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Isolation, Characterization, and Emulsifying Properties of Wattle Seed (*Acacia victoriae* Bentham) Extracts

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Extracts from either ground whole wattle seeds or uncoated cotyledons were obtained using water, alkali, or ethanol. These extracts were then analyzed for their protein molecular weight and electrophoretic profiles using sodium dodecyl sulfate−polyacrylamide gel electrophoresis and capillary electrophoresis, respectively. Water extracts and those from the cotyledons gave higher material yield and contained significantly more proteins than other extracts. Furthermore, the proteins ranged in molecular weight from 6 to 92 kDa, with the highest concentration between 27 and 61 kDa. Water extracts, even at very low protein concentrations (0.17−1.12%), formed stable emulsions, containing up to 50% canola oil, and these emulsions were affected by pH (4−9), ionic strength (0.25−1% NaCl), and retorting (115 °C for 30 min). The study showed that water-soluble wattle extracts have great potential as emulsifiers and stabilizers for the food industry, especially at low pH levels.

KEYWORDS: Wattle seed; Australian bushfoods; protein isolation; oil-in-water emulsions

INTRODUCTION

The wattle seed (*Acacia* spp.) is one of the major products from Australian native plants with significant economic potential because of its extensive availability from both soil cultivation and wild harvest (*1*). It is currently available commercially as a roasted product with a nutty, coffee, or chocolate aroma. This roasted product is used mainly as a food ingredient in beverages (used extensively as a coffee analogue) and sometimes as a flavoring agent in ice cream and dairy products (*2*).

Wattle seeds have a very hard and thick coat. Analysis of nonroasted seeds from several Acacia varieties (*3*) has, however, shown that the seeds are very rich in nutrients, such as proteins (∼20%), carbohydrates (∼35%), fiber (∼28%), and lipids (5−15%). Of these components, proteins are the most versatile of all because of the many functional properties to which they can be applied in foods (*4*). Proteins are known to have varying solubility in different solvents, and this has been a basis for their classification (*5*). Solubility in water is especially known to be very important in the application of proteins in food systems (*6*). However, to further study these wattle seed proteins, they must be isolated (e.g., by solubility in different solvents), and characterized, for example, by measuring their molecular-weight profiles and thermal properties.

Anecdotal evidence, from mainly processors of Australian native foods, including wattle seed, suggests that the wattle seed has very good emulsifying and foam stabilizing ability, which will make it very suitable in food formulations, such as desserts and dressings. In these products, the well-known flavor-enhancing abilities of the wattle seed would be expected to be complemented by its technological functionality. It has also been suggested that the seeds have a very high amount of complex carbohydrates (dietary fiber), which may confer a very low glycaemic index to products made from them, and that the fatty acid profile shows more unsaturated than saturated fatty acids, a potential for incorporation as healthy adjuncts into common foods, such as baked goods (*7*). Some of the polysaccharides from Australian Acacia gums have also been confirmed to have similar properties to the gum Arabic, a major hydrocolloid used as a thickener and stabilizer in the food industry (*8*). Unfortunately, these functional properties have yet to be systematically established, especially in terms of particular components responsible for different functional properties, and neither has the influence of processing conditions on these properties. In this study, therefore, we report on the isolation of proteins, using different solvents, from whole wattle seeds as well as from the uncoated cotyledons only. We also report on the molecular-weight profiles of the protein extracts and the application of the water extracts in the manufacture of oil-in-water emulsions under varying conditions of pH, ionic strength, and heating.

MATERIALS AND METHODS

Materials. Whole wattle seeds (*Acacia victoriae* Bentham) were supplied by Outback Bushfoods, Alice Springs, Australia. Canola oil was purchased from the local Woolworths supermarket. All other reagents and chemicals were supplied by Sigma-Aldrich, Castle Hill, NSW, Australia.

Extraction of Wattle Seed Flours. Whole wattle seeds or the dehulled cotyledons (uncoated cotyledons) were milled with a ZM 100 ultracentrifugal mill (Retsch GmbH, Haan, Germany) separately to pass through a 0.11 mm mesh and then extracted sequentially with distilled

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water, 0.1 M NaOH solution, and 70% ethanol. Each flour was extracted with 10 times the volume of the solvent for 3 times, stirring for 1 h each time before centrifuging at 3000*g* to separate the residue from the soluble portion (supernatant). The supernatants for each extracting solvent were then pooled, and each fraction was freeze-dried using a Christ-Alpha 1-4 freeze dryer (Biotech International, Germany). However, prior to freeze drying, the alkali fraction was further purified by isoelectric precipitation using 1 M HCl at pH 3.85 and washing with distilled water.

