volatile compounds profile

1. Introduction

China is the largest sweet potato producer in the world, the annual yield of 2018 was 53 million tons, which account for 57 % of the total yield (FAOSTAT, 2020). After the roots been harvested mainly as the starch resources, equal tons of SPL need to be dealt with. Sweet potato (*Ipomoea batatas* L.) leaves (cultivar: Yuzi No. 7) is chosen as a potential food additive because of excellent performance, not only in its basic nutrition, macro and trace elements, but also for its high TPC, TFC and outstanding antioxidant activity (Sun, Mu, Xi, Zhang, & Chen, 2014).

The purpose of basic research is for commercial applications, until now the products related to SPL included green beverage powder (Mu, Sun, Xi, & Zhang, 2013), SPL tea (Kim, 2012), pickled SPL (Wei et al., 2015). SPL as a nutritional by-product has been comprehensively studied (Hong, Mu, Sun, Richel, & Blecker, 2020), especially phenolic acids and flavonoids among it (Luo, Mu, & Sun, 2020; Liu, Mu, Sun, & Fauconnier, 2020), which possessing excellent antioxidant and antibacterial activities (Wang, Nie, & Zhu, 2016), having potential to extend the shelf life of products, making SPL a potential functional additive in staple food.

Bread as the western staple food, origins from Mesopotamia thousand years ago, supporting the intake of carbohydrate for human beings, with the average consumption of 70 kg per capita (Carocho et al., 2020). Flour, water, yeast and salt as the main components, become the bread after kneading, fermenting and

baking process. Herbs, spices and plant extract and waste could be used as the functional additives to increase the nutritional values of the breads (Vasileva et al., 2018). For example, adding sources rich in polyphenols to wheat flour could increase the antioxidant activity of the breads significantly (Dziki, Rózyło, Gawlik-Dziki, & Świeca, 2014).

When adding ginger powder (up to 6 %) to wheat flour to make bread, although the excellent TPC and antioxidant activity were obtained, however, this kind of bread showed the poor performance in the rheological property, including a tougher structure, and this bread sample was not sensorily acceptable (Balestra, Cocci, Pinnavaia, & Romani, 2011). The addition of quinoa leaves (1-5%) to wheat flour resulting in positively affected antioxidant activity of the fortified bread, while negatively affected its physical characteristics (Świeca, Sęczyk, Gawlik-Dziki, & Dziki, 2014). Furthermore, bread fortified with *Cistus* extract (2.5 %) increased TPC by 50 %, compared to the control, without significant adverse effect on the overall quality (Mikulec, Kowalski, Makarewicz, Skoczylas, & Tabaszewska, 2020). Adding of the green coffee extract (up to 2 %) to wheat flour didn't change physical and chemical characteristics of the bread significantly, but increased TPC and antioxidant activity by 12- and 6-fold compared to the control, separately (Mukkundur Vasudevaiah, Chaturvedi, Kulathooran, & Dasappa, 2017). The addition of green tea extract (2.5 %) could increase TPC by 4-fold without significant negative effect on volume and firmness of the crumb (Pasrija, Ezhilarasi, Indrani, & Anandharamakrishnan, 2015).

Therefore, the aim of present study was to evaluate the effect of SPL (addition of 1, 2, 3.5 and 5 %) on physical characteristics (specific volume, porosity, texture, color), chemical characteristics (TPC, TFC, antioxidant activity, volatile compounds) of the bread, and discuss the possibility of SPL applied in bakery products.

2. Material and methods

2.1. Material and reagents

SPL (variety of Yuzi No. 7) was harvested in Yubei District, Chongqing, China, between 29°84′ north latitude and 106°47′ east longitude, for 70 days of growing period (June to September, 2017), which had the highest TPC and strongest antioxidant activity among 40 varieties based on the previous study (Sun, Mu, Xi, Zhang, & Chen, 2014), washed, freeze-dried and grounded to fine powder and filtered through a 0.15mm sieve. The powder was seal in aluminum foil bags and stored at -18 °C before use, which contained 10.5 % of ash, 28.8 % of crude protein, 3.3 % of crude fat, 18.5 % of crude fiber and 39.0 % of carbohydrate.

2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and Folin-

Ciocalteu reagent were bought from Sigma-Aldrich, wheat flour with 13.7 % of water, 12.1 % of protein, 1.7 % of fat, 3.0 % of crude fiber, 68.5 % of carbohydrate and 0.2 % of ash (Molini Certosa, Italy), butter (Président, France), salt (La baleine, France) and instant dry yeast (Bruggeman, Belgium) were food grade and bought from local supermarket (Delhaize, Belgium), other chemical reagents were analytical grade and bought from Sigma-Aldrich (Belgium).

2.2. Farinograph test of the dough

Freeze-dried SPL powder substituted 0, 1, 2, 3.5 and 5 % of wheat flour to make a dough, separately. A 300 g mixing bowl equipped by the farinograph (Farinogragh-E, Brabender, Germany) was chosen as the blend container. The flour was mixed with distilled water at 63 rpm to reach a maximum dough consistency centered at the 500 Brabender Units (BU) line, which called standard water content. The results were reported on a 14 % moisture basis, when adding 0, 1, 2, 3.5 and 5 % levels of SPL, the water absorption of the flour was 58.3 %, 58.6 %, 58.9 %, 59.4 % and 60.4 %, respectively. Thus, adding SPL improves water absorption which is considered as an important economic criterion in the bread making industry.

2.3. Preparation of the bread

For standardization of the bread making, a bread formula was followed: 300 g of flour (by replacing 1, 2, 3.5 and 5 % of flour with freeze-dried SPL powder), 7.5 g of butter, 4.5 g of salt, 3 g of yeast and an amount of water determined by the water absorption as determined with the farinograph data (A %) (the added water must be at a specific temperature, that subtraction of the temperature of air and flour from 69 °C). First, put the flour and the yeast in the household mixer (Chef XL Sense Kitchen Machine, Kenwood, Aartselaar, Belgium), and stirred for 2 min at 248 rpm (position 2). Then, added the butter which cut into small pieces, and stirred for 2 min at 248 rpm (position 2). After that, added the water gradually in 1 min, at the exact temperature mentioned above, stirred for 2.5 min at 305 rpm (position 3). Last, added the salt and stirred for 2 min at 248 rpm (position 2) to make one big ball of the dough. First fermentation was carried out at 30 °C and 95 % relative humidity (RH) for 45 min in a proofer (Commercial Proofer, Andrew James, UK). Weighed ≈25 g of first fermented dough, kneaded a small ball and put it in the growth controller, which adjusted the height to 2.3 cm, in the meantime, weighed doughs of approximately 40 g, kneaded balls and put them in the oiled molds. Placed the growth controller and the molds in the fermentation chamber at 30 °C and 95 % RH. When the height of dough reaching to 4.0 cm in the growth controller while took the molds out of the chamber (in general, it usually took between 40-60 min), then baked in the oven (Pizzaoven

ET 205, Bartscher GmbH, Germany) preheated to 230 °C for 15 min. After baking, the breads were removed from the molds, cooled for 2 h at room temperature (20±1 °C) and packed in paper bag. Physical properties were measured after 18 h and finished within 6 h.

2.4. Physical parameters evaluation of the bread

2.4.1. Specific volume and porosity

Volume of the bread was determined by rapeseeds displacement method using standard volume-measuring apparatus (National Manufacturing Co., Lincoln, Netherland), and specific volume (cm^3/g) was the reliable quality of bread and calculated by dividing volume by weight (Conte, Del Caro, Balestra, Piga, & Fadda, 2018).The porosity was determined by modified method of Lazaridou, Duta, Papageorgiou, Belc, & Biliaderis (2007) with some modification. Breads were cut into 12 mm thick slices using a KRUPS Cutter and photographed by a digital camera (Nikon, ECLIPSE E400). The images were analyzed by Image J (Version 1.52p, National Institutes of Health, USA). The central part image of the crumb was selected and the area was determined. In order to obtain a black and white threshold, images were converted into 8-bit and performed by binary segmentation. The results were calculated as the porosity of the crumb.

2.4.2. Texture profile analysis

Texture Analyzer TA-XTi (Stable Micro Systems, UK) with the software "Texture Profile" was applied to measure texture profile of the crumb, in which equipped with a ball probe (P/0.75S). Breads were sliced into approximate 12 mm thick slices, and the analysis was carried out in the middle part of the slice. Test parameters included: test mode of compression, pre-test speed of 2 mm/s, to a trigger point force of 0.001 N, test speed of 2 mm/s, with the target mode of distance (10 mm), holding for 60 s then post-test speed of 2 mm/s. Hardness, cohesiveness, springiness and chewiness were calculated from the texture measurement (Texture Technologies Corp. and Stable Micro Systems, 2018).

2.4.3. Color of the crumb

Each loaf was cut vertically into 12 mm thick slices, crumb color (L*, a*, b*) was measured in each side of 3 central slices using colorimeter (Color Flex EZ, HunterLab, USA), with a standard light source, standard colorimetric observer (10°), and a standard hole (12.3 mm diameter). L* represented lightness in which 0 equaled blackness and 100 equaled whiteness, a* represented greenness/redness, and b* represented yellowness/blueness, where the smaller of the value lower than 0, the more greenness or yellowness of the color, the bigger of the value higher than 0, the more redness or blueness of the color, separately.

ΔE* as the overall index of the color difference between the SPL fortified bread (SPLB) and control bread, was calculated by the equation as follows:

$$\Delta E^* = \sqrt{(\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})} \quad (1)$$

Where ΔL^*, Δa^*, Δb^* referred to the color difference of lightness, greenness/redness and yellowness/blueness between SPLB and control bread.

