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Improving Oxidative Stability of Polyphenolic Fraction of Apple Pomace by Encapsulation Using Naturally Occurring Polymers

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Abstract

Polyphenolic compounds with relatively high antioxidant activity obtained from subcritical water extraction of apple pomace were assessed for encapsulation by spray drying technique, making use of polymeric substances co-extracted with the polyphenolic compounds. Comparative assessments were carried out of the directly encapsulated subcritical water extract (SWE) products with particles formed when encapsulated with the addition of hydroxyl propyl-β-Cyclodextrin (SWE + HPβ-CD). The powders were characterized for their physico-chemical properties such as, moisture content, density, particle size, hygroscopicity to assess their suitability within cosmetic formulations. The SWE and SWE + HPβ-CD encapsulated products resulted in different physical properties. Although the particle size was less than 4 μm for both products, the direct encapsulation (SWE) was highly hygroscopic and this property was significantly reduced with addition of HPβ-Cyclodextrin (SWE + HPβ-CD). Scanning electron microscopy (SEM) and Fourier Transform Infra-red (FT-IR) spectroscopic were employed to analyse the micronised powders to support evidence of encapsulation. Both techniques revealed the interaction between compounds in extract and the carrier HPβ-Cyclodextrin suggesting successful encapsulation. The effect of storage conditions on retention of antioxidant activity of the subcritical water extract was evaluated within 35 days for extracts with and without the carrier HPβ-Cyclodextrin. Hydroxyl propyl-β-Cyclodextrin offered protection against degradation of antioxidant compounds thereby potentially extending the shelf-life and making the encapsulated powder suitable for incorporation in cosmetic and pharmaceutical applications.

Keywords

Apple Pomace, Polyphenolic Compounds, Antioxidant Activity, Encapsulation, Hydroxyl Propyl-β-Cyclodextrin

1. Introduction

There is a growing concern that common synthetic preservatives such sodium benzoate, sodium sorbate, formaldehyde releasers and isothiazolinones may have hazardous effects such as hormonal and neurological disorders, human carcinogen linked to damaging long term effects [1]. This coupled with consumer demand for natural ingredient has driven increased interest in natural antioxidants and other active ingredients obtained from plants [2]. However topical formulations incorporating extracts derived from plant sources may be restricted due to their physical and chemical properties, which might affect the stability of the product and overall performance [3].

Polyphenolic compounds including Proanthocyanidins from grape seeds and coffee extracts have been shown to be strong antioxidants in vitro when compared with Carotenoids, Vitamins C and E [4]-[8].

Apples are a significant source of bioavailable polyphenolic compounds and are a common fruit [9]. The major phenolic compounds found in apples include; Chlorogenic acids, Epicatechins, Phloridzin, Procyanidins and the Quercetin conjugates [10]. Apple pomace a by-product of apple juice and cider productions have higher amounts of polyphenolic compounds compared to the apple flesh, and are also a potential source of carbohydrate, fibre and pectin [11] [12]. Approximately 9×10^6 tonnes of apple pomace are produced globally per annum [13]. A recent study has demonstrated that subcritical water, a green solvent, can support the efficient and effective extraction of polyphenolic compounds with relatively high antioxidant activity from apple pomace [14]. However phenolic compounds can undergo molecular transformation through oxidation when exposed to severe environmental conditions such as high temperature and humidity which potentially reduce the efficacy within cosmetic, nutraceutical pharmaceutical and food formulations. Therefore, the stabilisation of the high antioxidant activity is important to ensure efficacy before, and during applications. Encapsulation is a technique widely applied to stabilise and protect bioactive compounds against degradation, within nutraceutical, cosmetic pharmaceutical and food industries [15]. The technique involves the creation of barriers around active ingredients which modulate their interactions with the environment [16] [17]. The barrier material often referred to as the carrier can be natural, synthetic or modified polymeric substances such as lipids, proteins and carbohydrates.

Freeze and spray drying microencapsulation technologies are extensively used in the cosmetic pharmaceutical and food industries in recent times to stabilise

ingredients against oxidation, improve shelf-life, enhances solubility and bioavailability during applications [18]. The benefits of spray drying are: 1) low process cost; 2) wide choice of coating; 3) efficient encapsulation; 4) good product stability; 5) potential for continuous operation [15] [19].

The equipment setup generates particles from homogenized solution of active ingredients and coating materials [20] [21] [22] [23]. During operation, the active ingredient is dispersed within the coating material and is normally atomised by passing it through a nozzle with the help of a compressed gas system. Hot process air or nitrogen is delivered to meet with the atomised sample thereby causing rapid evaporation of the solvent from the particles which drops to the bottom of the drying chamber. The physical and structural properties of the particles are strongly influenced by the geometry of the nozzle and the viscosity of the feed [24].

Naturally occurring polymers have been successfully used as carriers when encapsulating bioactive compounds, For example, polyphenolic compounds such as Caffeic acid, Chlorogenic acid, Gallic acid, Quercetin, Kaemferol, Myricetin, and green tea flavonoids have been coated with soy protein [25]. Similarly, lipid nano-capsules were used as a membrane for quercetin delivery [26]. Direct formation of microparticulate polysaccharide powders from health promoting mushroom, *Ganoderma Lucidum,* was demonstrated after hydrolysing with subcritical water between 100˚C - 190˚C [27]. Micronisation of quercetin and its derivatives recovered from onion waste using pressurised hot water and supercritical antisolvent techniques have been demonstrated [28].

The current study builds on the fact that carbohydrates and polyphenolic compounds from plant materials have been shown to interact [29]. The objective of the research was to evaluate the feasibility of coupling extraction of polyphenolics with spray drying mediated encapsulation using co-extracted natural polymers, therefore, negating the need for an external carrier to ensure improved stability of the polyphenolic antioxidant compounds against oxidation. The natural occurring polymeric compounds such as hemicellulose, lignin and proteins have previously been co-extracted with polyphenolics under subcritical water condition [30]. As a comparative study HP β-Cyclodextrin, which oligomers of glucose are known to form inclusion complexes with polyphenolic, was mixed with the subcritical water extract before spray drying and evaluating the stability of the particles derived.

2. Materials and Methods

2.1. Apple Pomace Sample

Apple pomace a residue from cider production comprising Michelin, Dabinett, Yarlinton Mill, Chisel Jersey, Brown Snout, Vilberie and Harry Masters Jersey varieties, was supplied by Universal Beverages Limited (UBL) a subsidiary company of Bulmers, UK. The pomace sample was very heterogeneous comprising peels seeds, apple flesh and therefore was thoroughly mixed to ensure replicate

samples were representative of the population of samples.

The dry weight of all samples was determined using AOCS (American Oil Chemist Society Standard) standard protocol using a laboratory oven (STATUS international, UK) at 103˚C ± 3˚C. The frozen wet apple pomace sample was homogenized for 30 seconds using Moulinex domestic blending machine to minimize variability in batch-to-batch analysis. Portions of the homogenize apple pomace was freeze-dried using vacuum freeze dryer (Model number EQ03 by Vacuum and Industrial Products, UK).

2.2. Subcritical Water Extraction of Polyphenolics from Apple Pomace

Subcritical water-mediated extraction of polyphenolics from wet homogenised apple pomace was conducted using a Parr instrument (model 5521), which was a 300 ml stainless steel reactor vessel with a heating jacket. The vessel was connected with temperature and pressure sensors. Magnetic stirrer (1240 rpm) with integrated cooling system was attached to help enhance mass transfer. A back pressure regulatory valve was used to control the pressure inside the vessel. A nitrogen gas cylinder was used to pressurize the reactor. The setup is illustrated in Figure 1.