Characterization of Wattle Seed Extracts. The extracts were characterized by yield, proximate analysis, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), and capillary electrophoresis (CE) profiles. The total yield was obtained by weighing the freeze-dried isolates, while the proximate analysis was determined by standard Association of Official Analytical Chemists (AOAC) (*9*) methods. SDS−PAGE analysis of the protein isolates was determined by using the Laemmli buffer system (*10*) and running the samples on a precast gel (4−20% gradient) with a Bio-Rad Mini PROTEAN 3 system (Bio-Rad Laboratories, Hercules, CA). The bands were stained with Coomassie brilliant blue R-250. Molecular-weight markers ranging from 6.5 to 205 kDa were also run on the same gel, and the results were used to estimate the molecular-weight profile of the protein bands from the stained gels using the Genetools software (Syngene, Inc., Frederick, MD). The CE profile was determined on a Beckman P/ACE system 5510 (Beckman Coulter, Inc., Fullerton, CA) according to the method of Basha (*11*). The electrophoresis was conducted at 25 °C using an uncoated fused-silica capillary of 75 μm in internal diameter and 57 cm in total length. A total of 10 mg of each freeze-dried extract was dissolved in 1 mL of 0.01 M sodium phosphate buffer (pH 8.3) and injected by pressure for 30 s. Each separation run was for 10 min at 25 kV of voltage in 0.3% sodium tetraborate buffer, while the detector was set at 214 nm. The capillary was rinsed sequentially between successive electrophoretic runs with 0.1 M NaOH (2 min), 1 M HCl (1 min), distilled water (2 min), and the run buffer (2 min). Data were processed with the P/ACE system 5000 series software.

Emulsifying Properties of Wattle Seed Extracts. A typical oil-in-water emulsion was prepared using canola oil as the dispersed phase and an appropriate amount of supernatant of whole wattle seed extract in 0.01 M phosphate buffer (pH adjusted by either 1 M NaOH or 1 M HCl) as the continuous phase. After the oil was mixed with the volume of protein solution to give the appropriate oil contents of 20, 50, and 80% (v/v), respectively, in the final emulsion, the coarse emulsion was passed at no pressure through a two-stage high-pressure homogenizer (Niro Soavi, Parma, Italy) to form a pre-emulsion. Then, using a constant first-stage pressure of 14.3 MPa, the pressure was increased to 28.6 MPa using the second-stage control valve, after which the sample was collected. Each emulsion was passed 3 times through the homogenizer, to ensure complete dispersion of the oil. On the basis of measured values of solids and protein contents of the wattle seed extract, the 20, 50, and 80% oil-in-water emulsions contained 1.1, 0.43, and 0.17% (w/v) protein contents, respectively.

The effect of salt on the emulsions was studied in the 20 and 50% oil-in-water emulsions prepared at pH values of 4 and 5 by adding the appropriate amounts of NaCl to the water (buffer) phase to obtain 0.25, 0.5, and 1% (w/v) concentrations in the final emulsion. The 20% emulsions were also sterilized at 115 °C for 30 min in a vertical retort to study their stability to heat.

Table 1. Proximate Analysis of the Wattle Seed[a]

component	whole seed (%)	cotyledons (%)
water	2.13 ± 0.01	2.54 ± 0.01
protein (g N × 6.5)	18.20 ± 0.03	34.45 ± 0.12
fat	5.40 ± 0.02	10.31 ± 0.04
neutral detergent fiber	32.10 ± 0.23	16.76 ± 0.15
ash	3.70 ± 0.02	2.80 ± 0.02
carbohydrates (by difference)	38.47 ± 0.38	33.14 ± 0.32
water-soluble carbohydrates	5.81 ± 0.02	11.03 ± 0.05

[a] Data are means of three replicates with standard deviations. Results show significant differences ($p < 0.05$) between the components from each sample.