2.5. Chemical evaluation of bread

2.5.1. Bread extraction solutions

The powdered samples (15 g) of control bread and SPLB were extracted by 150 mL of 70 % ethanol for 15 min in an ultrasonic bath (RK 514 H, Bandelin, Berlin, Germany). Extraction procedure was repeated 2 times, extraction solutions were combined and volumed to 500 mL, then stored at 4 °C away from light.

2.5.2. Determination of polyphenols

Total polyphenols content (TPC) was measured by Folin-Ciocalteu method described by Xi, Mu, & Sun. (2015). 1.0 mL sample solution was mixed with 1.0 mL Folin-Ciocalteu reagent (10 times dilution), reacted at 30 °C for 30 min, then 2.0 mL of saturated Na_2CO_3 solution (10 %, v/v) was supplemented, fully mixed and incubated for another 30 min at 30 °C in the water bath. Absorbance of the solution was measured at 736 nm by UV-Vis spectrophotometer (Ultrospec 7000, BIOCHROM, UK), a standard curve for chlorogenic acid at the concentration of 0-0.05 mg/mL was determined. TPC was expressed in milligrams of chlorogenic acid equivalent per g of dry weight of bread powder (mg CAE/g DW).

2.5.3. Determination of flavonoids

Total flavonoids content (TFC) was measured by $NaNO_2$-$AlCl_3$-NaOH method described by Gomes et al. (2017) with some modification. 1.0 mL of extraction solution combined with 4.0 mL of distilled water, mixed with 0.3 mL of 5 % $NaNO_2$, stood still for 5 min, then added 0.3 mL of 10 % $AlCl_3$, stood still for another 5 min, finally complemented with 2.0 mL of 4 % NaOH, thoroughly mixed and incubated for 10 min. The absorbance of the solution was measured at 333 nm (maximum absorbance) by spectrophotometer. Quercetin was taken as the standard, the standard curve at the concentration of 0-0.10 mg/mL was ready. TFC was expressed in milligrams of quercetin equivalent per g dry weight of bread powder (mg QUE/g DW).

2.5.4. Evaluation of antioxidant activity

The antioxidant activity of control bread and SPLB was evaluated by the method of Li, Lin, Gao, Han, & Chen (2012) with some modifications. 5.0 mL ABTS (7.4 mM) and 5.0 mL $K_2S_2O_8$ (2.6 mM) were mixed well and kept away from light for 24 h at 4 °C to make $ABTS^{\cdot+}$ stocking solution, then diluted approximately 70 times to obtain the working solution with the absorbance of 0.70±0.02 at 734 nm. 2.0 mL $ABTS^{\cdot+}$ working solution reacted with 1.0 mL bread extraction solutions (adjusted to the suitable concentrations), according to the

absorbance at 734 nm after 6 min, $ABTS^{\cdot+}$ scavenging rate of the samples was calculated as follows:

$$Scavenging\ rate\ (\%) = \frac{A0-A}{A0} \times 100\% \quad (2)$$

Where A_0 was the absorbance of the blank at 734 nm, A was the absorbance of the mixture at 734 nm. The antioxidant activity of the samples evaluated by $ABTS^{\cdot+}$ scavenging capacity, which was expressed in IC_{50} value.

2.6. Collection and analysis of volatile compounds

2.6.1. Head space solid phase microextraction

Four fibers were tested, which were polydimethysiloxane/divinylbenzene (PDMS/DVB) (65 μm), divinylbenzene/carboxen/polydimethysiloxane (DVB/CAR/PDMS) (50/30 μm), carboxen/polydimethysiloxane (CAR/PDMS) (85 μm) and polydimethylsiloxane (PDMS) (100 μm), separately, all of them were bought from Supelco, Sigma-Aldrich (Bellefonte, Pennsylvania, USA). 2.0 g of sample was placed in 12 mL headspace glass vial sealed with silicone septa. After been equilibrating at 60 °C in the water bath for 15 min with a magnetic stirring of 700 rpm, the solid phase microextraction (SPME) fiber was injected into the headspace (HS) of the vial, 10 mm above the sample maintaining for 30 min, to absorb volatile compounds, following with the method of Azeem, Mu, & Zhang (2020) with some modifications. The SPME fiber was withdrawn back to the injector, then been pulled out of the vial and pierced into the inlet septum, when GC/MS was ready and absorption process was done.

2.6.2. Analysis of volatile compounds

The analysis of GC/MS was performed in a gas chromatograph (GC) (Agilent 7890A) combined with a mass spectrometer (MS) (Agilent 5975C, inert XL EI/CI MSD with Triple-Axis Detector), according to the method of Cui, Liu, & Li (2010) with some modifications. Volatile compounds of the bread were separated on a capillary column (Agilent VF-WAXms, 30 m × 0.25 mm × 0.25 μm). The injector port was heated to 300 °C, and helium was taken as the carrier gas, with the constant flow of 1.5 mL/min. The oven temperature was kept at 40 °C for 4 min, increased to 90 °C at 5 °C/min, holding for 5 min, then increased to 230 °C at 10 °C/min and holding for 7 min.

The MS was operated in electron impact (EI) mode with the electron energy of 70 eV using ion source temperature of 230 °C. The mass-to-charge ratio was set in the range of 40-500. The temperature of MS quadrupole and transfer line was 150 °C and 230 °C, respectively. The GC/MS peaks were identified by comparing their mass spectra with PAL 600 Mass Spectral Library database, in which the match quality higher than 80 % was selected as a positive identification. The content of volatile compounds was expressed as relative peak area, which could be calculated as follows:

$$Relative\ peak\ area = \frac{Peak\ area\ of\ the\ compound}{Total\ area} \times 100\% \quad (3)$$

2.7. Statistical analysis

Physical evaluation was carried out in 5 parallels, chemical evaluation was carried out in 3 parallels, results were expressed as mean±standard deviation (SD). Significant difference ($P < 0.05$) between the means was calculated by one-way analysis of variance (ANOVA) and Duncan's test. Correlation analysis between TPC and TFC, TPC and antioxidant activity, and TFC and antioxidant activity were calculated by Pearson's correlation analysis. SPSS (Version 24, USA) was used as the analysis software.

3. Results and discussion

3.1. Physical characteristics of the bread

The water absorption of SPLB was significantly higher than the control bread, and increased with the increasing level of SPL, which was increased by 2.1 % (from 58.3 % to 60.4 %) when the addition of SPL was increased from 0 % to 5 %. Similar tendency was found by Sui, Zhang, & Zhou (2016) that when the level of anthocyanin-rich extract from black rice increased from 0 % to 4 %, the water absorption of its fortified bread flour was increased by 4.8 %. The increased water absorption could due to the reason that SPL with high fiber content (18.5 g/100 g DW) had a high water-holding ability, which kept consistent with Ortiz de Erive, Wang, He, & Chen (2020). As data shown in Table 6-1, when the addition of SPL increased from 0 % to 5 %, the weight of bread increased from 25.6 g to 28.9 g. This change could prevent the proper expansion of dough.

The added SPL interfered gluten, resulting in a weak and more easy disruption of gluten network, inducing lower gas-holding capacity, compared with the control bread (Wang et al., 2003). The volume of the breads decreased significantly from 120.4 mL (control bread) to 63.6 mL (5 % SPLB), separately shrank 6 %, 10 %, 27 % and 47 % of its volume, compared with the control bread. Correspondingly, specific volume of the SPLB shrank from 4.7 mL/g to 2.2 mL/g, the addition of SPL significantly decreased the specific volume of the bread. Baiano, Viggiani, Terracone, Romaniello, & Del Nobile (2015) studied the breads fortified with phenolic extracts from chicory, cabbage, celery, fennel, olive leaf and grape marc, the result showed a decrease in the specific volume (ranging from 5.9 % to 21.0 %), compared with the control bread, indicating that the phenolic extracts might denature the gluten network.

The cross-section and their 8-bit and binary images (obtained by Image J software) of control bread (A1-A3), 1 % SPLB (B1-B3), 2 % SPLB (C1-C3), 3.5 % SPLB (D1-D3) and 5 % SPLB (E1-E3) were exhibited in the Figure 6-1. As

another important physical parameter, the porosity of crumb determined the quality of bread, which was 33.5 % and 32.3 %, respectively when adding 1 % and 2 % of SPL to the wheat flour, increased significantly compared with the control bread (24.3 %). However, when the supplement of SPL increased to 3.5 %, the crumb became disintegrating with the porosity of 44.6 %. Not mention adding 5 % SPLB, the crumb even aged significantly and the porosity decreased to 40.0 %, which was also given in Table 6-1. This tendency could be explained that when adding fiber-rich materials to flour to make bread, water could migrate from gluten network by incorporation with fiber during dough resting, which could lead to partial dehydration of gluten, finally made the network stiffer (Hemdane et al., 2017).

When bread was taken out of the oven, aging occurred immediately, especially for amylose. Water diffused from gluten network to starch, because the crystallinity of starch requiring water, which migrated from crumb to crust (Bosmans, Lagrain, Fierens, & Delcour, 2013). The Mechanism led to a significant increase in the firmness of crumb, while decreased crumb elasticity, which was shown in Table 6-1.