Wet homogenised apple pomace was loaded into the reactor according to the solid-to-solvent ratio of 4.5% (w/v). The reactor vessel was first purged for 10 - 15 seconds with nitrogen, before the extraction pressure inside the vessel was set to 50 bar and the gas valve (V-3) closed. The temperature of extraction was set to 150˚C at 20 minutes residency time. Once the reaction was finished, the gas valve (V-1) was closed and the heating and stirring turned off. Cooling system to the reactor was turned on and vessel quickly removed from the heating jacket into an ice bath to allow the reactor cool below 50˚C and depressurised to allow the extract to be collected.

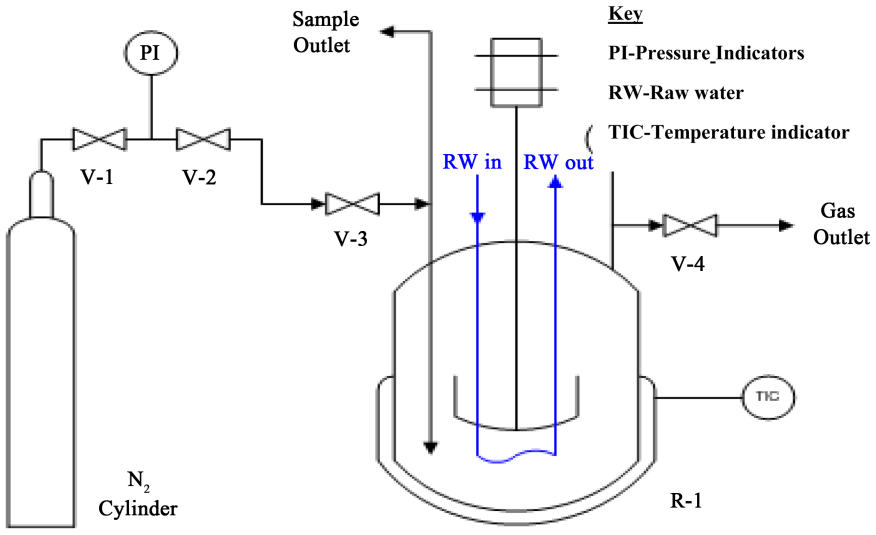

Figure 1. Setup for the subcritical water extraction of Apple pomace.

The extract was transferred into 500 ml Beckman centrifuge bottles and centrifuged at 4000 g for 10 minutes at a temperature 4˚C using a Beckman J2-20 centrifuge. Supernatant from the centrifugation step was further filtered under atmospheric pressure using Fisherbrand filter paper (QL 100).

2.3. Total Phenolic Content

Total phenolic content of the extract was determined using the Folin Ciocalteus's micro scale method proposed by Waterhouse, (2001) [31]. Gallic acid was employed as the standard. 20 µl aliquots of extract were pipetted into 3 test tubes and 1.58 ml of distilled water added to each sample. 100 µl of Folin-Ciocalteu reagent was then added and thoroughly mixed. 300 µl Sodium Carbonate solution was added to the mixture and vortexed thoroughly (Miximatic Vortex). The samples were incubated for 30 minutes at 40˚C in a water bath (Clifton). 300 µl of the solutions were pipetted into 96 Well F/B microplate and absorbance of sample read using a microplate spectrophotometer at 750 nm (Promega Glomax). Total phenolic content was expressed in mg Gallic acid equivalents per gram dry weight of apple pomace.

2.4. Identification of Polyphenolic Compounds by HPLC

Phenolic compounds in the subcritical water extracts were resolved according to the protocol described by [32]. Specifically, an Agilent 1100 series HPLC was fitted with a Prodigy 5 µm ODS3 100 A, C18 (250 × 4.6 mm I.D) column (Phenomenex, Torrance, CA, USA) and operated at 40˚C with a guard column. The mobile phase was 2% (v/v) of the glacial Acetic acid in water as eluent A and 0.5% of Acetic acid in 50:50 (v/v) of Acetonitrile and Water as eluent B. Eluent C was (100%) Acetonitrile. A 10 µl aliquot was injected and subject to a gradient elution profile at a flow rate of 1 ml/min. Phenolic compounds were identified by comparing retention times of external standards at their maximum absorbance and spectral data. The external standards made of 0.1 - 0.2 mg/ml of epicatechin (≥90%), (-) ± catechin hydrate, chlorogenic acid (≥95%), phloridzin dihydrate (≥99%), procyanidin B2 (≥90%), quercetin-3-β-D-glucoside (≥90%), quercetin-3-D-galactoside (≥97%), and 3,4-dihydroxybenzaldehyde (≥97%) from Sigma-Aldrich, UK.

2.5. Antioxidant Activity by ORAC Assay

The antioxidant activity of the extract was determined using a modification of the oxygen radical antioxidant activity assay (ORAC)protocol described by Huang *et al.*, (2002) [33]. The principle behind the ORAC assay is that, it is designed to monitor the decrease in the fluorescence of sodium fluorescein (protein target) probe in the presence of peroxyl radicals generated from thermal decomposition of 2,2[l]-azinobis (2-amidinopropane) dihydrochloride (AAPH) relative to Trolox-a water soluble vitamin E. A fluorescein working solution (150 µl) was added into a 96 well microplate in quadruplicate using a multichannel

pipette into the wells designated as, control, blank, sample and Trolox. 50 µl of Phosphate buffer (75 mM and pH 7.4) was added only into the control wells. Aliquot (25 µl of phosphate buffer, pH 7.4) was pipetted into the blank wells. 25 µl of phenolic sample was added into sample wells and Trolox standard solution (25 µl) added into Trolox designated wells. The solutions were thoroughly mixed using a Microplate Thermo-shaker PHMP series (Grant Instruments, Cambridge) set at 1000 rpm for 3 minutes and before incubating the microplate at 37˚C for 30 minutes. The reaction was started by adding 25 µl of 2,2[l]-azinobis (2-amidinopropane) dihydrochloride (0.414 g of AAPH in 5 ml of 75 mM phosphate buffer), warmed to 37˚C, to the blank/sample/Trolox wells and mixing 1000 rpm for 20 seconds. The microplate was then placed into a microplate reader (Promega) to monitor the fluorescence decay over a period of 45 minutes. ORAC data analysis performed using Microsoft Excel 2010 and results expressed as µmol of Trolox equivalents per g of dry weight apple pomace.

2.6. Preparation of the HP-*β*-Cyclodextrin-Polyphenolic Inclusion Complex

The procedure for preparing HP-*β*-Cyclodextrin-polyphenolic inclusion complex described by Gioxari *et al.*, 2010 was adopted with slight modifications [34]. The inclusion complex was prepared (4:1 in mass ratio) by weighing 56.5 g of HP-*β*-Cyclodextrin into 500 ml of the subcritical water extract of apple pomace containing 0.0283 g/ml of dry solids into 1000 ml Erlenmeyer flask and covered with an aluminium foil. The mixture was stirred for 4 hours at room temperature. 5 ml of the mixture was pipetted into 15 ml plastic vials and frozen at −20˚C for 24 hrs and then freeze-dried using a vacuum freeze dryer (Model number EQ03 by Vacuum and Industrial products). Freeze dried solid lumps were broken using glass rod and stored in a desiccator for future analysis. The remaining liquid mixture was then arranged for spray drying.