Characterization of Emulsions. The effect of the storage temperature on all emulsions was studied by dividing the emulsions into two groups and storing at either the refrigeration temperature (5 °C) or in an incubator at 25 °C for up to 7 days. Each preparation also had 0.05% (w/v) sodium azide added to prevent microbial contamination. Every day, the emulsions were visually examined for signs of creaming, oiling off, or other physical separation attributes. Creaming was indicated if there was any change in turbidity between the top and lower layers of the emulsions, while oiling off relates to the presence of any free oil on the surface of the emulsion stored in a test tube (*16*). Furthermore, the droplet-size distribution and, hence, the weight/volume average emulsion particle diameter (d_{43}), defined as $\sum N_i d_i^4 / \sum N_i d_i^3$, were measured by small-angle laser light scattering using a Mastersizer E (Malvern Instruments Ltd., Worcestershire, U.K.) (The terms N_i and d_i are defined as the number of particles in the ith class and the diameter of particles in the ith class, respectively). The presentation factor was 0303 (i.e., a refractive index of 1.414 and an absorption of 0.001), and a polydisperse model was chosen for the size distribution using a lens of 45 mm in focal length. Emulsion droplets were sized using distilled water as the dispersant (*12*).

Measurement of Viscosity. The viscosity of the wattle seed extract and the emulsions formed with each extract at different pH values was measured using the narrow gap rotational geometry of a Brookfield viscometer (Brookfield Engineering Laboratories, Middleboro, MA). These room-temperature measurements were made on the same day of extraction or emulsion preparation in triplicates, and average values were reported.

Statistical Analysis. All extractions and analyses were carried out at least in triplicates, and the means were reported. Data collected were subjected to analysis of variance, and means of treatments showing a significant difference ($p < 0.05$) were subjected to Fisher's least significant difference test.

RESULTS AND DISCUSSION

Extraction and Characterization of Proteins. The proximate analysis of both whole wattle seed and its dehulled counterpart was carried out. As shown in **Table 1**, whole wattle seed was higher in fiber, ash, and total carbohydrates than the cotyledon only. However, the cotyledon was significantly higher (almost double) in protein, fat, and water-soluble carbohydrates. The result for the whole seeds was in agreement with Brand et

Table 2. Recovery of Wattle Seed Extracts[a]

sample	extract	yield (g/100 g of sample)	protein content (%)	fiber content (wt %)	water-soluble carbohydrates (wt %)
whole seed	water	34.49 ± 0.31	26.00 ± 0.27	< 0.10	18.50 ± 0.14
	alkali	11.33 ± 0.10	52.65 ± 0.56	<0.10	ND
	ethanol	1.79 ± 0.04	13.00 ± 0.12	<0.10	10.71
	residue	49.74 ± 0.45	7.15 ± 0.07	59.58 ± 0.71	ND
cotyledons	water	67.77 ± 0.87	44.85 ± 0.40	<0.10	17.90 ± 0.15
	alkali	2.02 ± 0.01	41.60 ± 0.39	<0.10	ND
	ethanol	1.29 ± 0.01	5.85 ± 0.03	<0.10	9.89 ± 0.10
	residue	22.59 ± 0.30	2.60 ± 0.02	63.93 ± 0.91	ND

[a] Data are means of three replicates with standard deviations.