Texture was a major index for consumers to evaluate the taste of bread, in which hardness (N), adhesiveness, springiness, cohesiveness and chewiness (N) were chosen as the parameters. Hardness was the force needed to deform into the sample, which was about 1-, 2-, 2.5- 3-fold, compared with control bread, when bread was fortified by 1, 2, 3.5 and 5 % SPL, respectively, indicating bread became harder and harder when adding increased level of SPL to the flour, however, adding 1 % SPL won't have significant influence. The increase of bread hardness might be the result that the increase of its density, which could be verified by its specific volume. Bread fortified with anthocyanin-rich extract from black rice showed the same tendency that hardness increased significantly as the content of black rice extracts, only supplemented of 4 % black rice extracts increased 61 % of hardness for the crumb (Sui, Zhang, & Zhou, 2016).

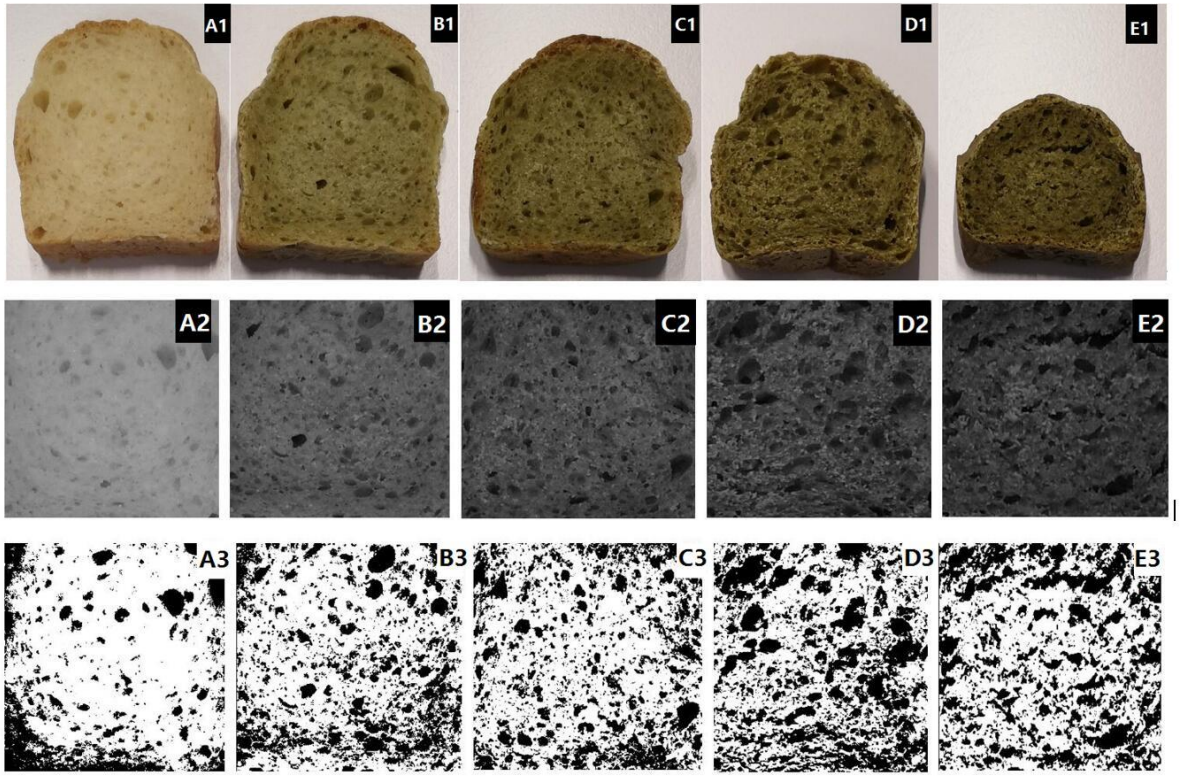

Figure 6-1 The cross-section of bread crumb (A1-E1): 8-bit image of bread crumb obtained by Image J (A2-E2): The binary image of bread crumb obtained by Image J (A3-E3). A: Control bread; B: 1 % SPLB; C: 2 % SPLB; D: 3.5 % SPLB; E: 5 % SPLB.
SPLB, sweet potato leaf fortified bread.

Table 6-1 The physical characteristics of sweet potato leaf fortified bread

Physical characteristics	Control bread	1% SPLB	2% SPLB	3.5% SPLB	5% SPLB
Weight (g)	25.61±0.47^{c}	25.86±0.72^{c}	26.41±0.51^{ab}	27.31±0.69^{ab}	28.90±0.90^{a}
Volume (mL)	120.4±2.9^{a}	113.8±3.5^{ab}	108.3±4.4^{b}	87.4±2.6^{c}	63.6±3.1^{d}
Specific volume (mL/g)	4.7±0.3^{a}	4.4±0.28^{a}	4.1±0.25^{ab}	3.2±0.27^{b}	2.2±0.14^{c}
Width (mm)	44.00±0.14^{ab}	43.19±0.42^{ab}	44.87±0.31^{a}	42.18±0.52^{b}	41.84±1.39^{b}
Height (mm)	49.06±0.21^{a}	49.44±0.56^{a}	44.49±0.38^{b}	40.61±0.75^{c}	30.97±0.33^{d}
Total area (mm^2)	2006±47^{a}	1929±52^{a}	1900±28^{a}	1590±14^{b}	1177±31^{c}
Porosity (%)	24.29±0.48^{d}	33.51±0.88^{c}	32.29±0.84^{c}	44.63±1.07^{a}	40.02±0.06^{b}
Crumb color property					
L*	75.77±1.01^{a}	61.19±0.71^{b}	54.97±1.08^{c}	49.90±0.71^{d}	45.92±0.66^{e}
a*	1.47±0.15^{a}	-0.72±0.07^{b}	-1.24±0.24^{bc}	-1.66±0.14^{cd}	-2.11±0.20^{d}
b*	22.52±0.50^{b}	25.76±0.29^{a}	26.66±0.42^{a}	27.08±0.43^{a}	26.70±0.74^{a}
⊿E		15.10^{d}	21.38^{c}	26.45^{b}	30.35^{a}
Crumb texture property					
Hardness (N)	3.00±0.039^{c}	3.25±0.087^{c}	6.13±0.24^{b}	7.51±0.42^{a}	8.58±0.72^{a}
Adhesiveness (N·s)	0.029±0.01^{c}	0.037±0.01^{c}	0.24±0.01^{b}	0.33±0.027^{a}	0.36±0.002^{a}
Springiness	0.91±0.02^{a}	0.86±0.03^{a}	0.79±0.01^{b}	0.7±0.01^{c}	0.62±0.02^{d}
Cohesiveness	0.78±0.01^{a}	0.73±0.01^{a}	0.66±0.02^{b}	0.58±0.01^{c}	0.51±0.03^{d}
Chewiness (N)	2.13±0.16^{b}	2.04±0.21^{b}	3.2±0.27^{a}	3.05±0.11^{a}	3.71±0.19^{a}

[a] Different letter (a-e) in the same parameter represented values were significantly different following with the increased SPL ($P < 0.05$).

Adhesiveness represented teeth sticky degree for food, in which produced a negative force when been chewed, the higher of the negative area, the more-sticky of the sample. The value of control bread and SPLB was quite small, as expected, not even reaching 1, represented the bread was not adhesive, even 5 % SPLB possessed the highest adhesiveness of only 0.36 N·s, following with 3.5, 2, 1 % SPLB and control bread (Table 6-1), which was consistent with the research done by Wang, Zhou, & Isabelle (2007) that there was an increase tendency in the adhesiveness of bread with increased level of green tea extract. Springiness was expressed as the elasticity of the bread, and when the addition of SPL was more than 1 % already decreased the crumb elasticity significantly, which might due to the weaker and less elastic gluten structure of SPLB.

Cohesiveness represented internal resistance of the bread structure, which a decrease of cohesiveness in the bread was observed with increased SPL level, according to the result shown in Table 6-1, illustrated high cohesiveness tended to form bolus rather than disintegrating during mastication process (Matos & Rosell, 2013), which kept consistent with the literature that the cohesiveness of 20 % brewers' spent grain bread, 10 % sugar beet pulp bread and 10 % apple pomace bread decreased significantly compared with control bread 24 h after baking (Torbica, Škrobot, Janić Hajnal, Belović, & Zhang, 2019).

Chewiness could be explained the energy needed when chewing the solid or semi-solid food, in which the higher of the value, the more difficult to masticate. The addition of SPL (1 %) did not cause significant change in chewiness compared with the control bread, but adding 2, 3.5 and 5 % SPL could significantly increase the chewiness by 43 %, 44 % and 74 %, compared with the control bread, which might due to the strengthening of the dough structure (Wirkijowska et al., 2020).

Color of the bread was an important sensory attribute to attract consumers, which was presented in Table 6-1. Supplement of SPL significantly affected the color of the crumb. As the increase of SPL level, the L* and a* value decreased significantly but the b* value increased remarkably, indicated a darker, greener and yellower crumb obtained when adding a growing level of SPL to wheat flour. The natural green-yellow of SPL (which was rich in chlorophyll and flavonoids) led to the coloration of crumb. Our result was consistent with the research done by Cacak-Pietrzak et al. (2019) that even if only a 1 % addition of *Cistus incanus* L. would significantly affect the color of fortified bread compared with control bread. Furthermore, ⊿E was taken to evaluate the total difference of color which ranging from 15.1, 21.4, 26.5 and 30.4 between 1, 2, 3.5 and 5 % SPLB compared with control bread, individually. The total color difference (⊿E*) was usually considered as a minor difference (0-1.5), a difference (1.5-3) and a major difference (>3) to the human eyes (Wirkijowska et al., 2020), the adding of up to only 5 % SPL significantly deteriorated the color of bread.

