Antioxidant phenolic compounds recovery from *Mangifera indica* L. by-products by supercritical antisolvent extraction

Miguel A. Meneses[a,b]; Giuseppe Caputo[b]; Mariarosa Scognamiglio[b]; Ernesto Reverchon[b]; Renata Adami[b]

a. Department of Agricultural and Food Sciences, Universidad Técnica Particular de Loja. San Cayetano alto sn. AP 1101608. Loja, Ecuador.

b. Department of Industrial Engineering, University of Salerno. Via Giovanni Paolo II, 132, 84084. Fisciano (SA), Italy.
e-mail: mameneses@utpl.edu.ec (M. A. Meneses), Tel. +593 7 2570275

ABSTRACT

Supercritical Antisolvent Extraction (SAE) was used for the recovery of antioxidant compounds from mango by-products. The liquid extract of mango by-product was obtained by solid-liquid extraction using acetone (70%) and subsequent adsorption/desorption in C18 cartridge with ethanol, then it has been processed by SAE technique using SC-CO_2 as antisolvent. The main operative parameters, temperature and pressure, were varied in the range 35-45°C and 8-15 MPa to allow the selective recovery and precipitation of phenolic compounds. The SAE process allows the recovery of about 90% of the phenolic compounds in the processed solution, moreover, when SAE experiments were performed at the operating conditions corresponding to the supercritical region of the vapor liquid equilibrium diagram for CO_2-ethanol system, it was possible to produce nanometric particles with improved dissolution properties, verified in aqueous solution for DPPH assay. The best recovery of polyphenols was obtained at 40°C and 10 MPa and the main phenols identified were mangiferin, isomangiferin, quercetin 3-O-galactoside, quercetin 3-O-glucoside, quercetin 3-O-xyloside, quercetin 3-O-arabinoside, quercetin and kaempferol. The product obtained was formed by a dry powder, with the morphology of spherical nanoparticles with a particle diameter of 143 ± 40 nm. The antioxidant activity of precipitated product was 851.9 µMol TE/g with an IC_{50} of 90 µg/mL. SAE technique is effective in the selective recovery of phenolic compounds without degradation of the antioxidant activity. Furthermore, the analyses on the nanometric particles indicate that SAE processing of natural extracts may be considered for improving the activity of natural antioxidant compounds.

1. Introduction

The market of functional ingredients/foods is increasing, as a consequence of the interest of customers in preventing illness and improving health using natural sources (Khan et al., 2012). Epidemiological evidence supports the health benefits related to a diet rich in fruits and vegetables, that provides prevention of cardiovascular diseases and certain types of cancer (Aruoma, 2003; Van Duyn and Pivonka, 2000). Mango (*Mangifera indica* L.) fruit has been recognized as good source of bioactive compounds that have antiviral, antibacterial, analgesic, anti-inflammatory and immune modulatory activities (Makare et al., 2001). Some studies showed phenolic compounds as enhancing health benefits (Berardini et al., 2004; Berardini et al., 2005a; Ribeiro et al., 2007); in particular, xanthones and flavonols show antioxidant effect and have anti-inflammatory activities. Among them, mangiferin, a xanthone-C-glucoside the major phenolic compound in mango, is an interesting active compound for the variety of pharmacological properties (Hou et al., 2012; Ling et al., 2009).

The main bio-wastes produced in the processing of mango fruits are peels and seeds that represent between 35 and 60 % of the whole fruit (Larrauri et al., 1996). These by-products have been recognized as natural source of bioactive phenolic compounds; since peels are not currently utilized for a commercial purpose, their use could be an important and sustainable opportunity for reducing pollution from bio-wastes (Ajila et al., 2007). Mango peels have been shown to be a rich source of flavonol O-glycoside and xanthone C-glycoside, gallotannins and benzophenone derivatives (Ajila et al., 2010; Berardini et al., 2004; Berardini et al., 2005b; Schieber et al., 2003a). The recovery of polyphenols from mango peels mainly depends on the extraction technique used and the relative solvents; the solvents frequently used are methanol, ethanol, ethyl acetate, acetone and mixtures of these solvents with water (Dorta et al., 2012). In all cases, post-processing steps are required to fractionate or to purify the compounds from co-extracted material, and to eliminate the toxic organic solvent. In previous studies, it has been found that total phenol content of mango peels is between 5.46 mg GAE/g DM (GAE=gallic acid equivalent, DM=dry matter) for fiber concentrates (Martinez et al., 2012) and about 110 mg GAE/g dry matter of peels (Ajila et al., 2007). Berardini et al. (2005b) reported the phenolic composition and the major compounds, such as mangiferin, mangiferin derivates, quercetin and quercetin derivates.

The main drawbacks related to classical extraction techniques are the thermal and chemical degradation of labile compounds and the residual of harmful solvent affecting the quality and safety of extract. Indeed, direct use of mango extracts in food or pharmaceutical industry is not allowed without quality guaranties of low residual of toxic solvents(Wijngaard et al., 2012). Therefore, it is highly desired to develop non-organic-solvent-based-extraction methods with high extraction efficiency.

The use of carbon dioxide (CO_2) in supercritical fluid extraction (SFE) is an alternative for extracting natural antioxidants from herbs and plants; it shows advantages in comparison with traditional extraction techniques, since degradation and decomposition of the active compounds are avoided by operating at reduced temperatures, in absence of light and oxygen. The process is non-toxic, no pollutant organic solvents are used and, as a consequence the post-processing of extracts necessary to separate organic solvents is not necessary (Reverchon and De Marco, 2006), moreover it is also possible to modulate the solvent power of supercritical carbon dioxide (SC-CO_2) to perform a selective extraction. However, CO_2 has a very limited capacity to dissolve polar and high molecular weight compounds. Supercritical antisolvent extraction (SAE) has been proposed to overcome the limits related to the extraction of polar compounds by SC-CO_2 (Martín et al., 2011; Reverchon and De Marco, 2006). SAE is based on the same principles of supercritical antisolvent technique, that has been extensively used to study the production of micronic and nanometric particles of pharmaceutical compounds with controlled particle size and distribution (Reverchon, 1999; Reverchon et al., 2008), however it has different approaches and purposes. When SAE is used on natural matters containing several families of compounds, some of them are extracted using a polar solvent, like, ethanol and when SC-CO_2 extracts the solvent by antisolvent mechanism, some compounds can result soluble or partially soluble in the mixture formed (ethanol-CO_2) and could be recovered in the separation step; whereas the remaining compounds are precipitated.

SAE technique has rarely been used for polyphenolic fractionation, Floris et al. (2010) used SAE for the recovery of antioxidant compounds present in a liquid solution of grape by-products using SC-CO_2 as antisolvent at moderate conditions of pressure and temperature (40ºC and 11 MPa). The same principle has also been used for the coprecipitation of green tea extracts (Sosa et al., 2011) and rosemary antioxidants

(Visentin et al., 2012) to get protection against degradation; in both cases, the process allowed the selective precipitation of 90% of polyphenols compounds together with a biodegradable polymer. The same procedure was used by Yang et al. (2011) to micronize polymeric procyanidins at 80°C and 25 MPa; the nanoscale material showed higher antioxidant activity than the starting material. The possibility to obtain precipitated/fractionated extracts as microparticles is a further advantage, indeed, it is known that the reduction of particle size is the first step for improving the dissolution rate of poorly water-soluble compounds for pharmaceutical or food applications, as it has been demonstrated in literature for phenolic compounds present in mango peel (Lee et al., 2012; Sahoo et al., 2011; Tzeng et al., 2011; Yen et al., 2010).