2.7. Spray Drying

The laboratory scale spray dryer (Model-SS07, by Lab plant Ltd, UK) was employed to produce the powders. All glassware was fitted to the unit and inlet temperature set to 200˚C and allowed for 15 minutes to warm up before setting the actual spray temperature. Inlet and outlet temperatures were set at 170˚C and 84˚C respectively. Sample feed was delivered at 3.6 ml/min using a variable speed peristaltic pump into a 0.5 mm two-fluid-stainless spray nozzle with an air flow rate of 180 g/min. The subcritical water extract of the apple pomace containing 0.0283 g/ml dry solids was sprayed from 140˚C to 170˚C. The previously prepared inclusion complex mixture was also sprayed at the same condition. Feeds were continuously stirred during spraying and dry powders were separated by a cyclone and collected in an insulated sample collection bottle. A portion of the dried powder was sampled for analysis and bulk packed in seal polyethylene bags and stored in a desiccator.

2.8. Determination of Powder Density

A gas displacement technique was employed to determine the density of the spray dried powder using an AccuPyc II 1340 gas Pycnometer (Micrometrics Instruments Corporation). A 1 cm^3 cup whose weight was previously determined using the microbalance (SART 1702 Germany) was filled with samples of the dried powder and reweighed to obtain accurate weight. The cup containing the powder was sealed in the instrument compartment and helium gas was admitted to serve as a displacement medium and expanded within the internal volume of powder. The solid phase volume of powder was computed from the changes in pressure during filling of sample chamber and that of the discharge empty chamber. Data were analysed using VI.05 software and density of powder was determined by dividing the average volume into powder weight.

2.9. Particle Size Measurement

Particle size and the distribution of particle size within the dried powders was determined using a HELOS/RODOS/VIBRI dispersing system (Sympatec GmbH, Clausthal-Zellerfel Germany) The setup consisted of HELOS (Helium-Neon-Laser optical system), a dry powder dispersion system RODOS, and a vibrating feeder VIBRI. Operations were controlled by software WINDOX 5 for evaluation of particle size and other analysis.

2.10. 2 Hygroscopicity Test

0.5 g of the dried powders (SWE and SWE + HPβ-CD) were weighed in triplicates using a microbalance instrument GR-202 (A & D Scientific Laboratory suppliers) and spread uniformly on glass petri dishes. The powder samples were kept at 23˚C in an incubator model SI-600R (Medline Scientific). 300 ml saturated solution of Sodium Chloride was placed inside the incubator to provide approximately 75.5% relative humidity and left for 7 days. Samples were reweighed after the 7 days and hygroscopicity HG of powders determined according to the equation below;

$$\mathrm{HG} = \frac{\Delta m / \left(M + M_i\right)}{1 + \dfrac{\Delta m}{M}}$$

where Δm was the increase in weight of powder after equilibrium. M was the initial weight of powder and M_i was the free water content of powder prior to exposure to the humid environment [35] [36] [37] [38].

2.11. Scanning Electron Microscopy

Environmental Scanning Electron Microscopy (XL 30 ESEM FEG Philips, Netherlands) was used to observe the morphology of the freeze-dried and spray dried powders. The samples were spread on ESEM-stub covered with sticky carbon tape, and sputter coated under high vacuum with gold using EMSCOPE SC 500 gold sputter coater. All samples were scanned at a voltage of 15 kV using XL 30

ESEM FEG electron microscope and images captured over a range of magnifications within the sample.

2.12. Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR analysis was performed to characterise the powders in molecular terms using Jasco FT-IR 6300 infrared spectrometer. Resolution of 4 cm^{-1} and 32 scans were used in a range between 4000 and 600 cm^{-1}. A background was performed before each sample analysis to scan the environment which was subtracted from the sample spectra to avoid any interference in the results.

2.13. Stability Studies

1.5 ml of subcritical water extract (SWE) and subcritical water extract with HPβ-Cyclodextrin (SWE + HPβ-CD) were measured into separate 2 ml Eppendorf tubes and incubated at 65˚C in a drying cabinet (Fisons Scientific instruments UK). Stability assessment in terms of antioxidant activity of all samples were determined every 7 days for 35 days by Folin Ciocalteu method. All measurements were done in triplicates and samples were not protected from external light.

3. Results and Discussion

3.1. Powder Production

Several inlet and outlet temperatures of spray drying operations were employed by trial and error to favour the generation of dried powders of the subcritical water extract. Dried powders were obtained from subcritical water extract (SWE) for inlet temperatures from 140˚C to 170˚C. There was difficulty in obtaining fine and dried powders below 140˚C and beyond 180˚C for the subcritical water extract whose solid concentration was only 2.75% (w/v). Wet products were observed below 140˚C due to insufficient drying and sticky brown products at 180˚C due to caramelisation reaction of the monomeric sugars in the extract at the high temperatures. However, spray drying of HPβ -Cyclodextrins encapsulated with the subcritical water extract (SWE + HP-β-CD) was without challenges. The incorporation of HPβ-Cyclodextrin raised the glass transition temperature of the subcritical water extracts thereby reducing the stickiness [35]. Previous reported research suggested, many challenges during spray drying of substances containing higher levels in glucose, fructose and sucrose without a wall material [38] [39] [40].

3.2. Characterisation of Powders

Colour of the directly encapsulated polyphenolic fraction (SWE) of the apple pomace with polymers co-extracted under the subcritical water-mediated hydrolysis was yellowish brown and those of the HP-β-Cyclodextrin (SWE + HPβ-CD) encapsulated reflected the colour of the directly encapsulated product in extract and were lightly brownish.

3.2.1. Moisture Content of Powder

Moisture content or residual water associated with solid raw materials for nutraceutical, pharmaceutical, food and cosmetic applications can significantly affect their physico-chemical properties. Rate of dissolution, flow and compactibility of powder, as well as degradation or deterioration are all affected due to prolong exposure to moisture [41]. The average moisture content of samples (g water/100g powder) is shown in Table 1.

Moisture content of directly encapsulated subcritical water extract (SWE) powder was 22.6% higher than HPβ-Cyclodextrin inclusion complex (SWE + HPβ-CD). Thus suggesting that increasing the solid content through the addition of HPβ-Cyclodextrin to the subcritical water extract reduces the amount of residual water of the powder. Previous studies have shown that the moisture content of spray dried powders is dependent on the type and concentration of the carrier material used [42] [43].

3.2.2. Hygroscopicity of Powders

Hygroscopicity is defined as the estimation of the ability of a substance to absorb moisture from a relatively high humid environment and is an important property to consider during storage of powder [44] [45] [46]. Spray dried powder of the directly encapsulated (SWE) had a higher hygroscopicity (9.30 ± 0.11 g/100g) compared with HPβ-Cyclodextrin encapsulated powder (5.08 ± 0.01 g/100g) (Table 1). The results showed the absorptive capacity of the micronised subcritical water extract in humid environment was approximately twice that of the spray dried powder HPβ-Cyclodextrins complexed with SCW extract. Inclusion of HPβ-Cyclodextrins decreased the hygroscopic nature of the extract. Many researchers have reported the reduction of hygroscopicity of extracts when carriers were added. As an example, the hygroscopicity of acai extract was reduced through the addition of the polysaccharide maltodextrin [38]. Moreover, the effect has been shown to be correlated with concentration [45]. Reduction of hygroscopicity of mango powder with maltodextrin has also been reported [36].