Retrogradation property of starch was highly related to the staling of bread

during storage, when amylose associated to the short-term (first day) retrogradation in the initial few hours, amylopectin re-crystallization associated to the bread firming in the next few days. Staling resulted in firming and toughening in the crumb, and losing of moisture and flavor (Zhu, 2014).

3.2. The content of polyphenols and flavonoids

The addition of SPL clearly contributed to the increase of antioxidative components of the obtained breads (Table 6-2), which were characterized by a higher content of polyphenols, ranging from 0.9 to 3.3 mg CAE / g DW of bread, compared with the control bread (0.5 mg CAE / g DW). The result was similar to bread supplemented with *Cistus incanus* L. (1, 2, 3, 4 and 5 %) with the increase of TPC by 17, 40, 65, 73 and 110 %, separately, compared with the control, and 1 % of this additive could already significantly increase TPC of bread (Cacak-Pietrzak et al., 2019). In the meantime, SPLB had significantly higher content of flavonoids, ranged from 0.5 to 2.1 mg QUE / g DW of the bread, compared with the control bread (0.2 mg QUE / g DW). The similar result was found when adding 0, 2.5 and 5 % of stinging nettle *(Urtica dioica)* leaves to wheat flour to make bread, TFC in which was 0.35, 0.55 and 0.63 mg catechin equivalent / g DW, respectively, supplemented only 5 % of stinging nettle could almost double TFC of bread (Đurović et al., 2020).

Table 6-2 Total polyphenols content, total flavonoids content and antioxidant activity of sweet potato leaf fortified bread

Sample	TPC (mg CAE/g)	TFC (mg QUE/g)	IC_{50} value of antioxidant activity (mg extract/mL)
Control bread	0.5 ± 0.036^{e}	0.2 ± 0.03^{e}	5.14 ± 0.22^{c}
1 % SPLB	0.9 ± 0.014^{d}	0.5 ± 0.04^{d}	1.61 ± 0.15^{b}
2 % SPLB	1.6 ± 0.042^{c}	0.8 ± 0.10^{c}	1.29 ± 0.10^{b}
3.5 % SPLB	2.7 ± 0.095^{b}	1.7 ± 0.13^{b}	0.71 ± 0.04^{a}
5 % SPLB	3.3 ± 0.099^{a}	2.1 ± 0.05^{a}	0.49 ± 0.02^{a}

[a] Different letter (a-e) in the same parameter represented values were significantly different following with the increased SPL ($P < 0.05$).

[b] SPLB, sweet potato leaf fortified bread; TPC, total polyphenols content; CAE, chlorogenic acid equivalent; TFC, total flavonoids content; QUE, quercetin equivalent.

3.3. The antioxidant activity of the bread

The antioxidant activity of the bread was followed the order of 5 % > 3.5 % > 2 % > 1 % SPLB > Control bread (Table 6-2). The result showed that a small amount addition of SPL to wheat flour greatly enhanced antioxidant activity of the bread, IC_{50} value of $ABTS^{\cdot+}$scavenging capacity decreased significantly from 5.1 to 0.5 mg extract/mL, correspondingly, which might due to the incorporation of polyphenols, possessing high antioxidant activity, such as flavonoids (astragalin, quercetrin, isoquercitrin and tiliroside) and non-flavonoids (4,5-, 3,5- and 3,4-chlorogenic acid) (Liu, Mu, Sun, & Fauconnier, 2019; Xi, Mu, & Sun, 2015). Similar result was found such as when adding 5 % of lavender and Melissa (which were rich in polyphenols and flavonoids) could increase the antioxidant activity of breads by about 10 times, evaluated by 2,2-diphenyl-1- picrylhy- drazyl (DPPH) radical scavenging ability and ferric reducing antioxidant power (FRAP) (Vasileva et al., 2018). 1, 2, 3, 4 and 5 % *Cistus incanus* L. was added to wheat flour to make bread with the ability of quenching $ABTS^{\cdot+}$ increased about 1, 2, 3, 4 and 5-fold, compared with the control (Cacak-Pietrzak et al., 2019). Different enhancing ability of antioxidant activity for the same amount of adding might due to the reason that the composition of polyphenols and flavonoids was different varied with the samples, meanwhile, the mechanism of antioxidant activity detecting methods mentioned above was different (Alam, Bristi, & Rafiquzzaman, 2013).

Coefficient higher than 0.9 could be considered as strong correlation, while $0.7 < r^2 < 0.9$ and $0.5 < r^2 < 0.7$ were represented high and moderate correlation, respectively, $r^2 < 0.5$ was considered poor correlation. In SPLB, strong positive correlation ($r^2 = 0.99$; $p < 0.05$) among TPC and TFC was noticed, while high negative correlation between TPC and IC_{50} value of $ABTS^{\cdot+}$ scavenging capacity ($r^2 = -0.78$; $p < 0.05$), TFC and IC_{50} value of $ABTS^{\cdot+}$ scavenging capacity (($r^2 = -0.76$; $p < 0.05$) were clearly observed. The results indicated that flavonoids in polyphenols were responsible for high antioxidant activity of SPLB. Our study was consistent with Đurović et al. (2020) that TPC, TFC and antioxidant activity had strong correlation pairwise, which r^2 of TPC and TFC, TPC and antioxidant activity, TFC and antioxidant activity was 0.90, 0.76 and 0.73, respectively.

3.4. The effect of different fibers on the volatile compounds of sweet potato leaf fortified bread

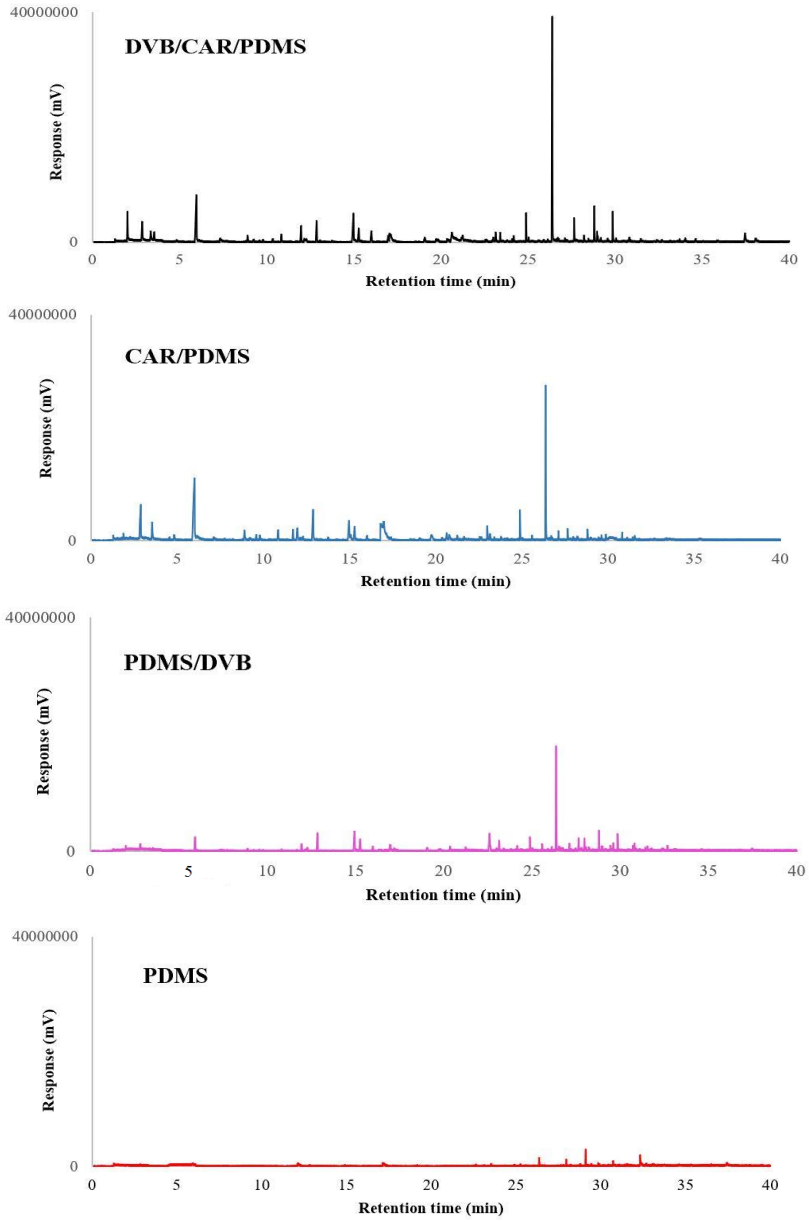

Figure 6-2 Total ion current gas chromatogram of HS-SPME adsorption and GC-MS analysis of volatile compounds from SPLB using different fibers.
HS-SPME, headspace solid phase microextraction; GC-MS, gas chromatography mass spectrum; SPLB, sweet potato leaf fortified bread; PDMS/DVB, polydimethysiloxane/divinylbenzene; DVB/CAR/PDMS, divinylbenzene/carboxen/polydimethysiloxane; CAR/PDMS, carboxen/polydimethysiloxane; PDMS, polydimethylsiloxane.