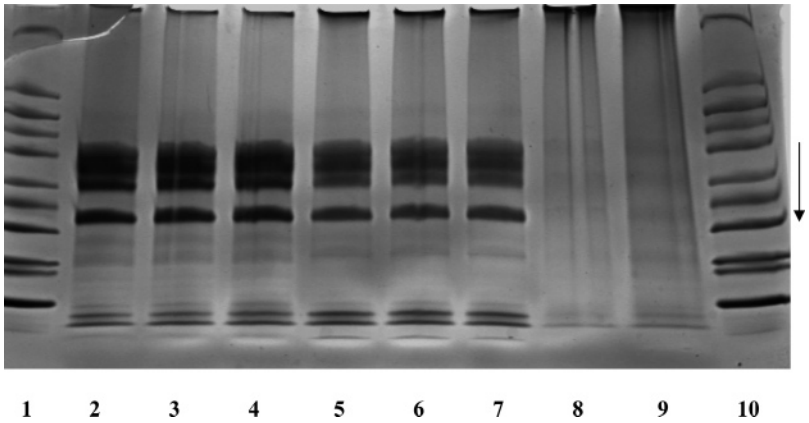

Figure 1. Molecular weight (MW) analysis by SDS–PAGE of protein extracts from wattle seed. Lanes 1 and 10, Sigma markers (MW from the top: 205.0, 116.0, 97.0, 84.0, 66.0, 55.0, 45.0, 29.0, 26.0, 24.0, 20.0, and 14.2 kDa); lanes 2 and 3, wattle seed powders from whole seed and uncoated cotyledons, respectively; lanes 4 and 5, water-soluble extracts from whole seed and uncoated cotyledons, respectively; lanes 6 and 7, alkali-soluble extracts from whole seed and uncoated cotyledons, respectively; lanes 8 and 9, ethanol-soluble extracts from whole seed and uncoated cotyledons, respectively. The arrow shows the direction of migration.

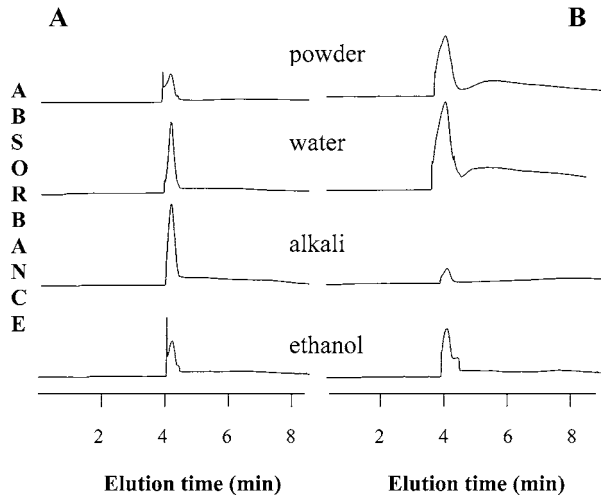

Figure 2. Capillary electropherograms of powders and extracts (as labeled) from whole wattle seed (A) and uncoated cotyledons only (B). Absorbance (arbitrary units) was measured at 214 nm. See the Materials and Methods for details of the CE protocol.

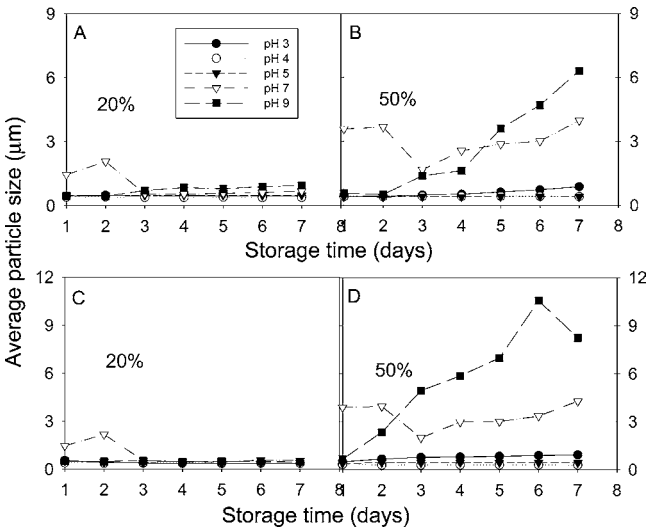

Figure 3. Effect of the storage time on the average particle size (d_{43}) of 20 or 50% oil-in-water emulsions formed with water extract from ground whole wattle seed. Emulsions were stored either at the refrigeration temperature (A and B) or incubated at 25 °C (C and D).

al. (*3*), who studied the composition of various Australian native plants, including several from the Acacia family. Results of the material yield after extraction (**Table 2**) also show a much higher fiber level, suggesting that most of the insoluble materials from the whole seeds were fiber. Incidentally, the residue obtained after extraction of the cotyledons, although much smaller in total material, had a much higher fiber content than that obtained from extracting the whole seed. This would suggest that it was much easier to extract the soluble components from the cotyledon than from the whole seed. **Table 2** also shows, conclusively, that there were more water-soluble components in both sets of samples than the other constituents extracted, especially in the cotyledon, whereby the water-soluble extract yielded 67.77 g of solids/100 g of sample compared to 34.49 g of solids/100 g of sample in the whole seed. It also appeared that alkali and ethanol were better able to extract dry matter from the whole seed than from the cotyledon. Furthermore, these alkali- and ethanol-soluble extracts contained significantly more proteins. The results would indicate the nature and properties of constituents in wattle seed, with the extracts being composed mainly of proteins and water-soluble carbohydrates.