The presence and concentration of low molecular weight sugars (Glucose, fructose) and organic acids (citric, malic and tartaric acid) are thought to account for the hydroscopic properties of spray dried powders. Furthermore, the high hygroscopicity, thermoplasticity, and low glass transition temperature (Tg) of these low-molecular-weight substances contribute to the stickiness of dried powders which is a phenomenon frequently encountered during spray drying [36] [39] [44] [46]. Powder stickiness can be reduced by the addition of carriers to increase glass transition temperature thereby eliminating the problem during

Table 1. Some physico-chemical properties of spray dried powders of subcritical water extract and extract encapsulated in HPβ-Cyclodextrin.

Powder Sample	Moisture (%)	Hygroscopicity (g/100g)	Particle size (μm)
SWE + HPβ-CD	5.59 ± 0.4	5.08 ± 0.01	3.46 ±0.04
SWE	7.22 ± 0.01	9.30 ± 0.11	3.41 ± 0.15

processing and storage [36] [38]. Hygroscopicity data of spray dried powder of subcritical water extract of apple pomace is not available for comparison. However, mean hygroscopicity value of 23 g/100g apple pomace residue was reported during vacuum drying at temperatures between 80˚C and 110˚C with hygroscopicity decreasing at higher temperatures [47]. The physical state of the drying powders under the spray dry operation was already changing rapidly with stickiness during collection consistent with previously reported investigations for sugar containing samples [39]. Stickiness of powders was lesser in the SWE + HPβ-CD compared to the auto-encapsulated subcritical water extract (SWE).

3.2.3. Density and Particle Size of Powders

Many methods for determining particle density of solids have been applied and reported in the literature [48]-[54]. However, the density of the encapsulated products evaluated by the gas displacement technique has been described as a reliable way to obtain the "true, absolute and apparent density" [55]. The average density of the powders recorded for 5 cycles at different times is shown in Figure 2.

Analysis of the SWE spry dried powder indicated that it had a density of 1.560 ± 0.001 g/cm^3 which was higher than the density of spray dried powders of the SWE + HPβ-Cyclodextrin complex (1.503 ± 0.003 g/cm^3). The increase in the volume of the SWE + HPβ-Cyclodextrins powders can be attributed to the conical structure of the cyclodextrin which supports a more open matrix when compared to the compact polymers co-extracted with polyphenolics under subcritical water-mediated hydrolysis. The cumulative distribution curves for both powder samples are presented in Figure 3.

The mean particle size of SWE and SWE + HPβ-CD were 3.35 μm and 3.42 μm respectively and while not significant ($p < 0.05$) the results do reflect the differences in density which has been attributed to the architecture of the matrix within each particle. The lack of significant difference in particle size can perhaps be explained by the fact the spray drying operating conditions were similar for both samples. As it has been illustrated that different particle sizes of powders

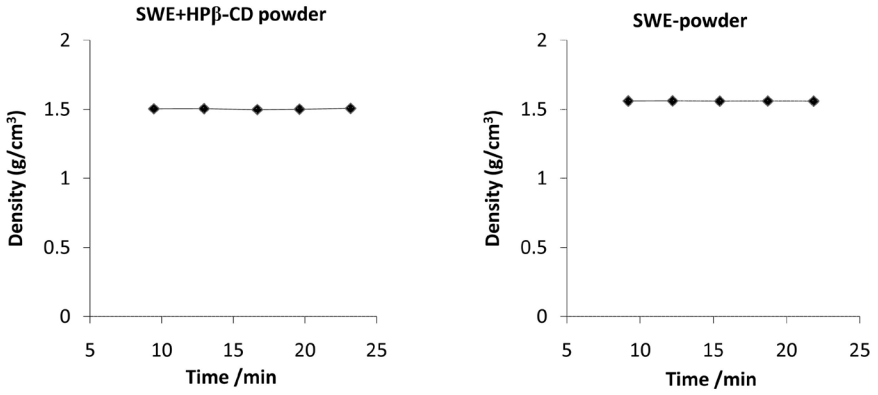

Figure 2. The density of powder as a function of time under 5 cycles with purge fill pressure of 19.50 psig and equilibrating rate at 0.020 psig per minute.

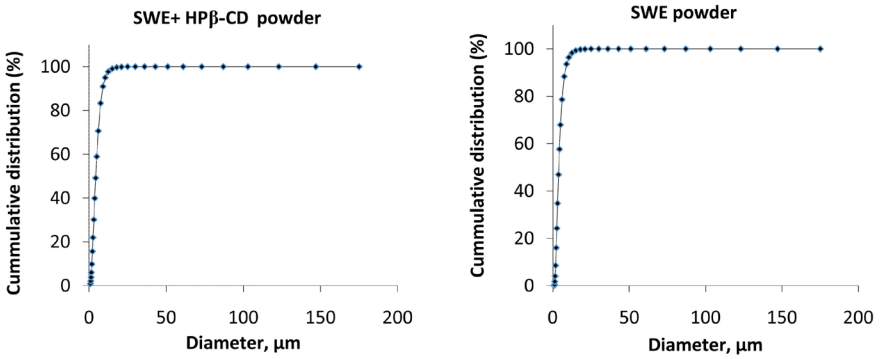

Figure 3. Cumulative distribution of particle size percent vs the upper limit of each size class from the HELOS and RODOS particle size analyser using density values of 1.50 g/cm^3 (SWE + HPβ-CD powder) and 1.56 g/cm^3 (SWE powder).

were produced when the spray drying operating conditions are varied [56]. The micronized powders are therefore suitable for cosmetic and pharmaceutical formulations due to the size obtained in micrometres. Physical appearance, feel and stability of cosmetic products are influenced by particle size of raw materials. Particle size analysis is an indicator of quality and performance because of size impacts flow and compaction properties. Particle sizes less than 0.1µm are not suitable for cosmetic formulations [57] [58].

3.2.4. Morphology of Powders

Scanning electron microscopy (SEM) was used to investigate the impact of the type of drying technique and carrier introduced. SEM images of freeze-dried and spray dried subcritical water extract (SWE) of the apple pomace revealed different morphologies. The SEM of freeze-dried subcritical water extract (**Figure 4**).

Smooth aggregates of varying sizes had formed and reflected the observations and reported for freeze-dried powders [35] [59] [60] [61]. The spray dried subcritical water extracts presented very different morphologies (**Figure 5**).

The particles formed under spray drying appear spherical and aggregate into a network. The morphology suggests the extract have been directly encapsulated and the "stickiness" of the particles supports the network of aggregates.

The morphology of pure HPβ-CD is shown in **Figure 6** and illustrates the smooth spherical structures which at high magnification reveal voids within the particle.

SEM images of the freeze-dried encapsulated subcritical water extracts with HPβ-Cyclodextrin (**Figure 7**) consisted of smooth spherical surface with less shrinking. Absence of cracks or pores in the freeze-dried microencapsulated SWE-HPβ-CD may be an indicator of preservation of the active ingredient as freeze-dried quercetin encapsulated in β-CD showed similar morphology and reported as such [62].

The ice formed within the encapsulated product during the freeze-drying process prevented the collapse and shrinkage of the particles [63].