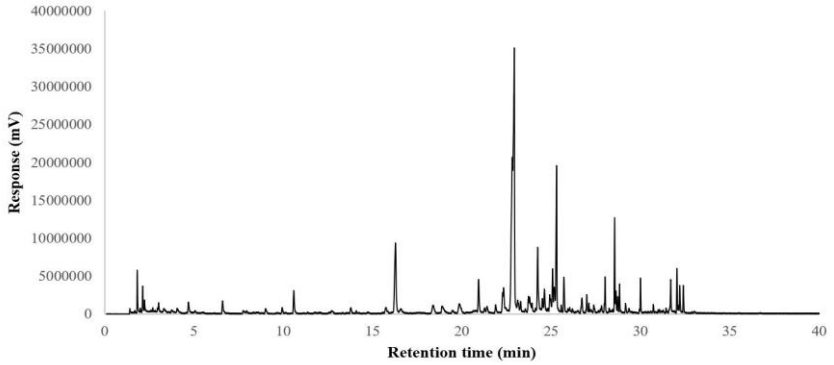

Figure 6-3 Total ion current gas chromatogram of HS-SPME adsorption and GC-MS analysis of volatile compounds from SPL.
HS-SPME, headspace solid phase microextraction; GC-MS, gas chromatography mass spectrum; SPL, sweet potato leaves.

The type and coating of SPME fiber are important for the analytical performance of HS-SPME analysis. Several types of coating, with a wide range of polarities, are commercially available (Vas & Vekey, 2004). Four coatings were tested, for the absorption of volatile compounds from SPLB, that different fiber had significantly various effect on the result (Figure 6-2). Extraction by DVB/CAR/PDMS fiber resulted in chromatogram with the best performance and highest response, followed by CAR/PDMS and PDMS/DVB fibers, PDMS fiber showed the poorest performance. The reason could be attributed to CAR/PDMS fiber was used for extraction of low molecular weight volatile compounds and polar compounds, while dual-coated fiber (DVB/CAR/PDMS) was recommended for flavor and odor extraction (Pico, Gómez, Bernal, & Bernal, 2016). Considering the actual effect, DVB/CAR/PDMS fiber was chosen in this study, which also exhibited the best performance in analyzing volatile compounds in SPL (Figure 6-3). The result kept consistent with the report from Cui, Liu, & Li (2010), who studied the changes of volatile compounds in SPL during fermentation. Therefore, it was decided to use DVB/CAR/PDMS fiber to analyze the volatile compounds in SPL, control bread and SPLB.

3.5. Volatile compounds in the breads

Volatile compounds in SPL, control bread and SPLB were shown in Table 6-3. β-caryophyllene was recorded maximum in SPL which was 25.64 %, followed by 2-butoxyethanol (6.05 %), bisabolene (5.41 %), ρ-cymene (4.44 %), α-humulene (3.36 %), benzaldehyde (2.87 %), limonene (2.73 %), 3,5-octadien-2-one (2.68 %) and other aldehydes, ketones, esters, alcohols, acids, hydrocarbons, etc. Our result kept consistent with the research done by Cui, Liu, & Li (2010), and demonstrated the odor characteristics of SPL was typically sweet, woody, green and herbal,

which was similar to characteristic odor compounds in tea (Yang, Baldermann, & Watanabe, 2013).

On the other hand, hexanoic acid (18 %), 1-hexanal (9.23 %), 1-nonaldehyde (6.96 %), 1-octanal (4.02 %), ethanol (3.46 %), 1-pentanol (3.45 %), ethyl acetate (2.76 %), pentanoic acid (2.65 %), acetic acid (2.21 %) and octanoic acid (2.00 %) were the main compounds with odor activity detected in control bread, proved the control bread possessed a typical sweet, acid, cheesy and fermented odor, which might be the credit for butter when preparing the bread, was quite different from the odor of SPL. When 1, 2, 3.5 and 5 % SPL was added to the flour to make bread. Most of volatile compounds in SPL were sensitive to the baking temperature, especially the alkenes, that were almost completely destroyed during bakery process, which eliminated their specific woody, herbal smell, illustrated the addition of SPL (up to 5 %) wouldn't significantly alter the odor of bread.

SPL (rich in flavonoids) exhibited strong inhibitory effect on Maillard-type volatile compounds, which 2-methylbutyraldehyde existed in control bread could not be detected anymore in SPLB, even addition amount of SPL was only 1 %. Meanwhile, furan derivatives, such as 2-pentyl furan, furfural was completely suppressed when adding only 1 % SPL. Furanic compounds was formed through the breakdown of glycosylamine in the early stage of reaction, while polyphenols in SPL exhibited significant inhibitory effect on this process. Mildner-szkudlarz, Ró, Piechowska, Wa, & Zawirska-wojtasiak (2019) illustrated the same reason, and pointed out that phenolic acids showed stronger effect than did flavonoids. Similarly, Oral, Dogan, & Sarioglu (2014) found out the addition of polyphenols reduced the formation of furfural in a glucose-glycine model system, when baking at 180 °C in the oven.

Hexanal, heptanal and pentanal were the major aliphatic aldehyde in wheat flour, in which reduced ≈ 30 % of pentanal and ≈ 7 % of hexanal, when supplement of only 1 % SPL, while increased heptanal significantly by ≈ 80 %. The study done by Birch, Petersen, & Hansen (2013) showed the similar result. These aldehydes originated from lipid oxidation which arousing unpleasant odor when the concentration increased.

1-butanol, 1-pentanol, 1-hexanol, 1-octanol, 2-phenylethanol and ethyl acetate in the crumb of control bread came from fermentation. When SPL was added in flour to make bread, the generation of fermentation products was significantly suppressed by ≈ 25-100 %. Meanwhile, when the amount of SPL supplement was higher than 2 %, the formation of ethyl acetate was completely suppressed, however, higher content of ethyl acetate in bread aroma was welcome, for it possessing a pleasant, sweet, fruity aroma (Birch, Petersen, & Hansen, 2013).

Table 6-3 Volatile compounds (% of total volatile compounds) in sweet potato leaf, control bread and sweet potato leaf bread

Volatile compounds	CAS#	Aroma description	SPL	Control bread	1 % SPLB	2 % SPLB	3.5% SPLB	5 % SPLB
Aldehydes								
2-Methylbutyraldehyde	96-17-3	Musty, chocolate, nutty		0.42±0.13^{a}				
3-Methylbutanal	590-86-3	Fruity, peachy	0.55±0.03^{a}					
1-Pentanal	110-62-3	Fermented, nutty, berry		1.89±0.05^{a}	1.12±0.09^{c}	1.25±0.01^{bc}	1.34±0.01^{b}	1.2±0.00^{bc}
1-Hexanal	66-25-1	Fresh, vegetable	0.74±0.03^{b}	9.23±0.40^{a}	8.53±0.23^{a}	9.06±0.26^{a}	8.85±0.28^{a}	8.7±0.29^{a}
1-Heptanal	111-71-7	Fresh, fatty, citrus		0.72±0.06^{c}	1.36±0.08^{a}	0.97±0.00^{b}	1.53±0.04^{a}	1.41±0.07^{a}
Trans-2-Hexenal	6728-26-3	Green, leafy						0.69±0.02^{a}
Tran-2-hexenal	6728-26-3	Green, banana, cheesy	1.20±0.21^{a}					
1-Octanal	124-13-0	Waxy, citrus, green		4.02±0.26^{a}	3.91±0.11^{a}	2.46±0.02^{c}	3.22±0.06^{b}	3.34±0.10^{b}
Caprylic aldehyde	124-13-0	Waxy, citrus, fatty	0.45±0.03^{a}					
Trans-2-Heptenal	18829-55-5	Vegetable, fresh, fatty		0.86±0.02^{d}	1.95±0.01^{cd}	3.36±0.22^{bc}	5.14±1.0^{b}	7.79±0.51^{a}
1-Nonaldehyde	124-19-6	Waxy, rosy, orange peel		6.96±0.50^{bc}	8.79±0.33^{a}	5.58±0.06^{c}	6.78±0.77^{bc}	7.97±0.15^{ab}
Trans-2-Octenal	2548-87-0	Cucumber, herbal, green		0.49±0.01^{c}	1.3±0.03^{b}	2.12±0.29^{a}	1.70±0.10^{ab}	1.68±0.21^{ab}
1-Decaldehyde	112-31-2	Sweet, citrus, floral		1.67±0.06^{a}	1.82±0.05^{a}	1.1±0.04^{b}	1.42±0.26^{ab}	1.4±0.02^{ab}
Citronellal	106-23-0	Floral, herbal, citrus	0.98±0.02^{a}					
Benzaldehyde	100-52-7	Bitter, almond	2.87±0.08^{a}	1.81±0.03^{b}	0.35±0.01^{c}	0.26±0.01^{c}	0.30±0.00^{c}	0.48±0.01^{c}
Trans-2-Nonenal	18829-56-6	Green, fatty, melon		0.73±0.02^{b}	0.44±0.01^{c}	0.52±0.02^{c}	0.72±0.01^{b}	0.94±0.04^{a}
Trans-2-Decenal	3913-81-3	Waxy, fatty, earthy		1.01±0.08^{c}	1.58±0.05^{b}	1.76±0.21^{ab}	2.03±0.08^{a}	2.18±0.10^{a}
β-Cyclocitral	432-25-7	Herbal, rose, tobacco	0.53±0.03^{a}					
2,4-Nonadienal	6750-03-4	Fatty, green, cucumber		0.2±0.00^{c}	0.43±0.01^{b}	0.61±0.05^{a}	0.50±0.02^{b}	0.43±0.03^{b}
2-Undecenal	2463-77-6	Fruity, orange peel		0.38±0.02^{b}	0.71±0.03^{a}	0.51±0.08^{b}	0.48±0.02^{b}	
Ketones								
Acetone	67-64-1	Fruity, apple, pear	0.91±0.02^{a}					