The application of SAE technique for the recovery of phenolic compounds from mango peels has been never performed before and it could overcome the drawbacks discussed for the conventional extraction techniques, leading to the complete elimination of solvent and the concentration of phenolic compounds in a single step and with a final product with the added characteristic of micronized particles. Therefore, in this work, SC-CO_2 was used as antisolvent to recovery the antioxidant compounds present in a mango peel extract, without damage their characteristics. A multi step procedure has been developed for the extraction from mango peel to eliminate interfering compounds: liquid–solid extraction using ethanol, then adsorption/desorption in a C18 column, finally SAE fractionation.

2. Materials and methods

2.1 Materials

By-products of canned fruits and juice from mango (*Mangifera indica* L. cv. Tommy Atkins and Haden) were provided by Agroficial (Ecuador), a fruit processing industry. Mango by-products corresponding to peels with added pulp were vacuum dehydrated at 37°C until a final humidity of about 10% before being ground to a particle size in the range of 200 to 250 μm.

Acetone (AC, purity 99.8%), ethanol (EtOH, 99.8%), methanol (MeOH, 99%), 2,2-diphenyl -1-picrylhydrazyl (DPPH), Folin-Ciocalteu 2N reagent, sodium carbonate, gallic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox 97%) were supplied by Sigma-Aldrich (Milan, Italy); carbon dioxide (CO_2, 99%) was

purchased from SON (Italy); Discovery® DSC-18 SPE cartridge 20 cm3/5g 55 µm for adsorption/desorption procedure was supplied by Sigma-Aldrich (Milan, Italy).
Polyphenol standards used were: mangiferin, quercetin, kaempferol and gallic acid from Sigma (Milan, Italy), and quercetin 3-O galactoside from Extrasynthese (Lyon, France).

2.2 Extraction of antioxidants from mango by-products

The extraction of antioxidant compounds was performed by solid-liquid extraction as described in literature (Schieber et al., 2003a). Aliquots of 5 g of mango by-products were mixed with 50 mL of aqueous acetone (80% v/v), after the addition of 0.5 g of ascorbic acid and the extraction was carried for 6 h under stirring at room temperature (25ºC). Then, the solution was centrifuged and the supernatant was separated while the solid residue extracted once again with 50 mL of solvent for 30 min. Both supernatants were combined and the organic solvent was removed by evaporation in vacuum at 30°C. The aqueous solution was dissolved in deionized water (1:4) and then filtered in a cartridge with a porosity of 5 µm. This procedure was optimized to obtain an efficient adsorption on the C18 cartridge.

2.3 Purification of phenolic compounds by adsorption and desorption

The C18 cartridge was activated adding 25 mL of ethanol and wetted with 30 mL of deionized water. Then, the extract obtained was flushed in the column. After washing with 30 mL of deionized water, the phenolic compounds were desorbed from the column with 30 mL of ethanol. The purified solution was kept at -4 ºC until the subsequent experiments and analysis were performed.

2.4 Supercritical Antisolvent Extraction (SAE)

A membrane high-pressure pump (LEWA, mod. LDB1 M210S) was used to deliver CO_2 and a HPLC pump (Gilson, mod. 305) to deliver the liquid solution (extract from the C18 cartridge). The precipitator was a stainless steel vessel (V=0.4 L, i.d.=50 mm). A 180 µm nozzle on the top of the precipitator allowed the injection of the liquid solution and a stainless steel filter with a porosity of 1 µm, located at the bottom, allowed the collection of the powder material. A separator, located downstream the pressure reduction valve, operating at 3 MPa was used to recover the liquid solvent.

Further information about the SAE apparatus can be found elsewhere (Martín et al., 2011). Figure 1 shows a schematic representation of the SAE equipment used for the experiments.

For SAE experiments, first CO_2 was pumped to the precipitator at fixed temperature until the desired condition of pressure were reached, then the pressure was regulated by a micrometric valve located between the precipitator and the separator. When constant flow rate of CO_2 was established, pure solvent was sent through the nozzle to the precipitator until steady state conditions for solvent and antisolvent system were reached. At this point the delivery of the ethanolic solution, previously treated with C18 cartridge, started. The fast extraction of the solvent by SC-CO_2 produced the precipitation of the solute. After the ethanolic solution was delivered, the precipitator was purged with pure SC-CO_2 at the process conditions, to wash away residual solvent solubilized in the supercritical antisolvent. If the final purge with pure SC-CO_2 was not performed, the solvent contained in the antisolvent condensed during depressurization, solubilizing or modifying the precipitate. Finally, the precipitation vessel was depressurized and the precipitated compounds were collected. The solvent and solubilized compounds were recovered in the separator operated at 3 MPa.

The recovery for the process was determined by weighting the total amount of precipitated compounds collected in the precipitation vessel and the compounds condensed with ethanol in the separator, both fractions were related to the initial amount of extract dissolved in ethanol.

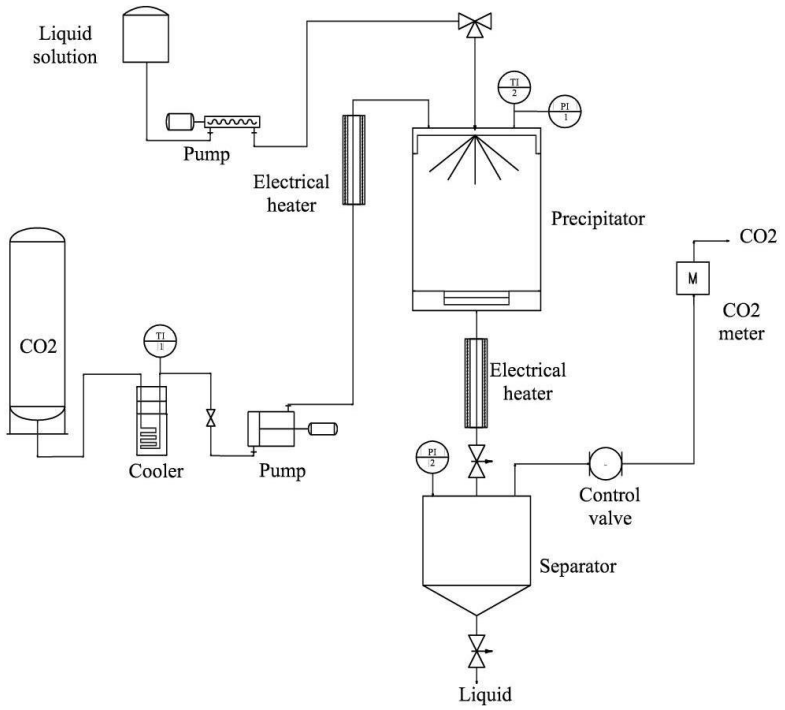


Figure 1. Schematic representation of SAE apparatus

2.5 Particle size distribution

The surface morphological examination of the unprocessed and processed mango extract was performed using a scanning electron microscope (SEM) (LEO 420 version V2.04, Germany): powders were dispersed on a carbon tab previously stuck to an aluminum stub (Agar Scientific, United Kingdom); then, were coated with gold (layer thickness 250 Å) using a sputter coater (mod. 108 A, Agar Scientific, Stansted, United Kingdom).