Results of SDS−PAGE analysis of the extracts from whole wattle seed are shown in **Figure 1**. It is in agreement with the percentage of protein data in **Table 2**, in that ethanol extracts (lanes 2 and 3) had very faint bands, indicating a much lower protein content compared to alkali (lanes 4 and 5) and water extracts (lanes 6 and 7). The total number of bands as indicated in the whole seed and uncoated cotyledons (lanes 8 and 9) was 12, which is slightly less than the 14 bands commonly reported for soybean protein after SDS−PAGE analysis (*4*). Results also indicate that most of the protein bands found in the whole seeds are also in the cotyledon, with the only difference being that the outer shell appears to only reduce the intensity of the bands. This relative intensity between bands of whole seed extracts and those from the uncoated cotyledons persists in most samples, also in agreement with the percentag of protein data, as shown in **Table 2**. Interestingly, most of the bands found in the alkali extracts were also present in the water extracts. The ethanol extracts were not well-defined (very faint), most likely because of their low protein concentration and purity. In both water and alkali extracts, the major bands were in the 27.6−60.9 kDa range. These results suggest that the proteins in the alkali-soluble

Table 3. Viscosity (in mPa)[a] of Wattle Extracts and Their Emulsions at Different pH Values

pH	extract (supernatant)	20% oil-in-water emulsion	50% oil-in-water emulsion
3	2.81 ± 0.12	4.91 ± 0.28	29.51 ± 1.12
4	3.40 ± 0.15	2.22 ± 0.10	14.06 ± 0.92
5	48.22 ± 2.32	64.70 ± 3.78	479.02 ± 10.82
7	810.03 ± 25.18	140.04 ± 8.90	966.03 ± 28.01
9	3.82 ± 0.20	50.12 ± 3.01	230.52 ± 11.87

[a] Data are means of three replicates with standard deviations.

extracts were possibly conjugated multiple units of the proteins in the water extracts, which were reduced to similar sizes during the experimental conditions employed for the SDS−PAGE analysis, e.g., reduction in disulfide bonds.

Results of CE analysis appear to confirm the data obtained from other protein analyses, with the extracts from cotyledons showing generally larger peak areas because of its higher protein concentration compared to the corresponding whole seed extracts. As shown in **Figure 2**, all extracts appeared to have similar retention times of around 4 min. The proteins appear as one major peak under CE analysis, which is typical of seed proteins (*11*). There were also some minor peaks, but these were not properly defined in this experiment probably because the experiments were performed under nondenaturing conditions. Storage proteins from rice were shown by Agboola et al. (*13*) to have several major peaks when the CE was carried out under denaturing conditions.

Emulsifying Properties of Water Extracts from Whole Wattle Seed. The emulsifying properties of the wattle seed have been studied using only the water extract from whole ground seeds because emulsifiers are expected to be soluble in the continuous phase of emulsions (*14*), in this case, the oil-in-water emulsion system. The extracts from the cotyledons were not used at this stage because of the difficulty in separating the uncoated cotyledons of the seeds from their outer coatings. Emulsions containing 20 or 50% oil were generally liquid, although 50% oil emulsions were more viscous. Comparatively, those emulsions containing 80% oil were gel-like in nature,