Volatile compounds	CAS#	Aroma description	SPL	Control bread	1 % SPLB	2 % SPLB	3.5% SPLB	5 % SPLB
3-hydroxy-2-butanone	513-86-0	Buttery, fatty, milky		0.38±0.02^{a}				
Methyl heptanone	110-93-0	Fruity, apple, banana	0.32±0.00^{a}					
3-Octen-2-one	1669-44-9	Earthy, herbal, mushroom		1.39±0.04^{c}	2.07±0.17^{b}	2.14±0.04^{b}	2.26±0.02^{b}	3.44±0.12^{a}
3,5-Octadien-2-one	30086-02-3	Fruity, green, grassy	2.68±0.09^{a}					
Acetophenone	98-86-2	Sweet, pungent, hawthorn	0.70±0.06^{a}					
β-Lonone	79-77-6	Flora, woody, orris	0.99±0.04^{a}					
2(3H)-Furanone	104-61-0	Fruity, peachy		0.30±0.01^{b}	0.38±0.03^{a}	0.28±0.05^{b}		
5-pentyl-2(5H)-furanone	21963-26-8	Minty, fruity		0.44±0.13^{b}	0.86±0.03^{a}	0.95±0.16^{a}	0.73±0.01^{ab}	0.50±0.02^{b}
β-ionone epoxide	23267-57-4	Berry, woody, violet	0.52±0.03^{a}					
Esters								
Ethyl acetate	141-78-6	Fruity, pineapple	0.14	2.76±0.11^{b}	3.31±0.08^{a}			
Vinyl Hexanoate	3050-69-9	Pineapple		0.52±0.02^{cd}	0.71±0.03^{b}	0.90±0.05^{a}	0.56±0.02^{c}	0.45±0.02^{d}
Methyl dihydrojasmonate	24851-98-7	Flora, jasmin, oily		0.25±0.01^{a}				
Alcohols								
Ethanol	64-17-5	Alcoholic	0.34	3.46±0.23^{bc}	1.92±0.07^{d}	2.91±0.12^{c}	3.94±0.14^{ab}	4.35±0.35^{a}
1-Butanol	123-51-3	Whiskey, fruity, banana		1.18±0.09^{a}				
1,8-Cineole	470-82-6	Camphor, herbal, fresh	0.26±0.04^{a}					
1-Pentanol	71-41-0	Fermented, fusel, oily		3.45±0.20^{a}	1.04±0.00^{a}	1.03±0.00^{a}	0.86±0.05^{a}	0.74±0.01^{a}
1-Hexanol	111-27-3	Oily, fruity, alcoholic		0.51±0.03^{a}				
2-Penten-1-ol	1576-95-0	Oily, apple, fermented	0.28±0.02^{a}					
Cis-3-hexen-1-ol	928-96-1	Grass, herbal	0.55±0.05^{a}					
2-Butoxyethanol	111-76-2	Sweet, fruity	6.05±0.43^{a}					
1-Octen-3-ol	3391-86-4	Mushroom, earthy, green		0.58±0.03^{c}	0.9±0.03^{bc}	1.21±0.12^{b}	2.01±0.27^{a}	2.27±0.10^{a}
Linalool	78-70-6	Citrus, woody, rose	0.40±0.02^{a}					

Volatile compounds	CAS#	Aroma description	SPL	Control bread	1 % SPLB	2 % SPLB	3.5% SPLB	5 % SPLB
1-Octanol	111-87-5	Waxy, citrus, floral		1.08±0.06^{bc}	1.32±0.07^{ab}	0.93±0.10^{c}	1.36±0.07^{a}	1.08±0.04^{bc}
Anethol	104-46-1	Sweet, anise, licorice	0.26±0.04^{a}					
2-phenylethanol	60-12-8	Sweet, floral, rosy		0.80±0.03^{a}	0.2±0.01^{e}	0.31±0.03^{d}	0.42±0.02^{c}	0.67±0.01^{b}
Santalol	11031-45-1	Sandalwood, woody	0.13±0.01^{a}					
α-Costol	65018-15-7			0.25±0.02^{a}				
Valerenenol	84249-42-3	Citrus, berry	0.19±0.01^{a}					
2-phenoxyethanol	122-99-6	Rosy, cinnamyl		0.58±0.03^{a}				
(-)-Spathulenol	77171-55-2	Honey	0.82±0.00^{a}					
Alkenes								
β-Myrcene	123-35-3	Herbal, woody, rosy	0.67±0.00^{a}					
Limonene	138-86-3	Citrus, herbal, camphor	2.73±0.25^{a}	1.39±0.04^{b}	1.45±0.06^{b}	0.96±0.00^{c}	1.46±0.03^{b}	1.64±0.10^{b}
β-Thujene	28634-89-1	Herbal	0.44±0.01^{a}					
Sabinene	3387-41-5	Woody, citrus, pine	0.38±0.01^{a}					
γ-Terpinene	99-85-4	Woody, lemon, herbal	0.29±0.01^{a}					
ρ-Cymene	25155-15-1	Cumin, thyme	4.44±0.38^{a}					
α-Cubebene	17699-14-8	Woody, camphor	1.07±0.08^{a}					
α-Copaene	3856-25-5	Woody	1.34±0.01^{a}					
β-Cubebene	13744-15-5	Citrus, fruity, radish	0.43±0.06^{a}					
β-Caryophyllene	87-44-5	Sweet, woody, clove	25.64±0.40^{a}					
δ-Cadinene	483-76-1	Thyme, herbal, woody	0.19±0.01^{a}					
γ-Cadinene	39029-41-9	Herbal, woody	0.74±0.06^{a}					
(+)-Aromadendrene	489-39-4		0.78±0.02^{a}					
α-Humulene	6753-98-6	Woody	3.36±0.14^{a}					
α-Amorphene	483-75-0	Woody	1.16±0.03^{a}					

Volatile compounds	CAS#	Aroma description	SPL	Control bread	1 % SPLB	2 % SPLB	3.5% SPLB	5 % SPLB
α-Terpinolene	586-62-9	Woody, lemon	0.15±0.01[a]					
1,6-Cyclodecadiene	23986-74-5	Woody	1.36±0.08[a]					
β-Selinene	17066-67-0	Herbal	1.63±0.07[a]					
α-Selinene	473-13-2	Amber	1.45±0.15[a]					
Bisabolene	495-62-5	Woody, citrus,	5.41±0.08[a]					
δ-Cadinene	483-76-1	Thyme, herbal, woody	1.40±0.01[a]					
α-Curcumene	644-30-4	Herbal	0.14±0.03[a]					
α-Muurolene	31983-22-9		0.16±0.01[a]					
Calamenene	483-77-2	Herbal, spicy	0.65±0.10[a]					
α-Calacorene	21391-99-1	Woody	0.17±0.01[a]					
β-Caryophyllene oxide	1139-30-6	Sweet, fresh, woody	1.82±0.30[a]					
Furans								
2-pentyl furan	3777-69-3	Fruity, green, earthy		0.32±0.01[a]				
Furfural	98-01-1	Bready, nutty, caramellic		0.78±0.01[a]				
Acids								
Acetic acid	64-19-7	Vinegar	0.67±0.02[d]	2.21±0.09[c]	2.37±0.11[bc]	2.88±0.38[b]	4.19±0.04[a]	1.12±0.06[d]
Pentanoic acid	109-52-4	Cheese, acid		2.65±0.10[bc]	2.33±0.06[d]	3.03±0.02[a]	2.85±0.05[ab]	2.43±0.09[cd]
Hexanoic acid	142-62-1	Sweety, Fatty, cheesy		18±1.0[c]	19.92±0.89[bc]	26.80±1.86[a]	23.95±0.32[ab]	18.20±0.77[c]
Butanoic acid	109-21-7	Fruity		0.71±0.02[a]				0.41[b]
Heptanoic acid	111-14-8	Cheesy, fermented, pineapple		1.08±0.05[c]	2.59±0.11[a]	2.45±0.19[ab]	2.72±0.05[a]	2.18±0.08[b]
Octanoic acid	124-07-2	Acidic, brandy		2.00±0.07[d]	4.78±0.20[a]	3.58±0.51[bc]	4.08±0.15[ab]	3.10±0.21[c]
2-Heptenoic acid	18999-28-5	Green			0.53±0.02	0.64±0.10	0.74±0.03	0.62±0.02
Nonanoic acid	112-05-0	Waxy, cheesy		2.35±0.10[c]	4.21±0.18[a]	3.33±0.50[ab]	2.87±0.14[bc]	2.93±0.09[bc]

[a] SPL, sweet potato leaves; SPLB, sweet potato leaf fortified bread.

[b] Different letters (a-e) in the same volatile compound represented values were significantly different following with the increased SPL ($P < 0.05$).

4. Conclusions

Even just 1 % of SPL could significantly change the color of bread, compared with the control. SPL negatively influenced the texture of crumb by increasing the hardness and chewiness when adding more than 1 % of SPL to the flour to make bread. Specific volume of the bread shrank almost 50 % when adding only 5 % SPL, meanwhile collapsing of the pore in the crumb became serious, while TPC and TFC increased 6 times and 10 times, and antioxidant activity enhanced 10 times, separately. Although the typical odor of alkenes in SPL would be removed after baking, however, flavonoids and phenolic acids existed in SPL were excellent antioxidants which would suppress the generation of representative odor of bread, the increase in antioxidant activity came at the expense of odor loss. In summary, considering the influence of SPL on the physical properties and chemical properties of bread, small amount of addition ($\leq$ 1 %) should be a better choice for bakery products.