Figure 8 shows the particle morphology generated when HPβ-CD is complexed

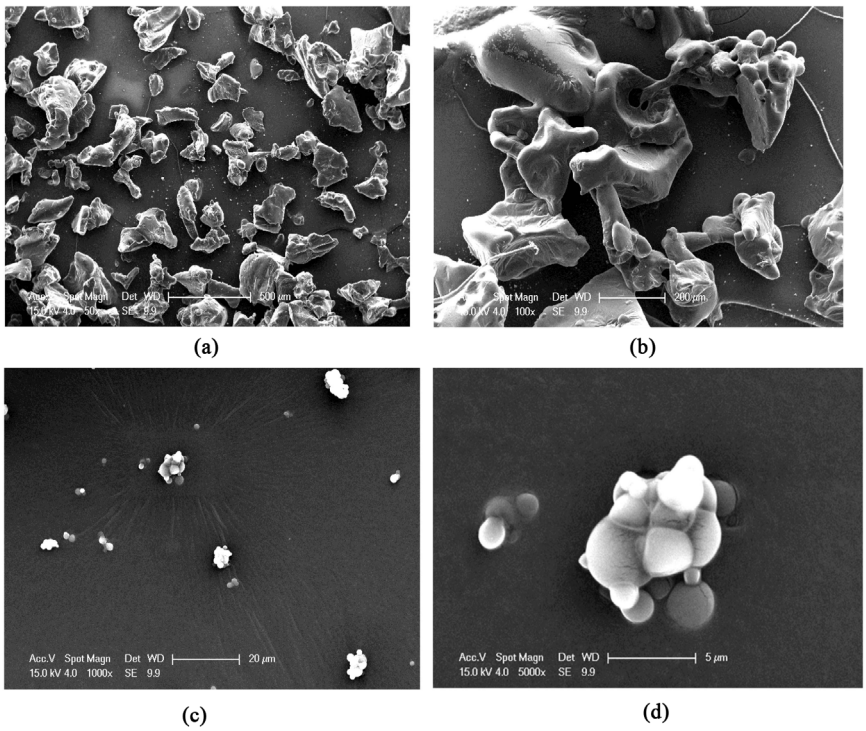

Figure 4. SEM images of freeze dried encapsulated phenolic fraction with natural polysaccharides co-extracted under subcritical water extraction of apple pomace (SWE); with Magnifications; (a) =50×; (b) =100×; (c) =1000× and (d) =5000×.

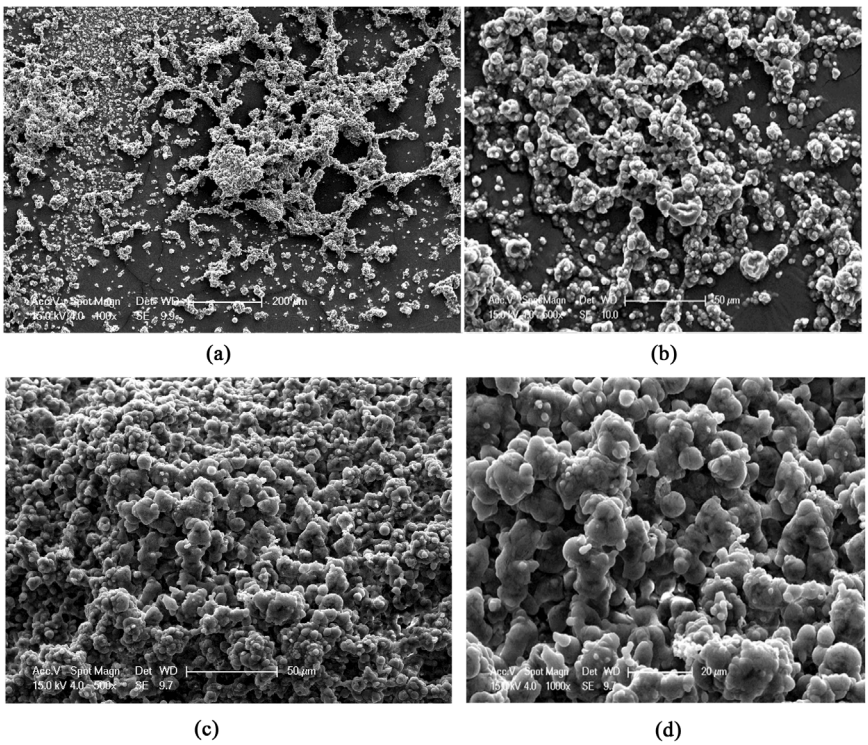

Figure 5. SEM images of spray dried encapsulated phenolic fraction with natural polysaccharides co-extracted under subcritical water extraction of apple pomace (SWE) with Magnifications; (a) =100×; (b) =200×; (c) =500× and (d) =1000×.

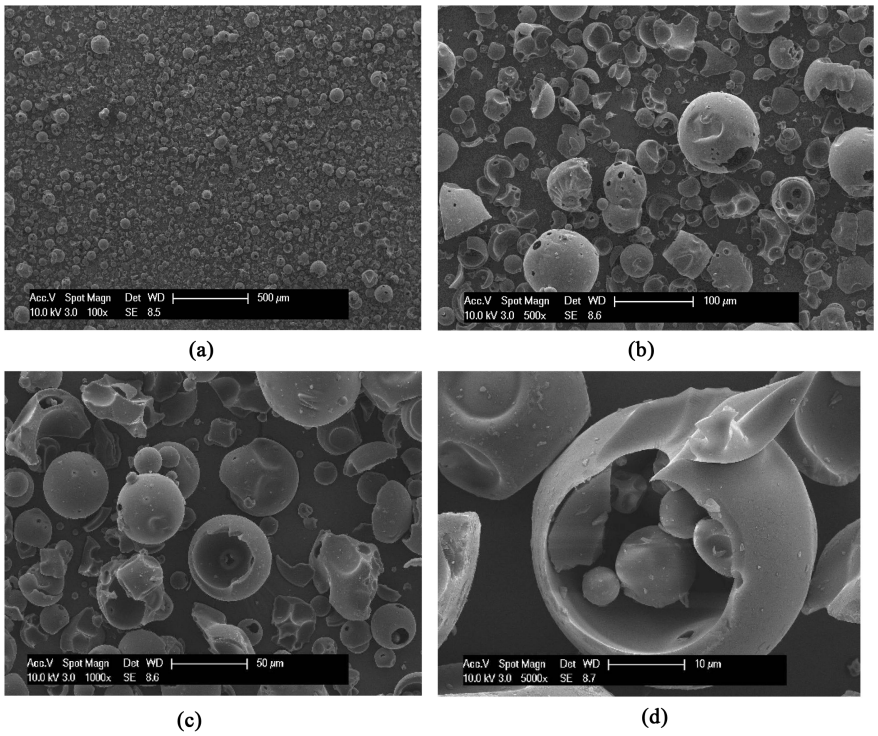

Figure 6. SEM images of pure HPβ-Cyclodextrins Powder with magnifications; (a) =100×; (b) =500×; (c) =1000× and (d) =5000×.