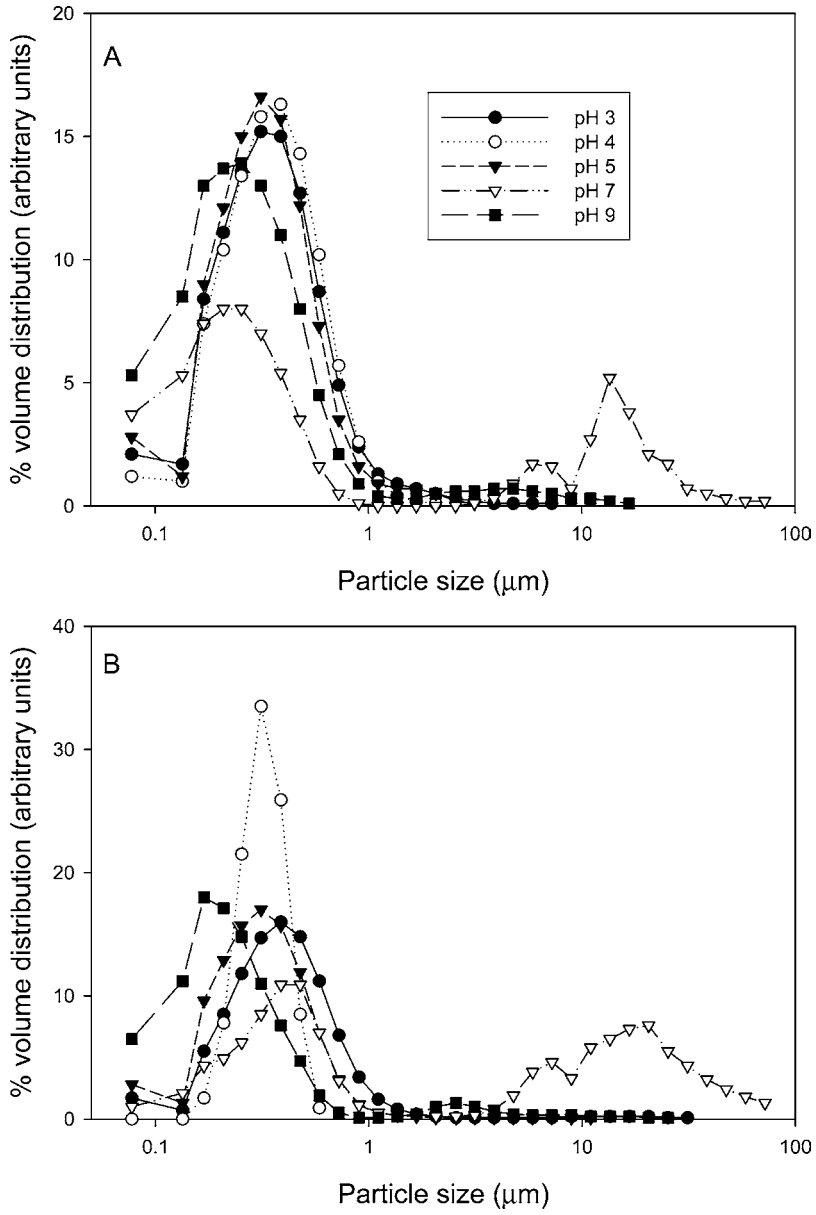

Figure 4. Particle-size distribution of 20% (A) and 50% (B) oil-in-water emulsions formed with water extract from ground wattle seed at different pH values.

oiling off within 24 h, irrespective of the storage temperature, salt level, or pH. As shown in **Table 3**, the viscosity of the extract was highly dependent upon the pH, a situation, which, in turn, affected the ease of formation of the emulsions and their stability under the various conditions used in this study. According to Walstra (*14*), viscosity of the continuous phase adversely affects the ability of the oil particles to deform and break down into smaller globules; consequently, the large particle thus formed at high viscosity may destabilize faster. This pH-induced effect on viscosity of the serum phase of the emulsions could be due to changes to the protein (proximity to the isoelectric point) or the carbohydrate components of the extracts (*4*).

The relationship between the viscosity of the extracts and that of corresponding emulsions at 20 and 50% seems to show a general trend toward increasing viscosity as the amount of oil incorporated increased. However, this was not the case at pH 7, whereby the extract had considerably higher viscosity (810 mPa) than the 20% oil-in-water emulsion (140 mPa). The reason for this observation is not understood, but the isoelectric point of the proteins in the extracts could be close to pH 7. As proteins get closer to their isoelectric pH, the amount of interaction between them increases, and this aggregation may lead to an increased viscosity, turbidity, and often precipitation from solution (*5*). There could conceivably be two competing forces for the establishment of emulsion viscosity in our system: the effect of homogenization on extract viscosity and the amount of oil incorporated. It is, thus, probable that, at pH 7 and for the 20% oil-in-water emulsion, homogenization weakened the interactions between the components more than the influence of oil increased the viscosity. Furthermore, at other pH values, the extract viscosity was most likely too low for homogenization to make any significant contribution toward viscosity reduction, while the oil content effect predominated. It must be noted though that, of all of the 20% oil-in-water emulsions, the highest viscosity was still recorded at pH 7.