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ChapterⅦ. General discussion, conclusion and perspective

The research undertaken in this thesis demonstrated that sweet potato leaves (SPL) are an excellent flavonoids source and could be added to the flour, to make the bread possessing high antioxidant activity. However, the addition should be limited to 1 % to avoid some adverse effects. The different sections of the thesis aimed at studying (i) optimization of ultrasonic-microwave synergistic extraction (UMSE) of flavonoids from SPL, (ii) effects of processing and storage conditions on the stability of SPL flavonoids (SPLF), (iii) simulated digestion of SPLF and sustained release of its maltodextrin nanoparticles, and (iv) physical-chemical properties and volatile compounds of flavonoids-rich SPL fortified bread (SPLB).

1. General discussion

1.1. The variety of sweet potato chosen for the research of flavonoids from the leaves

China is the largest sweet potato producer in the world, the annual yield of 2018 was 53 million tons, which account for 57 % of the total yield (FAOSTAT, 2020). After roots been harvested as starch resources, equal tons of SPL need to be dealt with. A comprehensive research has been carried out, focusing on SPL of 40 varieties around China, Yuzi No.7 was selected as the variety in this study for its highest total polyphenols content (TPC) (12.30 g chlorogenic acid equivalents (CHAE) /100 g dry weight (DW)) and strongest antioxidant activity (0.82 mg ascorbic acid equivalents (ACE)/ mg DW), in which the correlation coefficient between these two parameters was high (0.76, $p < 0.0001$). Meanwhile there was plenty of nutrients in Yuzi No.7, the content of moisture, crude protein, crude fiber, crude fat, carbohydrate and ash was 87.5, 21.1, 10.7, 5.2, 57.3, 8.7 g/100 g (DW), respectively (Sun, Mu, Xi, Zhang, & Chen, 2014). Polyphenols are classified into two major branches: flavonoids and non-flavonoids (Khan, Zill-E-Huma, & Dangles, 2014), as non-flavonoids part (phenolic acids) of polyphenols in SPL has been comprehensively studied, while flavonoids part begin drawing arising attention for its specific structure (two aromatic rings (A ring and B ring) connected by pyran ring (C ring), often containing hydroxyl, methoxy, methyl, isoamyl group and other substituents), which possessing antioxidant, anti-bacterial, anti-cancer activity etc. (Chen et al., 2011).

1.2. Selection of extraction methods of flavonoids from sweet potato leaves

UMSE is the extraction method combined with ultrasonic and microwave, which is economic and time-saving, comparing with other new extraction technologies such as pressurized liquid extraction, supercritical fluid extraction,

enzyme assisted extraction and pulsed electric field extraction (Vivekananda, Yogesh, & Hemalatha, 2007). The biggest advantage of ultrasonic extraction is the yield acceleration, which chlorogenic acid from artichoke leaves through ultrasonic extraction by 50 % ethanol, was close to the yield under boiling condition (Saleh et al., 2016). On the other hand, when extracted polyphenols from frankincense tree leaves, the traditional reflux extraction took 2 hours to achieve the maximum extraction rate, while microwave extraction only took 60 s, which saving more than 99 % of the time (Dahmoune et al., 2014).

1.3. Selection of extraction solvent for sweet potato leaf flavonoids

Water, methanol (50, 70, 90 %), ethanol (50, 70, 90 %), acetone (50, 70, 90 %) were chosen as extraction solvents for SPLF, turned out that 70 % ethanol exhibited best performance, followed by 90 % acetone, 90 % ethanol, 70 % acetone and 90 % methanol. Meanwhile antioxidant activity of extracts was determined, which 50 % acetone extract possessed the highest DPPH· scavenging activity, followed by 70 % acetone, 50 % ethanol, 70 % ethanol and 70 % methanol. Water showed the poorest extraction performance both in total flavonoids content (TFC) and antioxidant activity (Fu et al., 2016). Considering the extraction performance for SPLF and the safety of solvents, ethanol is the best option.

1.4. Optimization of ultrasonic-microwave synergistic extraction of flavonoids from sweet potato leaves

Our experiment optimized the extraction parameters of UMSE for SPLF. The optimal parameters calculated by response surface methodology (RSM) included a 1:40 (g/mL) of solid-liquid ratio, 57 °C of extraction temperature, 76 s of extraction time, 72 % (v/v) ethanol, and extracted 2 times, the highest yield of flavonoids from SPL was obtained, which was 5.13 g/100 g (DW). After been selected through petroleum ether and then ethyl acetate, the crude polyphenols extract was further purified to SPLF with the flavonoid purity of 76.1 %, equivalent to sonication extraction at room temperature for 12 h (Fu et al., 2016). High performance liquid chromatography (HPLC) analysis results showed that SPLF mainly consisted of astragalin (473.8 mg/g, DW), quercetrin (86.5 mg/g, DW), 4, 5-caffeoylquinic acids (CQA) (76.4 mg/g, DW), isoquercitrin (62.4 mg/g, DW), tiliroside (18.8 mg/g, DW), quercetin (12.5 mg/g, DW), 3, 4, 5-CQA (6.5 mg/g, DW), caffeic acid (CA) (6.1 mg/g, DW), kaempferol (6.0 mg/g, DW), myricetrin (5.9 mg/g, DW) and rhamnetin (4.3 mg/g, DW), which possessed high antioxidant capacity, 20 μg/mL SPLF could reduce 76.2 % of Fe^{3+} or scavenge 91.5 % of $ABTS^{\cdot+}$, similar to the *Gingko biloba* extract and propolis flavone (Zhang et al., 2010; da Cruz Almeida et al., 2017).

1.5. Effects of processing and storage conditions on the stability of sweet potato leaf flavonoids

Since SPLF been extracted, purified and evaluated the antioxidant activity of fresh state. It is important to figure out the stability of SPLF after different processing (thermal and non-thermal (high hydrostatic pressure (HHP))) and various storage conditions (pH, light and temperature), which were the critical indexes for the food products to transport from factories to consumers.

HHP treatment, as a novel non-thermal processing technology, has been successfully used to extend the shelf life for food products with minimal impact on their nutritional and sensory values (Huang et al., 2017). Industrial application of HHP is performed in the pressure range of 100-800 MPa. Food products are usually laid in a pressure-resistant stainless steel cavity, and pressurized at room temperature (RT) (≈20 °C) or low temperature by using a kind of liquid (usually water) as pressure transfer medium (Chawla, Patil, & Singh, 2011).

The results showed that the stability of SPLF after heat and HHP treatment was high. Heat treatment at 75 °C for 90 min did not cause any significant destruction, which kept consistent with the study done by Zeng et al. (2019) that TFC was decreased less than 5 % after 90 min when thermal treated at 80 °C. HHP treatment at 200, 400, 600 MPa for 30 min neither cause significant effect on SPLF, which was in good agreement with Patras, Brunton, Da Pieve, & Butler (2009) that the anthocyanin content in strawberry and blackberry had no significant difference after pressurizing at 400, 500, 600 MPa for 15 min at RT (≈20 °C).

The stability of SPLF under acid environment was good, which more than 60 % of TFC and antioxidant activity could be preserved after 16 weeks of storage, compared with neutral or alkaline environment. A decrease of TFC in *Passiflora quadrangularis* leaf extracts under alkali hydrolysis was 2.6-fold higher than the decrease under acid hydrolysis, which was clearly demonstrated in the study done by Echeverry, Medina, Costa, & Aragón (2018). SPLF kept away from light exhibited better retention of TFC (30 %) and antioxidant activity (10 %) after 16 weeks of storage. In the same way, Rabadán, Álvarez-Ortí, Pardo, & Alvarruiz (2018) reported that TPC of almond, walnut and pistachio was significantly lower under light storage at RT compared with dark storage. No significant difference in SPLF was observed for the samples stored at -18 °C, 4 °C and RT, and more than 80 % of TFC and 75 % of antioxidant activity was preserved under these conditions after 16 weeks, which was similar to the findings of Bradwell, Hurd, Pangloli, McClure, & Dia (2018) who reported that the TFC in sorghum phenolic extracts had no significant difference at 4 °C and 22 °C during the first 6 weeks of storage.

1.6. Selection of encapsulation method and wall material for sweet potato leaf flavonoids

The stability of SPLF after processing and storage was high, except towards pH (neutral or alkaline environment), later we need to evaluate the bioaccessibility of SPLF after *in vitro* digestion, however the pH was 7.0, 3.0 and 7.0, respectively in simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and whether nanoparticles could protect SPLF and postpone its release in simulated gastrointestinal digestion. Nanoencapsulation is a process of packaging aimed active material with a coating to form nanometer to micrometer particles (Fang & Bhandari, 2010). Spray-drying and freeze-drying are the most common used encapsulation methods. Spray drying is spraying a liquid into the chamber with a hot gas stream to obtain small granules by evaporating of solvent (Fatnassi et al., 2013). However, high drying temperature could damage sensitive compounds such as anthocyanins, lycopene, β-carotene, colors and flavors, which freeze-drying might be the better choice. Spent coffee grounds encapsulated by spray-drying and freeze-drying methods, proved that maltodextrin as the wall material was more appropriate for preserving flavonoids, especially when performed by freeze-drying, which extra reserved 60 % of flavonoids and 30 % of antioxidant activity (Ballesteros, Ramirez, Orrego, Teixeira, & Mussatto, 2017).