Particle size (PS) and particle size distribution (PSD) of precipitated powder were measured from SEM images using the Sigma Scan Pro image analysis software (version 5.0, Aspire Software International, USA). Approximatively 1000 particles were analyzed in the elaboration of each PSD. Histograms representing the PSD were fitted using Microcal Origin Software (version 8.0 Origin Lab Corporation, USA).

2.6 HPLC analysis

The separation and identification of phenolic compounds was performed in an Agilent HPLC series 1100 instrument equipped with ChemStation software. A Synergi Hydro-RP column (150 mm x 3.0 mm i.d., 4 µm, Phenomenex) with a C18 ODS guard column (4.0 mm x 2.0 mm i.d.) operated at room temperature. The elution program used was according to the method explained by Berardini et al. (2005a) with some modifications. The mobile phase consisted of two solutions: 2 % acetic acid in water (solution A) and 0.5% acetic acid in water/acetonitrile (50:50, v/v) (solution B), the gradient used for separation was: 0 - 25% B (15 min), 25 - 30 % B (35 min), 30 - 80% B (10 min) at 80% B (12 min), 80-100 B (3 min). The system was left to stabilize for 5 min between consecutive injections. All fractions were dissolved in methanol and filtered, using a 0.45 µm filter.The injection volume was 10 µL with a solvent flow 0.6 mL/min. The detection was performed at 370 nm. The compounds were identified by comparison with the relative retention time of polyphenol standard in methanol, and with reference to a chromatogram from literature (Berardini et al., 2005a).

2.7 Total phenol content

Total phenolic content (TPC) was determined by the Folin Ciocalteu method adapted from the one by Thaipong et al. (2006). 150 µL of methanolic extract solution were mixed with 2400 µL of water and 150 µL of 0.25N Folin-Ciocalteu. The mixture was allowed to react for 3 minutes and then 300 µL of 1N Na_2CO_3 solution was added and well mixed. The solution was incubated at room temperature in dark for 2 h. The absorbance was measured at 725 nm and the results were expressed as mg GAE/kg DMP using a standard curve. Additional dilution was done if the absorbance value measure was over the linear range of the standard curve.

2.8 Antioxidant assay

The method described by Brand-Williams et al. (1995), based on the reduction of DPPH radical was used with some modifications. For each extract, before and after the process, different concentrations were analyzed between 0.01-1 mg/mL in methanol and physiological solution. 5 mg of extract were dissolved in 1 mL of solvent, the solution was mixed by sonication during 10 minutes and then was filtered in a 0.45µm filter,

from this solution the other concentrations were prepared. An aliquot of 150 µL of extract solution was added to 2.85 mL of DPPH solution (1.1 ± 0.02 units in absorbance). The decrease in absorbance was determined at 515 nm after 24 h of reaction, as described by Thaipong et al. (2006). The radical scavenging activity (RSA) was defined by inhibition concentration (IC_{50}), that is the amount of mango extract necessary to decrease the initial DPPH concentration by 50%. The IC_{50} was calculated from the plots for all SAE extract. Furthermore, a trolox calibration curve was used to express the antioxidant activity as µMol equivalent of trolox (TE) by g of sample in this case concentrations of 1 mg/mL were used for measurements.

3. Results

3.1 Extraction of antioxidants from mango

Preliminary extraction tests were performed using absolute ethanol; but the whole process for the recovery of antioxidants, including SAE, was relatively unsuccessful because of the co-extraction of other compounds contained in the mango peels. Sugars and pectins contained in high quantities in mango peels (Berardini et al., 2005b) exerted not only a barrier and competition for the extraction of polyphenolic compounds, also could represent an operative problem when the solution was treated by SAE process, as discussed elsewhere (Floris et al., 2010). To overcome this difficulty, the extraction was performed with acetone (80%) and a successive adsorption/desorption step (Soto et al., 2011) was used to separate the fraction of polyphenolic compounds from sugars moiety, as described in the section 2.3. This procedure has been used in previous studies for the separation of polyphenols from fruit by-product extracts (Berardini et al., 2005b; Floris et al., 2010; Schieber et al., 2003b), phenolic compounds are absorbed in the stationary phase while other compounds pass through the resin without retention. A washing step with only water was necessary to avoid the precipitation of residues with high molecular weight.

3.2 Total Phenol content

The initial extraction with acetone allowed to obtain a phenolic content of 29545 mg GAE/kg DMP. This result corresponds to 2.9% with respect to the dry matter. If the TPC of mango by-products is compared with literature it can be noted that there is a certain variability, from 57240 mg GAE/kg DMP (Ribeiro et al., 2008) to 109700 mg

GAE/kg DMP (Ajila et al., 2007). This difference probably corresponds to variations in mango cultivar, exposition to UV radiation, ripening stages, storage conditions and because of the samples used in this study were obtained as industrial by-product.

The adsorption process showed to be effective for the elimination of co-extracted sugar moieties and selective for recovering of the phenolic fraction. With the extraction/adsorption process it was possible to recover 22150 mg GAE/kg DMP with a concentration of 888.63 ± 102.38 mg GAE/g dry purified extract. The procedure described in section 2.3 has been used to purify all the samples before fractionation by SAE.

3.3 SAE technique

The influence of pressure and temperature, the main parameters of the supercritical antisolvent process, on the fractionation efficiency of phenolic compounds was studied. The process temperature is a relevant parameter when polyphenolic compounds are processed, since they are degraded by exposure to light and high temperatures; therefore, the maximum temperature used was 45°C. The precipitator pressure was selected taking into account the vapor-liquid equilibrium diagram (VLE) of the system CO_2-EtOH (Figure 2) to obtain the operating points above the mixture critical point (MCP). Pressure and temperature were studied in the range from 8 to 15 MPa and 35 to 45°C. The operating pressure also influences the solubility of the compounds contained in the liquid solution in SC-CO_2. When pressure is increased, the solubility of some compounds is increased in the solution SC-CO_2-EtOH, and the fractionation of the inlet solution between the precipitator and the separator can be observed. In this work, the operating parameters such as SC-CO_2 mass flow rate and liquid solution flow rate were fixed to obtain a 0.98 molar fraction (x_{CO2}) in the system CO_2-EtOH; the liquid solution was injected at 1 mL/min. The operating conditions were selected to work in correspondence to the supercritical region of the vapour-liquid binary diagram (Figure 2), looking for a good compromise between SC-CO_2 solvent power and selectivity. The operating conditions of SAE experiments are reported in Table 1.