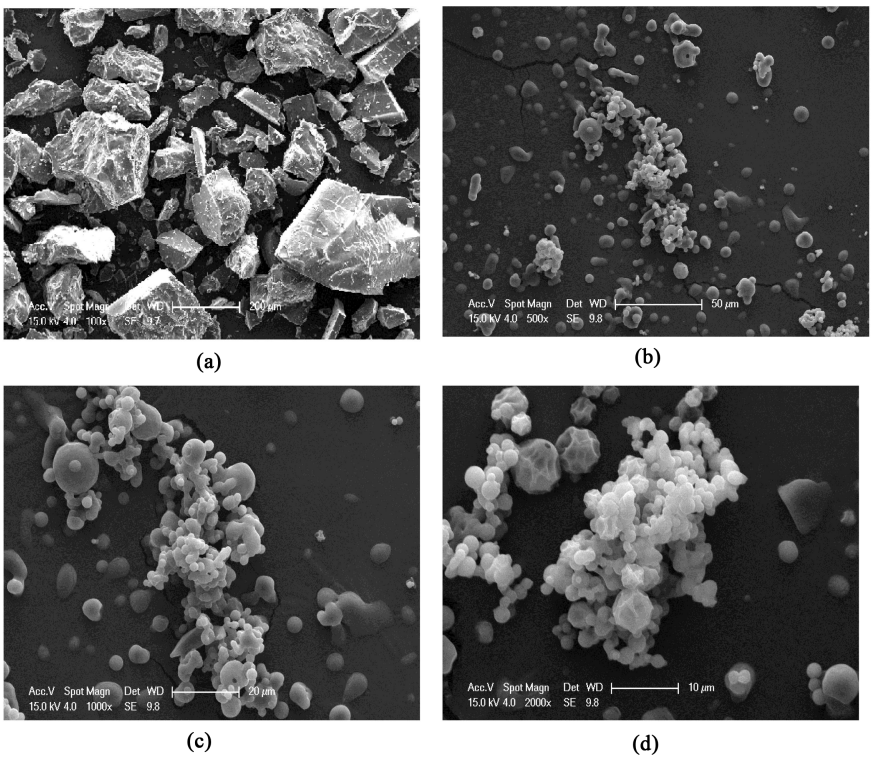

Figure 7. SEM images of freeze dried encapsulated subcritical water extract of apple pomace with HP-β-Cyclodextrins (SWE + HP-β-CD); with Magnifications; (a) =100×; (b) =500×; (c) =1000× and (d) =2000×.

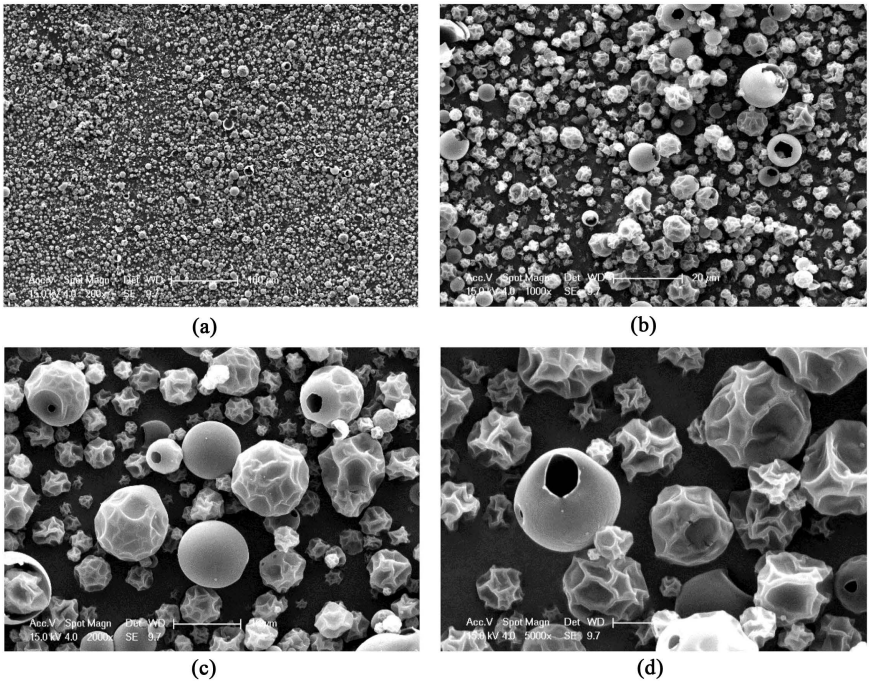

Figure 8. SEM images of spray dried encapsulated subcritical water extract of apple pomace with HP-β-Cyclodextrins (SWE + HP-β-CD) with Magnifications; (a) =200×; (b) =1000×; (c) =2000× and (d) =5000×.

with SWE priority and during spray drying. The SEM images clearly indicate that during processing discrete spherical particles of varying sizes are formed, creating an overall morphology distinct from that formed when the SWE is sprayed dried without cyclodextrin *i.e.* a free flowing powder.

However, increased magnification revealed the formation of "dents" or shrinkage in the spray dried particles. The mechanism of atomisation and combined effects of drying rates taking place at the initial drying stages could be responsible for the observed particle morphology [64] [65]. Viscoelasticity of the carrier before expansion of droplets also could possibly contribute to the deflated morphology. Rapid solidification due to high rate of drying, and formation of crust on particle surface hinders the inflation of the microcapsules hence, the shrunken surface [66] [67]. Crust formation on surfaces of powder suggests incomplete encapsulation [68]. Structural collapse and blow-holes on the surface of spray dried microencapsulated β-Cyclodexrin had been reported [67] [69]. The presence of holes on the spherical or cylindrical surfaces within microencapsulated structure could also indicate incomplete encapsulation because similar structures were seen in the pure HP β-Cyclodextrin SEM images (**Figure 6**). The subcritical water extract is made up of complex mixture of molecules with varying sizes and could have an influence in the morphology of encapsulated HPβ-Cyclodextrin. The natural polymers co-extracted could exceed the host capacity of HPβ-Cyclodextrin. The shape of HPβ-Cyclodextrin is toroidal with a hollow structure which changes during incorporation [70].

3.2.5. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectroscopy was employed to identify potential interactions between the carrier HP-β-CD and the subcritical water extract (SWE) complexes during both freeze drying and spray drying as a way to confirm encapsulation. The application of FT-IR was based on the fact that; vibrational spectra are unique physical properties of molecules. Therefore, it is possible to overlay spectra of pure and complexed powders to determine changes induced during encapsulation [71].

Infrared spectra of pure HPβ-Cyclodextrin and spray and freeze-dried HPβ-Cyclodextrin complexed with SWE, are shown in **Figure 9**.

No new peak was observed in the FTIR spectrum of binary systems, confirming that there were no new chemical interactions and no new covalent bonds had formed.

The FTIR spectrum of pure HPβ-Cyclodextrin revealed all the functional groupings recorded in **Table 2**, except for carboxylic acid group (C=O) (1760 - 1769 cm^{-1}).