Freeze-drying is a process of removing water from materials, through sublimation and desorption, which has the advantage of keeping low temperature during the process, avoiding thermal deterioration, which is an excellent method for encapsulation of heat sensitive bioactive compounds (Sanchez, Baeza, Galmarini, Zamora, & Chirife, 2013). For example, green tea polyphenols were encapsulated by maltodextrin, β-cyclodextrin and the combination of both, to protect their antioxidant activity against conditions of high temperature and alkaline environments. Nanoparticles prepared by freeze-drying coated with maltodextrin exhibited higher encapsulation efficiency (EE) (≈76 %), higher catechin content (1.1-folds compared with spray-dried encapsulates) and more excellent antioxidant activity (IC_{50} value of 54 μg/mL) (Pasrija, Ezhilarasi, Indrani, & Anandharamakrishnan, 2015). Similarly, Yamashita et al. (2017) studied the effect of freeze-drying on the encapsulation of blackberry extract (rich in anthocyanin), that using maltodextrin as the wall material obtained higher EE (76 %). This is the reason why freeze-drying and maltodextrin were selected as the encapsulation technique and wall material to prepare SPLF nanoparticles.

1.7. Simulated digestion of sweet potato leaf flavonoids and sustained release of its maltodextrin nanoparticles

Nanoparticles of SPLF prepared by freeze-drying encapsulation with 20 mg/mL maltodextrin, showed the highest stability with zeta potential of -41.6 mV, mean particle size of 277.4 nm, polydispersity index (PDI) of 0.417 and EE of 59.0 %, confocal laser scanning microscopy (CLSM) and infrared spectrum also provided the evidence. After simulated oral, gastric and intestinal digestion, the retention

of TFC was 75.4 %, 55.0 % and 45.9 % respectively, and the retention of antioxidant activity was 75.9 %, 65.0 % and 56.2 % separately. Nanoparticles could extra preserve about 15, 30, 30 % of TFC and 16, 16, 10 % of antioxidant activity in each digestion phase, compared with the sample without encapsulation, which could be intuitively observed by scanning electron microscopy (SEM). Similar results showed that catechins from green tea were very unstable after salivary, gastric and intestinal simulated digestions, about 80, 60 % and less than 10 % of catechins could be retained after each digestion phase, the harsh environment in gastrointestinal tract such as pH, enzymes, simulated digestion juices etc. were highly detrimental to the bioactivity of catechins (Shim et al., 2012). After been encapsulated by maltodextrin, the retention of anthocyanin in jussara (*Euterpe edulis* Martius) fruit increased 10 % compared with the non-encapsulated one, when passing through simulated digestion (Bernardes et al., 2019).

1.8. Physical-chemical properties and volatile compounds of flavonoids-rich sweet potato leaf fortified bread

Since SPL possessing high content of flavonoids and excellent antioxidant activity, which is a promising food additive with nutrition and function. SPL replaced 0, 1, 2, 3.5 and 5 % (w/w) of flour to make bread. 1 % SPL changed the bread color significantly with ΔE of 15.1, which could be clearly distinguished by human eyes. The hardness and chewiness of the crumb increased with increased SPL level, while specific volume shrank 32 % compared with the control (when the addition of SPL reached to 3.5 %), and pores in the crumb became deteriorating and disintegrating. TPC and TFC of the bread increased 6 and 10 times, and antioxidant activity enhanced 10 times, when adding 5 % SPL to the flour to make bread. Flavonoids were relatively stable during baking, such as baking onion merely decreased quercetin content by 1.3 % (Chassy et al., 2015). The addition of SPL won't alter flavor of the bread significantly, for the baking process destroyed most of the volatile compounds in SPL, especially alkenes with grass and herbal odor. However, the generation of typical bread odors (malty, bready) would be largely suppressed. Overall, SPL (no more than 1 %) could be supplemented to the flour to make bread with high TPC (2-fold), high TFC (2.5-fold) and excellent antioxidant activity (3-fold), without obviously adverse effect on physical parameters. Similar results were found in the bread fortified with 2.5 % of cistus extract, which increased TPC by 52 %, compared with the control, without significantly adverse effect on the overall quality of the bread (Mikulec, Kowalski, Makarewicz, Skoczylas, & Tabaszewska, 2020). The quality of bread fortified with 2 % of black rice extract (rich in anthocyanin), was not significantly different from the control, however, when the replacement of black rice extract increased to 4 % causing less elasticity and higher density of the bread (Sui, Zhang, & Zhou, 2016). 5 % of bee pollen fortified bread could increase TPC by 54 % and

antioxidant activity by 26 %, compared with the control (Conte et al., 2020).

However, the addition of SPL (just 1 %) completely suppressed the formation of 2-penthy furan and furfural, similar to the effect of grape marc extract, which influenced the flavor of bread in the negative way, made the smell mediocre. Many polyphenols were potential trappers of di-carbonyl compounds from sugar fragmentation, for the involvement of methylglyoxal and glyoxal in the formation of flavor compounds, such as pyrazines, pyridines, pyrrolines, pyrazinones, furanones, 3-methylbutanal, phenylacetaldehyde, thiazoles and thiazoline (Wang & Ho, 2012). Polyphenols might inhibit flavor formation during Maillard reaction and lipid oxidation, for their scavenging of free radicals, the increase in antioxidant activity came at the expense of odor loss (Rizzi, 2003).

2.Conclusion and perspectives

The general objective of this research was to get a comprehensive investigation on flavonoids branch of polyphenols for SPL, including the content of SPLF, the composition of SPLF, the antioxidant activity of SPLF, the stability of SPLF, encapsulation and digestion of SPLF. Evaluated the possibility of SPL (which rich in flavonoids) been fortified to the bread to obtain high antioxidant activity, by determining its effects towards physical and chemical qualities of the bread. All these works provided the basic information of SPL as a potential additive to fortify the nutrition and function of foods.

In order to achieve this objective, many analytical methods been applied, from traditional methods (HPLC, GC-MS) to some innovative technics (UMSE, CLSM), statistical tools were also relevant (RSM).

According to the summary of previous work done in our laboratory, Yuzi No. 7 was chosen as the experimental cultivar of SPL, for its highest TPC and strongest antioxidant activity, among 40 different cultivars grown in China. The stability towards heat and HHP treatments was high, except in neutral or alkaline environment, which would be seriously destroyed. However, this pH (3.0 and 7.0) was exactly the pH of salivary and intestinal digestion juice. When encapsulated by maltodextrin, SPLF could be protected to a certain degree during simulated oral digestion, and transported to the following phase. Furthermore, 1 % of SPL fortification could significantly accelerate TFC and antioxidant activity of bread without unacceptable physical characteristic or odor deterioration.

SPLF has been relatively comprehensively studied in this thesis, further questions were arousing that to what extent could simulated digestion experiment demonstrate the bioavailability of SPLF, and the mechanism of SPLF on bread odor and physical-chemical properties. A commercial product of SPL green juice powder (Cultivar: Simon No.1, Ningzi No.1 or Xinxiang No.1)

has been developed, which is convenient to store and transport and long in shelf life, retains the original color of SPL, and is smooth in taste and good in comprehensive palatability which being drunk. SPL green juice powder beverage contains nutritional and functional ingredients such as protein, polyphenol, dietary fiber, fat and mineral elements, and can make up for the insufficient intake of vegetable nutritional ingredients of people in daily life. Similarly, more products fortified with SPL would be developed in the future, to meet consumers' demands for the nutrition and function of food.

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Appendix - publications

1. Articles

(1) Shi, G., **Liu, J.**, Liu, Y., & Tian, X. **(2016)**. Separation and purification and in vitro anti-proliferative activity of leukemia cell K562 of *Galium aparine* L . petroleum ether phase. ***Saudi Pharmaceutical Journal***, *24*(3), 241–244.

(2) Shi, G. Q., Yang, J., **Liu, J.**, Liu, S. N., Song, H. X., Zhao, W. E., & Liu, Y. Q. **(2016)**. Isolation of flavonoids from onion skin and their effects on K562 cell viability. ***Bangladesh Journal of Pharmacology***, *11*, 18–25.

(3) **Liu, J.**, Mu, T., Sun, H., & Fauconnier, M. L. **(2019)**. Optimization of ultrasonic–microwave synergistic extraction of flavonoids from sweet potato leaves by response surface methodology. ***Journal of Food Processing and Preservation***, *43*(5), 1–10.

(4) **Liu, J.**, Mu, T., Sun, H., & Fauconnier, M. L. **(2020)**. Effects of processing and storage conditions on the stability of sweet potato (*Ipomoea batatas* L.) leaf flavonoids. ***International Journal of Food Science and Technology***, *55*(5), 2251–2260

(5) **Liu, J.**, Mu, T., Sun, H., & Fauconnier, M. L. *In-vitro* digestion of sweet potato leaf flavonoids and sustained release of its maltodextrin nanoparticles (Revised and re-submitted)

(6) Physical-chemical properties and volatile compounds of flavonoids-rich sweet potato leaf fortified bread (ready to submit)

2. Conference presentation

(1) Agricultural waste and residue management for a circular bio-economy: shared China and EU impact-oriented solutions. **China-EU Young Scholars Forum.** 22/10/2018.

(2) 25th National Symposium for Applied Biological Sciences (NSABS): **Poster Presentation** of own project, **1st author** (Gembloux) entitled "Effects of processing and storage conditions on the stability of sweet potato (*Ipomoea batatas* L.) leaf flavonoids 31/01/2020.