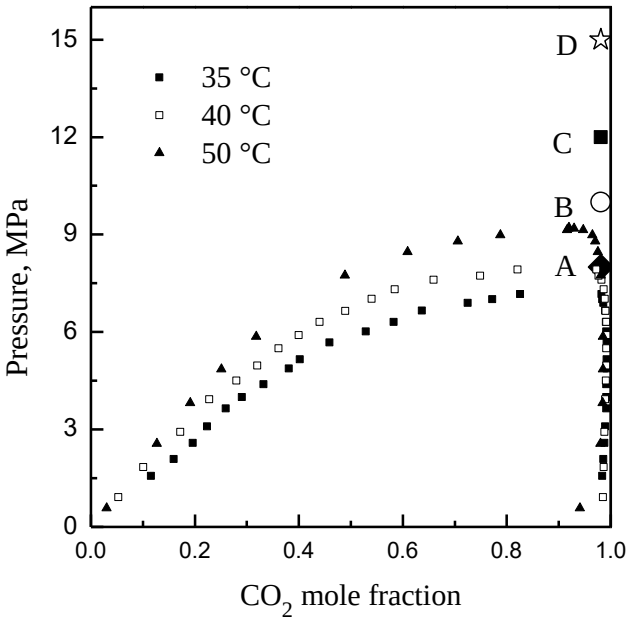


Figure 2. CO_2 VLEs adapted from Chang et al. (1997) at 35°C and 40°C, and from Joung et al. (2001) at 50°C. A (8 MPa), B (10 MPa), C (12 MPa) and D (15 MPa) correspond to the operating points of SAE experiments

3.4 Polyphenol fractionation

The HPLC analyses showed that ethanolic mango solutions produced by extraction and used in SAE experiment had a mean polyphenolic composition of mg/kg DMP of 218.24 ± 2.70 for mangiferin, 3.62 ± 0.03 for isomangiferin, 9963.88 ± 36.94 for quercetin 3-O-galactoside, 5953.20 ± 18.28 for quercetin 3-O-glucoside, 1550.29 ± 6.31 for quercetin 3-O-xyloside, 480.80 ± 4.38 for quercetin and 77.20 ± 2.42 for kaempferol. The chromatographic profile was similar to the one reported in literature, as characteristic of mango peel, with differences in the quantity and presence of compounds: Berardini et al. (2005a) reported the phenolic composition of mango peels (mg/kg DMP) to be in the range 11.2-1297 for mangiferin, 76.5-1467.7 for quercetin 3-O-galactoside, 77.4-1045.3 for quercetin 3-O-glucoside, 10.2-278.6 for quercetin 3-O-

xyloside and 1.7-19.3 for quercetin without detection of kaempferol; however, only 35% percent of the total phenolics were recovered in the proposed process (Berardini et al., 2005b). The differences in phenolic content with those reported in literature can be related to variations on the cultivar, processing and conservation of by-product samples.

The results obtained in this work were recalculated and expressed on the basis of the dry unprocessed extract as showed in Table 2. In SAE experiments, it was possible to recover between 72 and 85 % of inlet phenolic compounds. It can be observed that temperature and pressure had different effect on polyphenols. Since mangiferin is the main polyphenolic compound in mango peel, the effect of CO_2 density will be described for this compound assuming that, because of the similar solubility in SC-CO_2 (ref), all the other ones showed the same behavior. When the temperature of the antisolvent was increased from 35 to 45°C, for all pressures, the best results were observed at 10 MPa with a recovery of 94.5 to 94.8% (w/w) for mangiferin. For a fixed temperature, an increase in pressure from 8 to 15 MPa showed a higher recovery of mangiferin at 10 MPa. In every case, the experiments performed at 15 MPa of pressure leaded to the lower recovery of polyphenols, these results corresponded to the highest density of CO_2 and can be related to the lower antisolvent effect of SC-CO_2 at these operating conditions.

Table 1. Recovery of phenolic compounds in SAE precipitates

N	Pressure	Temperature	Antisolvent density	extract precipitated /total extract	Phenolic compounds[a] % w/w							
	MPa	°C	kg/m^3	% (w/w)	Mangiferin	Isomangiferin	Q-3-O-galactoside	Q-3-O-glucoside[b]	Q-3-O-xyloside[b]	Q-3-O-arabinoside[b]	Quercetin	Kaempferol
1	8	45	242.2	71.6	92.4 ± 0.3	90.6 ± 0.2	79.3 ± 0.1	88.1 ± 0.5	84.9 ± 0.5	89.6 ± 0.2	79.3 ± 0.1	98.8 ± 0.7
2	8	40	282.3	67.6	92.4 ± 1.6	88.9 ± 1.2	78.0 ± 1.2	87.5 ± 1.5	83.5 ± 1.4	91.0 ± 1.9	86.0 ± 0.8	95.7 ± 1.3
3	8	35	431.6	67.8	90.9 ± 0.4	87.0 ± 0.3	76.3 ± 0.1	84.3 ± 0.1	82.1 ± 0.1	87.1 ± 0.1	70.9 ± 0.5	97.9 ± 0.6
4	10	45	496.6	71.0	94.5 ± 1.6	90.3 ± 1.2	81.0 ± 1.9	88.5 ± 1.8	86.9 ± 1.9	92.2 ± 1.6	76.4 ± 1.2	96.4 ± 0.5
5	10	40	622.6	74.3	94.8 ± 0.7	92.2 ± 0.5	80.8 ± 0.1	89.0 ± 0.2	86.6 ± 0.1	91.7 ± 0.2	77.6 ± 1.0	97.0 ± 10.1
6	10	35	710.8	71.6	94.5 ± 0.4	89.2 ± 0.8	79.7 ± 0.3	87.4 ± 0.5	86.4 ± 0.2	92.2 ± 0.1	79.5 ± 2.5	98.2 ± 0.7
7	15	45	741.2	64.8	79.7 ± 2.2	77.5 ± 1.6	68.6 ± 1.4	76.3 ± 1.6	72.8 ± 1.4	78.3 ± 1.3	75.1 ± 1.1	86.3 ± 2.1
8	15	40	780.1	63.7	85.4 ± 2.0	80.7 ± 1.5	72.2 ± 1.4	79.6 ± 1.6	78.1 ± 1.6	83.5 ± 2.0	70.7 ± 1.8	89.4 ± 2.1
9	15	35	815.3	66.3	88.1 ± 0.3	83.2 ± 0.2	73.8 ± 0.1	81.0 ± 0.1	79.8 ± 0.1	85.8 ± 0.6	70.6 ± 0.3	93.7 ± 0.5

a. % w/w compounds on the precipitated product/compounds in the unprocessed extract

b. quantified as Q-3-O-galactoside equivalent

The antisolvent effect of SC-CO_2 depends on the capacity to dissolve the liquid solvent, in this case ethanol, and is related to the density of SC-CO_2. As can be observed in the results (Table 1), when density of SC-CO_2 increased until 710 kg/m^3, the recovery of mangiferin in the precipitated powder also increased, at higher densities the recovery decreased.

The highest recovery of polyphenol compounds was obtained at 10 MPa and 40°C, as showed in Table 2, where the amount of polyphenols (expressed as mg/Kg of DM) in the different fractions recovered in the SAE process, that is the powder on the precipitator and the ethanolic solution on the separator, are reported. The values are expressed as mg of polyphenol compound by kg of dry extract. Nevertheless, the process allows recovery almost all polyphenolic compounds; in all experiments SC-CO_2 has shown to be effective for precipitating a dried powder rich in antioxidant compounds, as microparticles or agglomerates.