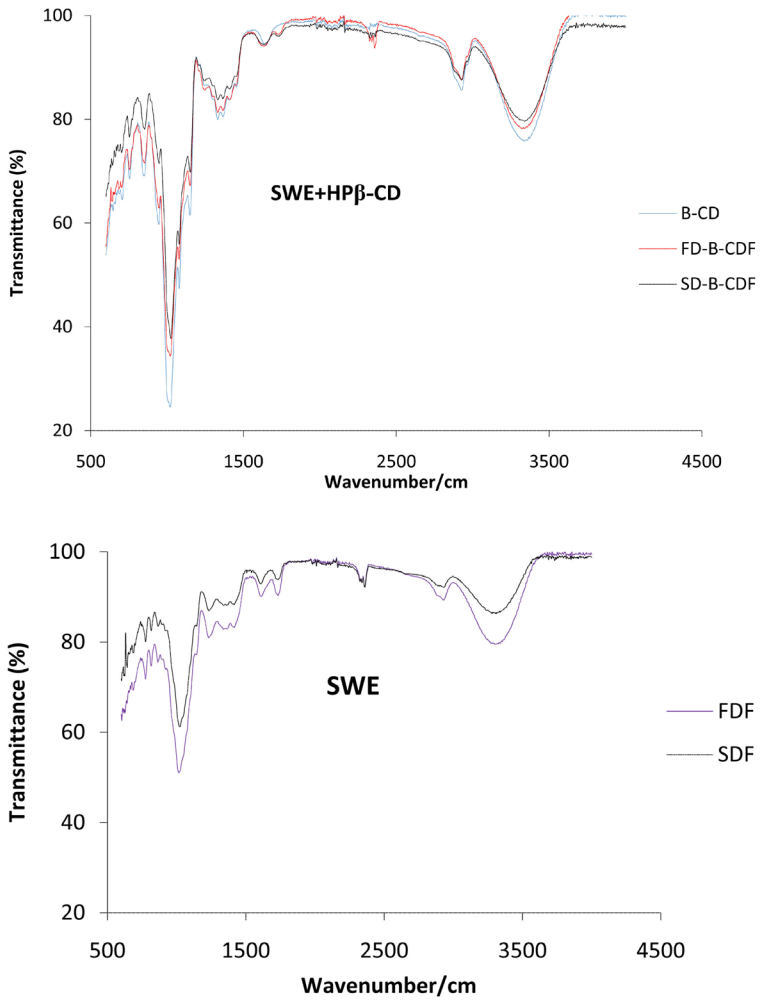

Figure 9. FTIR-spectra of HPβ-Cyclodextrin (B-CD), spray dried HPβ-Cyclodextrin with SWE (B-CD-SDF), Freeze dried HPβ-Cyclodextrin with SWE (FD-B-CDF), spray dried SWE (SDF) and Freeze dried SWE (FDF).

Table 2. Infrared Absorption characteristics of selected functional groups [72].

Functional group	Bond	Wavenumber (frequency) cm^{-1}
Alcohols and phenols	O-H stretching	3500-3200
Alkanes	C-H stretching	3000-2850
General carbonyls	C=O stretching	1760-1665
Carboxylic acids	C=O stretching	1760-1769
Esters and others	C-OH stretching	1320-1000

However, the C=O was relevant in the direct encapsulated subcritical water extract (SWE) of the apple pomace and was present when complexed with HPβ-Cyclodextrin (**Figure 9**). While no new peaks were observed, the spectrum of the HPβ-Cyclodextrin-SWE complex showed a shift in the frequencies (**Table 3** and **Table 4**) and the intensity, and was attributed to interactions involving hydrogen bonding.

Carboxylic acid group (C=O) is distinctive to polyphenolic compounds and a strong peak was reported at 1740 cm^{-1} when quercetin was encapsulated in a surfactant Poloxamers [73]. A shift of the ester (C-OH) band from 1183 - 1206 cm^{-1} ($\Delta\delta$ = +23) for dimethyl-β-Cyclodextrin [74] and 1180 to 1154 cm^{-1} ($\Delta\delta$ = +26) for Proxicam complexed with β-Cyclodextrin [75]. The results so far confirmed interaction between HPβ-Cyclodextrin and the phenolic extract but not very indicative enough to support evidence of encapsulation because FTIR spectroscopic method is less clarifying technique compared to single crystal diffraction (SCXRD) and powder X-ray diffraction (PXRD) suitable for detecting inclusion complexes [76] [77] [78] [79].

3.3. Antioxidant Stability

The antioxidant activity determined using the ORAC assay of the liquid subcritical water extract of the apple pomace was compared to that of the spray dried powder to establish the impact of "direct" encapsulation. The spray drying had a marked impact and resulted in 46.5% loss of antioxidant activity (**Table 5**).

A decrease in polyphenolic content of elderberry juice by 25% had been reported after spray drying and decreases further with increasing inlet temperature [80]. Similar results of decreases of 28% to 50% in antioxidant activity were reported after spray drying of grape pomace extracts [81]. Also, approximately 42% loss in antioxidant activity was reported during spray drying of *Momordica cochinchinensis,* fruit powder [82]. The subcritical water extraction was performed at 150˚C and the extracts were then spray dried at 170˚C, therefore it was possible the increased in temperature had a negative effect on both the antioxidant activity and polyphenolic content. Degradation of active compounds with increasing temperature had already been reported [60] [83]. In contrast, the HPβ-CD-SWE complex only lost 3.2% of its antioxidant activity after the spay drying process, suggesting that, the HPβ-CD demonstrated significant protection of the polyphenolic antioxidant compounds (**Table 6**).

Table 3. Comparison between frequencies of pure HPβ-CD and corresponding inclusion complex for spray dried powder.

Bond	Frequency		Change $\Delta\delta$
	HPβ-CD	SWE + HPβ-CD	
O-H	3338.18	3341.07	+2.89
C-H	2925.48	2926.45	+0.97
C=O (general carbonyl)	1643.05	1627.63	−15.42
C=O carboxyl	-	1731.76	+1731.76
C-OH	1020.16	1024.98	+4.82

Table 4. Comparison between frequencies of pure HPβ-CD and corresponding inclusion complex for freeze dried powder.

Bond	Frequency (cm^{-1})		Change $\Delta\delta$
	HPβ-CD	SWE + HPβ-CD	
O-H	3338.18	3317.93	−20.25
C-H	2925.48	2928.38	+2.90
C=O(general carbonyl)	1643.05	1634.38	−8.70
C=O carboxyl	-	1726.94	+1726.94
C-OH	1020.16	1021.12	+0.96

Table 5. Antioxidant activity changes of subcritical water extract before and after spray drying.

Antioxidant activity	Subcritical water extract		Percentage loss in antioxidant activity (%)
	Before spray drying	After spray drying	
TPC (mg/l) GAE	574.1 ± 13.9	318.8 ± 11.2	44.6
ORAC (μmolTE/g) DW	1517.6 ± 93	811.7± 20	46.5

Table 6. Antioxidant activity changes of subcritical water extract with HPβ-CD before and after spray drying.

Antioxidant activity	Subcritical water extract with HPβ-CD		Percentage loss in antioxidant activity (%)
	Before spray drying	After spray drying	
TPC (mg/l) GAE	530.0 ± 4.4	513.5 ± 16	3.2

3.4. Storage Antioxidant Stability

The caking nature and relatively high hygroscopicity of the directly encapsulated powder (SWE) from subcritical water extract was a potential challenge to the stability studies to be conducted in the solid form. However, liquid forms were utilised during analysis, because molecular encapsulation can form both in solid and in solution [84]. Solid and liquid forms of β-Cyclodextrin inclusion complexes with, hesperetin, hesperidin, naringin and narigeninn have been reported

using FT-IR, NMR, DSC and X-ray techniques to support evidence of encapsulation [85] [86].

Changes in antioxidant activity of SWE and SWE + HPβ-CD were monitored at facilitated conditions of 60˚C to observe time effect on the retention of polyphenolic antioxidant activity. A control experiment employing Chlorogenic acid standard was set up similar to the HPβ-Cyclodextrin complex. Folin Ciocalteu method proposed among standardised assays for quality control and antioxidant activity determination was adopted and applied.

The changes in antioxidant activity overtime are presented in Figure 10.