Table 2. Content (mg/kg of DM) of phenolic compounds of the Inlet solution, SAE (40°C, 10 MPa) precipitated and residue products

Phenolic compounds, as mg/kg DM	Ethyl alcohol extract	SAE precipitated	SAE condensed
Mangiferin	218.2 ± 2.70	206.8 ± 1.58	10.9 ± 0.14
Iso Mangiferin	3.6 ± 0.03	3.3 ± 0.02	0.2 ± 0.00
Quercetin 3-O-galactoside	9963.9 ± 36.94	8857.4 ± 0.50	498.2 ± 1.85
Quercetin 3-O-glucoside*	5953.2 ± 18.24	5295.6 ± 9.27	476.3 ± 1.46
Quercetin 3-O-xyloside*	1550.3 ± 6.31	1343.1 ± 1.22	111.6 ± 0.45
Quercetin 3-O-arabinoside*	737.7 ± 5.19	676.3 ± 1.22	50.2 ± 0.35
Quercetin	480.8 ± 4.38	373.3 ± 4.59	43.3 ± 0.39
Kaempferol	77.2 ± 2.42	74.9 ± 7.81	1.5 ± 0.05
Total	18984.9	16830.8	1192.2

* Calculated as Quercetin 3-O-galactoside equivalent

3.5 Particle size distribution

SEM analyses showed that the fraction collected in the precipitator was formed by micrometric particles. Figure 3 shows that at lower density of SC-CO_2 (420 kg/m^3) two different morphologies were obtained: sub-microparticles in the upper walls and agglomerates on the lower part of the precipitator. It can be hypothesized that the precipitation occurred in two phases, a supercritical phase on the upper side of the

precipitator that produced spherical microparticles, while a film like precipitated (agglomerates) was obtained in a liquid phase at the bottom of precipitator. Similar behavior was observed for SAS processing of a low molecular weight inulin (Reverchon et al., 2000).

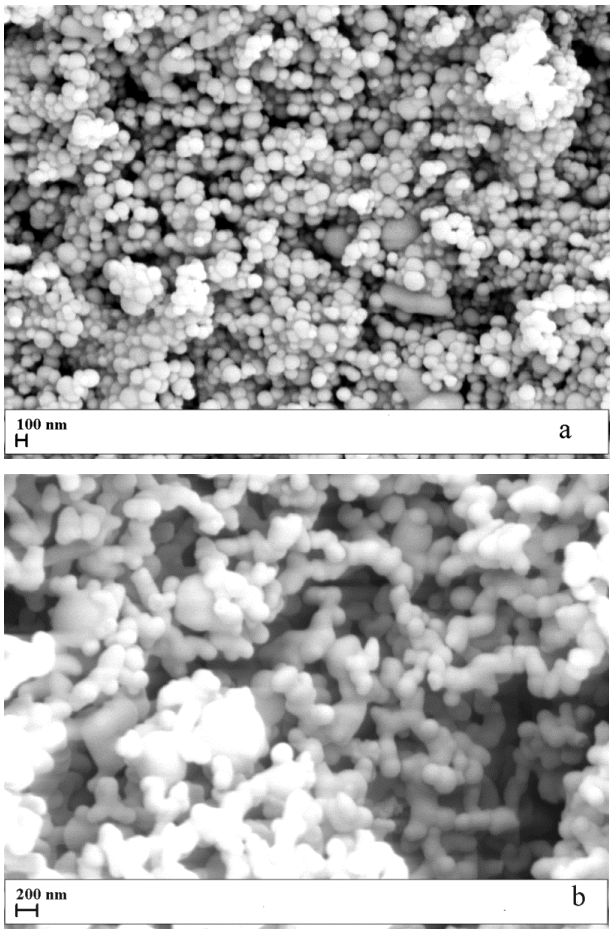


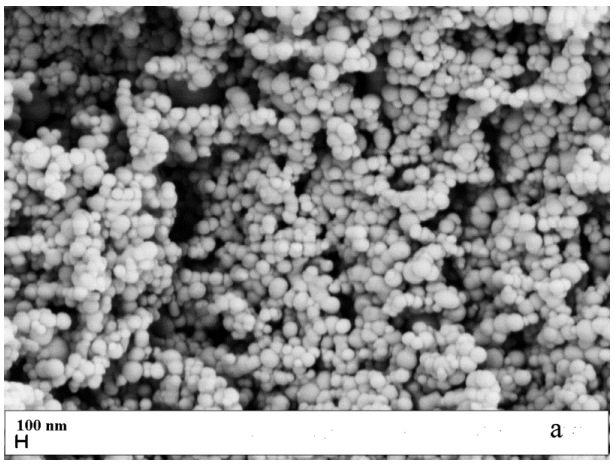
Figure 3. SEM images show a) microparticles (8MPa, 35°C) and b) agglomerates (8 MPa, 45 °C) in the upper and bottom of SAE precipitator

When pressure was increased from 8 to 15 MPa at a fixed temperature of 35°C a change in the overall behavior was observed. When the pressure was 15 MPa, only microparticles were obtained (Figure 4) at all the SC-CO_2 densities tested. The effect of SC-CO_2 as antisolvent is evident in the case of mango polyphenols: for densities higher

Commento [RA1]: Sub-micro o nano?

than 700 kg/m^3 it is possible work in a complete supercritical region obtaining spherical microparticles.

The approach based on pseudo-binary diagrams for the discussion of the results obtained by SAE neglects equilibrium involving a third component, assuming that the equilibrium is not influenced by the presence of the solid compound. The solubility behavior of the ternary system ethanol-CO_2-solute can be different than a binary system ethanol-CO_2 or solute-CO_2, going from co-solvent to antisolvent behavior depending on the conditions of CO_2 (De Gioannis et al., 2004). This behavior can be emphasized if the solid involved is composed by several families of compounds. The modification of VLE is usually noted in case of solutes that have a strong interaction with ethanol. The simplest modification of the VLE in the case of ternary non-cosolvency (antisolvent) systems, with respect to the corresponding binary system, consists in the increase of the pressure at which the ternary mixture becomes supercritical (Reverchon and De Marco, 2004). This fact explains the two morphologies observed for the particles obtained at lower pressures and therefore, in this work it can be hypothesized that polyphenolic compounds from mango by-products strongly influence the VLEs. Thus, in the selection of operating conditions for SAE, it is advisable to select an operating pressure far from the MCP pressure of the binary system, to avoid the risk of working at subcritical conditions.



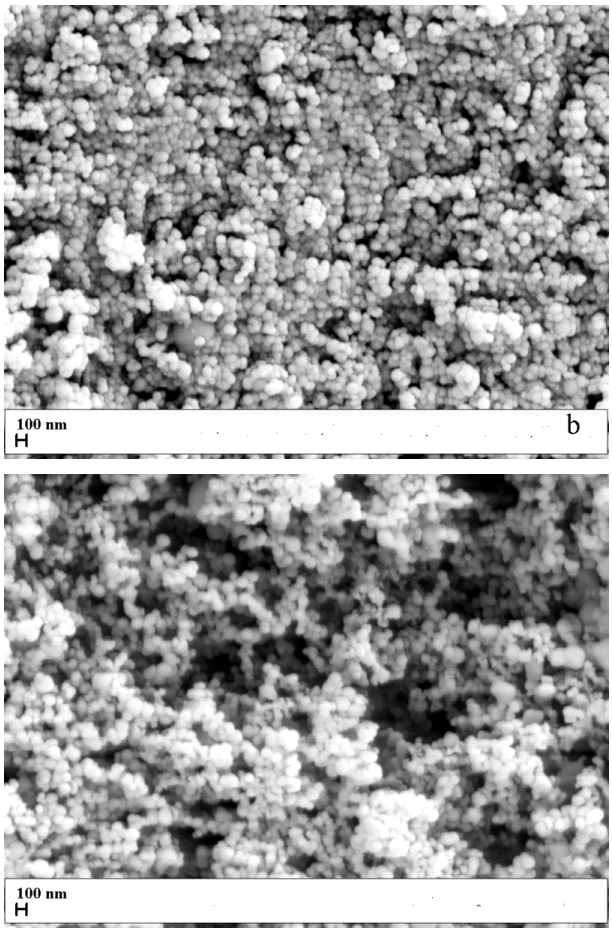


Figure 4. SEM photomicrographs of mango products precipitated at 15 MPa at (a) 35°C, (b) 40°C and (c) 45°C.

Figure 5 shows the particle size distribution (PSD) of the micronized powder at 10 MPa and different temperatures. It is possible to observe that the PSDs are similar: they have the mean diameters at 100-150 nm and all the particles are smaller than 400nm, suggesting that the precipitation took place at supercritical conditions. In correspondence to the highest recovery of mangiferin and total polyphenol content (10 MPa and 40°C), the precipitated material was formed by nanometric particles with a mean diameter of 143 ± 40 nm. In the precipitation of antioxidant compounds the operating conditions of SAE should take into account the density of SC-CO_2 as main parameter, always related to the temperature to avoid thermal damage.

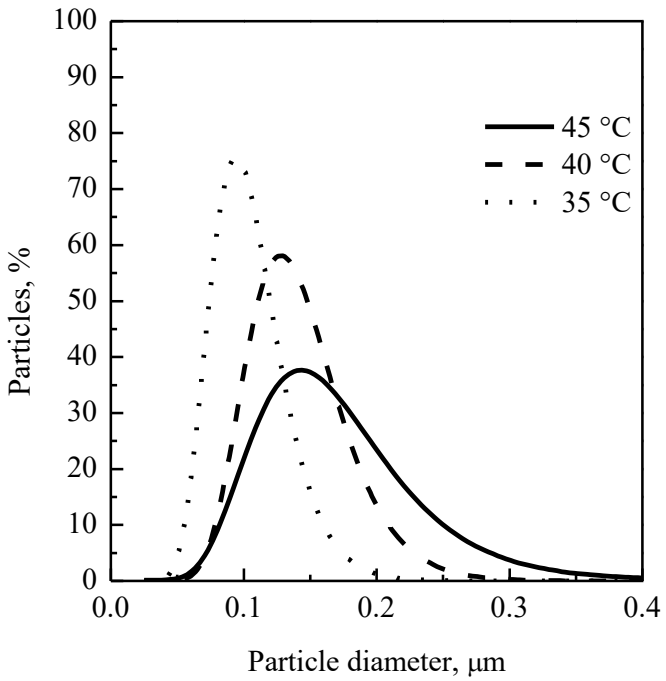


Figure 5. PSD in terms of frequency of number of particles of precipitated powder at 10 MPa

3.6 Antioxidant Activity

The polyphenols are reported to have low solubility in water systems (Mallick et al., 2007), as occurs for mangiferin (Han et al., 2010) and for kaempferol (Barve et al., 2009) with 1.2 and 2% respectively. The radical scavenging activity (RSA) of unprocessed and SAE processed mango extract was measured in ethanol and aqueous solution as shown in Figure 6. For each sample, seven concentrations were tested from 0 to 1 mg/mL. The RSA showed high dependence on the solvent used for dilution of unprocessed and processed extracts. It was observed that the RSA in aqueous solution for DPPH assay is higher for processed than for unprocessed mango peel extract.

In Table 3 are reported the antioxidant activities expressed as trolox equivalent for unprocessed extract and for SAE precipitated products. The variations in trolox equivalent of SAE processed extracts compared with unprocessed extract correspond to the recovery percentage of phenolic compounds in the SAE precipitated material as

shown in Table 1. Moreover, the extraction/adsorption process has been effective in the concentration of phenolic compounds with the relative antioxidant activity (from 851.9 to 883.8 µMol TE/g) if compared with literature. For example, Martinez et al. (2012) reported the antioxidant activity of a mango peel powder to be 47.1 µMol TE/g.

Table 3. Antioxidant activity as trolox equivalent and IC_{50} for unprocessed and processed products

N experiment	Pressure	Temperature	Antioxidant activity	Mean particle size	IC_{50} (mg/mL)	
	bar	°C	µMol TE/g dP*	µm	physiologic solution	ethanolic solution
Unprocessed extract	-	-	883.8 ± 1.6	-	0.59	0.04
1	8	45	858.5 ± 0.5	0.227 ± 0.10	0.10	0.04
2	8	40	863.3 ± 0.8	0.176 ± 0.05	0.10	0.05
3	8	35	857.6 ± 0.9	0.124 ± 0.03	0.08	0.05
4	10	45	868.1 ± 0.8	0.181 ± 0.09	0.11	0.04
5	10	40	851.9 ± 1.6	0.143 ± 0.04	0.09	0.05
6	10	35	864.0 ± 0.8	0.101 ± 0.03	0.11	0.05
7	15	45	869.8 ± 0.8	0.107 ± 0.02	0.09	0.07
8	15	40	867.1 ± 1.0	0.090 ± 0.02	0.09	0.05
9	15	35	861.8 ± 0.4	0.081 ± 0.02	0.09	0.05

* µMol TE/ g dP: micro mol Trolox equivalent per gram of dry precipitated product

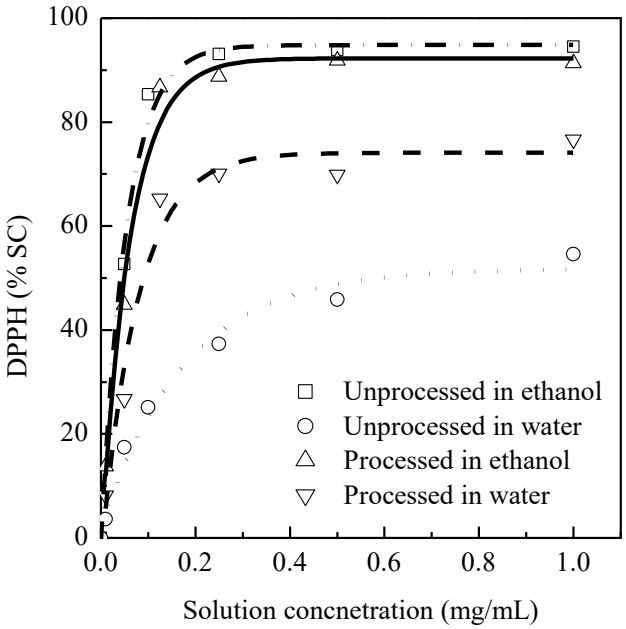


Figure 6. Radical scavenging activity of SAE processed mango extract obtained at 40°C and 15 MPa.