Antioxidant activity decreases with time at the constant temperature 60˚C. A 44% decrease in antioxidant activity of the directly encapsulated SWE was observed compared with 25% decrease of the subcritical SWE + HPβ-CD over the 35 days period. The initial antioxidant activity of the HPβ-Cyclodextrin included complexes (both subcritical water extract and standard Chlorogenic acid) were slightly below the direct encapsulated SWE and the Chlorogenic acid standard. However, there was a sharp increase in antioxidant activity within the 7 days' period and then decrease afterwards. One-way analysis of variance (ANOVA) of the antioxidant activity was tested against time for each treatment with post hoc Tukey comparisons at 95% confidence. The null hypothesis was, all means (antioxidant activity) were equal against the alternative hypotheses that, at least one mean (antioxidant activity) was different. There were no significant changes in antioxidant activity for subcritical water extracts within the first 7 days ($p > 0.05$). However, antioxidant activity varied significantly after the 7 days ($p < 0.05$) and no significant changes were observed between 14 - 35 days ($p < 0.05$). For the control experiment using the standard Chlorogenic acid, antioxidant activity significantly changed after 7 days ($p > 0.05$) and no significant differences in antioxidant activity measured from 14 - 28 days ($p < 0.05$). The 35th-day antioxidant activity of Chlorogenic acid was significantly different and may be attributed to high antioxidant activity of degradation products of Chlorogenic acid. For the HPβ-CD encapsulated complexes, antioxidant activities at day 7

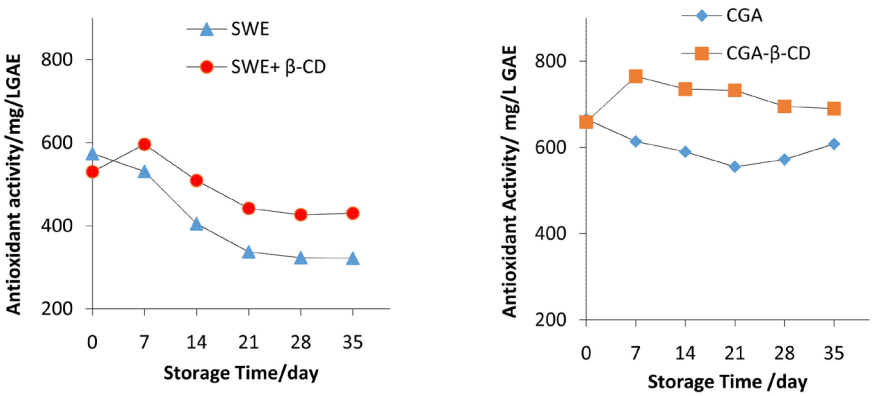

Figure 10. Antioxidant activity for subcritical water extract (SWE), extract with Cyclodextrin (SWE + HPβ-CD), Chlorogenic acid (CGA) and Chlorogenic acid with Cyclodextrin (CGA-HPβ-CD) following storage at 60˚C for 35 days.

were significantly higher compared to day 1 (initial) antioxidant activities ($p < 0.05$). No significant differences in antioxidant activities for days, 21, 28 and 35 for SWE + HPβ-CD encapsulated products were observed ($p > 0.05$).

Likewise, there was no significant difference in antioxidant activities for days 7, 28, 35 for HPβ-CD encapsulated Chlorogenic acid standard ($p > 0.05$).

Clearly, only first 7 days' antioxidant activities were comparable for both encapsulated and non-encapsulated subcritical water extracts samples. It implies that there was no significant difference between antioxidant activities for both treatments within the first 7 days ($p < 0.05$).

However, antioxidant activity for day 14, 21, 28 and 35 of HPβ-CD encapsulated subcritical water extracts were significantly higher than corresponding samples without HPβ-CD ($p > 0.05$). Unexpectedly, only day 7 antioxidant activity of control experiment of standard Chlorogenic acid was similar to day 7 antioxidant activity of Chlorogenic acid + HPβ-Cyclodextrin ($p < 0.05$).

There was a significant variation of the antioxidant activity after 7 days ($p < 0.05$) of Chlorogenic acid with HPβ-Cyclodextrin. Antioxidant activities of days 7, 14, 21, 28 and 35 of standard Chlorogenic acid samples were significantly lower ($p < 0.05$), compared to antioxidant activities of days 7, 14, 21, 28 and 35 of the complex of Chlorogenic acid with HPβ-CD.

The hydroxyl Propyl Cyclodextrin offered good protection of the polyphenolic antioxidant compounds in the subcritical water extracts against degradation, and was confirmed in the control experiment using standard phenolic compound Chlorogenic acid. Cyclodextrin was considered as secondary antioxidant and had been reported to have protective effect on ascorbic acid and phenolic compound 2,2,5,7,8-pentamethylchroman-6-ol (PMC) [87]. Many other reports of protective effect of Cyclodextrin on antioxidant activity of bioactive compounds are available [34] [88] [89].

Protective mechanism of the Cyclodextrin against degradation and oxidation were apparently due to the complexation of the subcritical water extracts into its hydrophobic cavity. Therefore, the HPβ-Cyclodextrin can be employed as a carrier to prolong shelf-life of the phenolic antioxidant compounds and to mask any undesired taste and colour of SWE for applications in nutraceutical, pharmaceutical industries. The polyphenolic compounds identified in the SWE is shown in **Figure 11**.

4. Conclusions

Encapsulation of subcritical water extract was successfully demonstrated by spray drying technique with and without an external carrier. Particle sizes of powders suitable for cosmetic formulations were achieved. However, spray drying methods negatively affected antioxidant activity of the extracts. Total antioxidant activity of the auto-encapsulated (SWE) was approximately 50% less than the liquid subcritical water extract. Micronised products obtained were found to be hygroscopic which would negatively affect their applications in

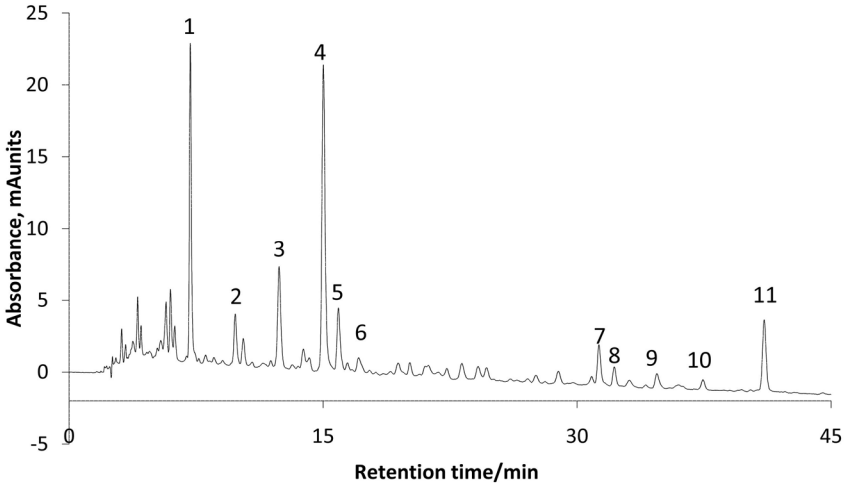

Figure 11. Chromatogram of subcritical water extract (SWE) at 150 °C for 30 minutes; 1: 5HMF; 2-furfural, 3: Protocatechuic aldehyde; 4: Chlorogenic acid; 5: (isomer of Chlorogenic acid); 6: caffeic acid; 7: Quercetin-3-galactoside; 8: Quercetin-3-glucoside, peak 9 and 10 not identified; 11: Phlorodzin.

cosmetic and pharmaceutical formulations and cost of storage. Hydroxyl propyl-β-Cyclodextrin as a carrier has decreased the hygroscopicity of the subcritical water extract, mask the brown colour, and in addition, demonstrated protective effect against oxidation and degradation, thereby prolonging the shelf life of the antioxidants compounds. Fourier transform infra-red spectroscopy (FTIR) and Electron scanning microscopy (SEM) were selected to characterised powders to support evidence of encapsulation of the subcritical water extract into the hydrophobic cavity of the Cyclodextrin. Both techniques have revealed some level of interaction between the host HPβ-CD and subcritical water extract.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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