The IC_{50} values of RSA were 0.04 mg/mL for unprocessed mango extract and between 0.04 and 0.07 mg/mL for processed mango extract dissolved in ethanol; these similar results showed the preservation of antioxidant power during SAE processing, due to the inert environment in the precipitator that avoids the degradation of compounds sensible to oxygen and light and also to the moderate conditions of temperature. Taking into account that the human body is an aqueous system, the DPPH assay was performed in a physiological solution to simulate the behavior of antioxidant powders in a living system. The IC_{50} values found in the aqueous system were 0.59 mg/mL for unprocessed and between 0.08 and 0.11 mg/mL for processed mango extract; a lower IC_{50} means a higher free radical-scavenging activity of the compounds dissolved. The enhancement of antioxidant power showed for processed products is probably due to the faster dissolution rate in water of micronized phenolic compounds because of the reduced PSD compared to the unprocessed material.

It is not unusual that a decrease of particle size improves the RSA, for example it has been observed for nanoparticles of kaempferol (IC_{50} 0.015 mg/mL) (Tzeng et al., 2011), quercetin (0.004 mg/mL) (Wu et al., 2008), curcumin (IC_{50} 0.018 mg/mL) (Yen et al., 2010) and resveratrol (Lee et al., 2012).

If compared with other results reported in literature for IC_{50}, the antioxidant micronized in this work showed higher RSA than extracts of mango leaves 0.17 mg/mL and 0.49 mg/mL in ethanol and water solution (Ling et al., 2009), also higher with respect to those reported by Ribeiro et al. (2008) for mango peel with IC_{50} of 2 mg/mL and for gallic acid and BHA with 18.8 and 7.70 % of RSA at 0.1 mg/mL.

Therefore, the micronization of mango natural extracts allows to enhance the dissolution rate in physiological solution. The epidemiological evidence suggests a considerable potential of phenolic compounds from mango peels to be developed as functional ingredients and the increase on solubility could permit the use in pharmaceutical or nutraceutical applications.

4. Conclusions

The supercritical antisolvent extraction technique is effective for the elimination of organic solvent from mango ethanolic extract and for the recovery of antioxidant compounds as micronized powder without affecting the polyphenol content and the related antioxidant activity. The precipitated product contains about 90% of the total phenolic compounds and the antioxidant activity has an IC_{50} of 0.11 mg/mL, five times the unprocessed extract (IC_{50} of 0.59 mg/mL), because the nanometric size of precipitates improves the dissolution rate of mango extract in aqueous solution. Moreover, SAE process is environmental friendly and can be used for processing of food ingredients. The by-products of mango industry are an invaluable source of polyphenols and by the process proposed in this work they can be recovered as concentrated dry powder with an increase of their bioavailability. The content of antioxidants in SAE precipitated from mango peels is considerable and the RSA related is also high, showing better results compared to literature. These data put the bases for extensive studies on the utilization of micronized polyphenols from mango by-products and their application for nutraceutical and functional products.

Acknowledgements

The authors would like to thank Agroficial for providing the mango by-products. M.A.M. acknowledges the doctoral scholarship from SENESCYT, Ecuador.

References

Ajila, C.M., Bhat, S.G., Rao, U.J.S.P., (2007). Valuable components of raw and ripe peels from two Indian mango varieties. Food Chemistry 102(4), 1006-1011.

Ajila, C.M., Rao, L.J., Rao, U.J.S.P., (2010). Characterization of bioactive compounds from raw and ripe Mangifera indica L. peel extracts. Food and Chemical Toxicology 48(12), 3406-3411.

Aruoma, O., (2003). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutation Research. Fundamental and molecular mechanisms of mutagenesis 523-524(February - March 2003), 9-20.

Barve, A., Chen, C., Hebbar, V., Desiderio, J., Saw, C.L.L., Kong, A.N., (2009). Metabolism, Oral Bioavailability and Pharmacokinetics of Chemopreventive Kaempferol in Rats. Biopharmaceutics & Drug Disposition 30(7), 356-365.

Berardini, N., Carle, R., Schieber, A., (2004). Characterization of gallotannins and benzophenone derivatives from mango (Mangifera indica L. cv. 'Tommy Atkins') peels, pulp and kernels by high-performance liquid chromatography/electrospray ionization mass spectrometry. Rapid Communications in Mass Spectrometry 18(19), 2208-2216.

Berardini, N., Fezer, R., Conrad, J., Beifuss, U., Carle, R., Schieber, A., (2005a). Screening of mango (Mangifera indica L.) cultivars for their contents of flavonol O- and xanthone C-glycosides, anthocyanins, and pectin. Journal of Agricultural and Food Chemistry 53(5), 1563-1570.

Berardini, N., Knodler, M., Schieber, A., Carle, R., (2005b). Utilization of mango peels as a source of pectin and polyphenolics. Innovative Food Science & Emerging Technologies 6(4), 442-452.

Brand-Williams, W., Cuvelier, M.E., Berset, C., (1995). Use of a free radical method to evaluate antioxidant activity. LWT - Food Science and Technology 28(1), 25-30.

Chang, C.J., Day, C.Y., Ko, C.M., Chiu, K.L., (1997). Densities and P-x-y diagrams for carbon dioxide dissolution in methanol, ethanol, and acetone mixtures. Fluid Phase Equilibria 131(1-2), 243-258.

De Gioannis, B., Vega-Gonzalez, A., Subra, P., (2004). Anti-solvent and co-solvent effect of CO2 on the solubility of griseofulvin in acetone and ethanol solutions. Journal of Supercritical Fluids 29(1-2), 49-57.

Dorta, E., Lobo, M.G., Gonzalez, M., (2012). Reutilization of Mango Byproducts: Study of the Effect of Extraction Solvent and Temperature on Their Antioxidant Properties. Journal of Food Science 77(1), C80-C88.

Floris, T., Filippino, G., Scrugli, S., Pinna, M.B., Argiolas, F., Argiolas, A., Murru, M., Reverchon, E., (2010). Antioxidant compounds recovery from grape residues by a supercritical antisolvent assisted process. The Journal of Supercritical Fluids 54(2), 165-170.

Han, D.D., Chen, C.J., Zhang, C., Zhang, Y., Tang, X., (2010). Determination of mangiferin in rat plasma by liquid-liquid extraction with UPLC-MS/MS. Journal of Pharmaceutical and Biomedical Analysis 51(1), 260-263.

Hou, S.Y., Wang, F., Li, Y.M., Li, Y., Wang, M.Q., Sun, D.J., Sun, C.H., (2012). Pharmacokinetic study of mangiferin in human plasma after oral administration. Food Chemistry 132(1), 289-294.

Joung, S.N., Yoo, C.W., Shin, H.Y., Kim, S.Y., Yoo, K.-P., Lee, C.S., Huh, W.S., (2001). Measurements and correlation of high-pressure VLE of binary CO2–alcohol systems (methanol, ethanol, 2-methoxyethanol and 2-ethoxyethanol). Fluid Phase Equilibria 185(1–2), 219-230.

Khan, R.S., Grigor, J., Winger, R., Win, A., (2012). Functional food product development – Opportunities and challenges for food manufacturers. Trends in Food Science & Technology In Press(0).

Larrauri, J.A., Rupérez, P., Borroto, B., Saura-Calixto, F., (1996). Mango Peels as a New Tropical Fibre: Preparation and Characterization. LWT - Food Science and Technology 29(8), 729-733.

Lee, C.W., Yen, F.L., Huang, H.W., Wu, T.H., Ko, H.H., Tzeng, W.S., Lin, C.C., (2012). Resveratrol nanoparticle system improves dissolution properties and enhances the hepatoprotective effect of resveratrol through antioxidant and anti-inflammatory pathways. J Agric Food Chem 60(18), 4662-4671.

Ling, L.T., Yap, S.-A., Radhakrishnan, A.K., Subramaniam, T., Cheng, H.M., Palanisamy, U.D., (2009). Standardised Mangifera indica extract is an ideal antioxidant. Food Chemistry 113(4), 1154-1159.

Makare, N., Bodhankar, S., Rangari, V., (2001). Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice. Journal of Ethno-Pharmacology 78(2-3).

Mallick, S., Pattnaik, S., Swain, K., (2007). Current perspectives of solubilization: Potential for improved bioavailability. Drug Development and Industrial Pharmacy 33(8), 865-873.

Martín, L., González-Coloma, A., Adami, R., Scognamiglio, M., Reverchon, E., Porta, G.D., Urieta, J.S., Mainar, A.M., (2011). Supercritical antisolvent fractionation of ryanodol from Persea indica. The Journal of Supercritical Fluids 60(0), 16-20.

Martinez, R., Torres, P., Meneses, M.A., Figueroa, J.G., Perez-Alvarez, J.A., Viuda-Martos, M., (2012). Chemical, technological and in vitro antioxidant properties of mango, guava, pineapple and passion fruit dietary fibre concentrate. Food Chemistry 135(3), 1520-1526.

Reverchon, E., (1999). Supercritical antisolvent precipitation of micro- and nano-particles. The Journal of Supercritical Fluids 15(1), 1-21.

Reverchon, E., Adami, R., Caputo, G., De Marco, I., (2008). Spherical microparticles production by supercritical antisolvent precipitation: Interpretation of results. The Journal of Supercritical Fluids 47(1), 70-84.

Reverchon, E., De Marco, I., (2004). Supercritical antisolvent micronization of Cefonicid: thermodynamic interpretation of results. The Journal of Supercritical Fluids 31(2), 207-215.

Reverchon, E., De Marco, I., (2006). Supercritical fluid extraction and fractionation of natural matter. The Journal of Supercritical Fluids 38(2), 146-166.

Reverchon, E., Della Porta, G., De Rosa, I., Subra, P., Letourneur, D., (2000). Supercritical antisolvent micronization of some biopolymers. The Journal of Supercritical Fluids 18(3), 239-245.

Ribeiro, S.M.R., Barbosa, L.C.A., Queiroz, J.H., Knödler, M., Schieber, A., (2008). Phenolic compounds and antioxidant capacity of Brazilian mango (Mangifera indica L.) varieties. Food Chemistry 110(3), 620-626.

Ribeiro, S.M.R., Queiroz, J., Lopes Ribeiro de Queiroz, M., Campos, F., Pinheiro Sant'Ana, H., (2007). Antioxidant in Mango (Mangifera indica L.) Pulp. Plant Foods for Human Nutrition 62(1), 13-17.

Sahoo, N.G., Kakran, M., Shaal, L.A., Li, L., Muller, R.H., Pal, M., Tan, L.P., (2011). Preparation and characterization of quercetin nanocrystals. Journal of pharmaceutical sciences 100(6), 2379 - 2390.

Schieber, A., Berardini, N., Carle, R., (2003a). Identification of flavonol and xanthone glycosides from mango (Mangifera indica L. cv. "Tommy Atkins") peels by high-performance liquid chromatography-electrospray ionization mass spectrometry. Journal of Agricultural and Food Chemistry 51(17), 5006-5011.

Schieber, A., Hilt, P., Streker, P., Endreß, H.-U., Rentschler, C., Carle, R., (2003b). A new process for the combined recovery of pectin and phenolic compounds from apple pomace. Innovative Food Science & Emerging Technologies 4(1), 99-107.

Sosa, M.V., Rodríguez-Rojo, S., Mattea, F., Cismondi, M., Cocero, M., (2011). Green tea encapsulation by means of high pressure antisolvent coprecipitation. The Journal of Supercritical Fluids 56(3), 304-311.

Soto, M.L., Moure, A., Dominguez, H., Parajo, J.C., (2011). Recovery, concentration and purification of phenolic compounds by adsorption: A review. Journal of Food Engineering 105(1), 1-27.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., Byrne, D.H., (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of Food Composition and Analysis 19(6-7), 669-675.

Tzeng, C.W., Yen, F.L., Wu, T.H., Ko, H.H., Lee, C.W., Tzeng, W.S., Lin, C.C., (2011). Enhancement of Dissolution and Antioxidant Activity of Kaempferol Using a Nanoparticle Engineering Process. Journal of Agricultural and Food Chemistry 59(9), 5073-5080.

Van Duyn, M.A.S., Pivonka, E., (2000). Overview of the Health Benefits of Fruit and Vegetable Consumption for the Dietetics Professional: Selected Literature. Journal of the American Dietetic Association 100(12), 1511-1521.

Visentin, A., Rodríguez-Rojo, S., Navarrete, A., Maestri, D., Cocero, M., (2012). Precipitation and encapsulation of rosemary antioxidants by supercritical antisolvent process. Journal of Food Engineering 109(1), 9-15.

Wijngaard, H., Hossain, M.B., Rai, D.K., Brunton, N., (2012). Techniques to extract bioactive compounds from food by-products of plant origin. Food Research International 46(2), 505-513.

Wu, T.-H., Yen, F.-L., Lin, L.-T., Tsai, T.-R., Lin, C.-C., Cham, T.-M., (2008). Preparation, physicochemical characterization, and antioxidant effects of quercetin nanoparticles. International Journal of Pharmaceutics 346(1–2), 160-168.

Yang, L., Huang, J.-m., Zu, Y.-g., Ma, C.-h., Wang, H., Sun, X.-w., Sun, Z., (2011). Preparation and radical scavenging activities of polymeric procyanidins nanoparticles by a supercritical antisolvent (SAS) process. Food Chemistry 128(4), 1152-1159.

Yen, F.-L., Wu, T.-H., Tzeng, C.-W., Lin, L.-T., Lin, C.-C., (2010). Curcumin Nanoparticles Improve the Physicochemical Properties of Curcumin and Effectively Enhance Its Antioxidant and Antihepatoma Activities. Journal of Agricultural and Food Chemistry 58(12), 7376-7382.