Emulsions were studied by measuring the d_{43} particle size average and size distribution under the influence of the pH, salt concentration, and retorting. As shown in **Figures 3** and **4A**, 20% oil-in-water emulsions made at pH values of 3, 4, 5, and 9 were smaller in size (emulsion particles ranged between 0.1 and 10 μm) and consequently more stable compared to the emulsions formed at pH 7 (a significant group of particles ranged between 10 and 100 μm). This is apparently due to the negative effect of high serum viscosity on the particle size as discussed above. The same trend was observed for 50% oil-in-water emulsions at pH values of 3, 4, and 5, albeit in much higher average size ranges (parts **B** and **D** of **Figure 3** and **Figure 4B**). Significantly though, 50% emulsions at pH 7 had high particle size averages that declined for the first 3 days, after which they slightly increased in size for the remaining 4 days of observation. This increase was, however, accompanied by the emulsion breaking and oiling off on the surface. Comparatively, 50% oil-in-water emulsions at pH 9 consistently increased in average size throughout most of the observation period (parts **B** and **D** of **Figure 3**) and were not accompanied by any significant oiling off. The 50% oil-in-water emulsions were also relatively unstable enough for the storage conditions to have an effect, especially at pH values of 7 and 9. It would appear that the higher oil content and the increased interfacial area created without an accompanying increase in the amount of surfactant (protein level reduced from 1.1% for 20% oil emulsion to 0.43% for 50% oil emulsion) led to such a significant change in the stability of 50% oil-in-water emulsions.

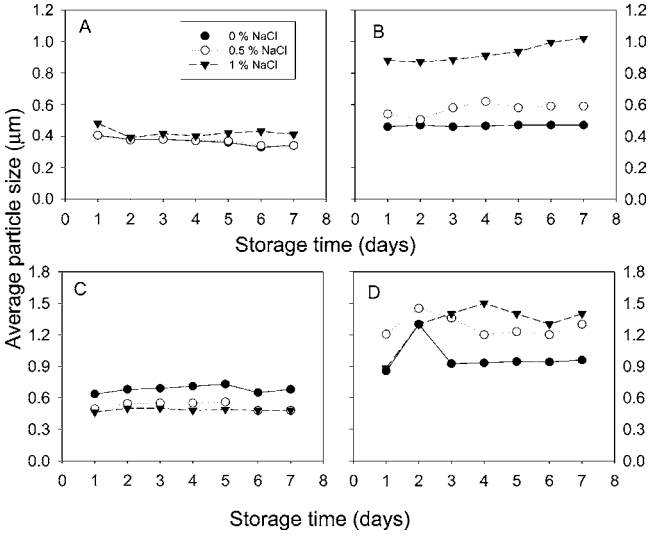

Figure 5. Influence of the NaCl content and retorting (bottom) on the stability (d_{43} average size) of wattle seed extract-stabilized 20% oil-in-water emulsions formed at pH 4 (A and C) and pH 5 (B and D).

Protein-stabilized emulsions generally have a lowered average particle size and good stability if the surface coverage is adequate (*12*, *15*). The results in this study would also suggest that the water-soluble extract of wattle seed contains materials suitable for stabilizing even high oil content emulsions, most likely the proteins and soluble carbohydrates.

The influence of the salt concentration and retorting on the stability of 20% oil-in-water emulsions formed at pH values of 4 and 5 are shown in **Figure 5**. In general, emulsions formed at pH 4 formed smaller particles (lower d_{43} average size) and were more stable under storage than those at pH 5, probably because of the increased viscosity of the extract at the higher pH as noted above (see **Table 3**). The influence of increasing the NaCl concentration was not very significant at pH 4 (**Figure 5A**), probably because of the inherent stability of the emulsions, but at pH 5, we clearly see a significantly less stable emulsion as the salt concentration increased to 0.5% and also at 1% (**Figure 5B**). Overall though, the emulsions were still stable (no breaking and no oiling off), even though the size average increased. It would appear that salt screening of the electrical double layer of the protein-covered emulsion droplets reduced electrostatic repulsion between the droplets, leading to flocculation and probably coalescence of the droplets (*16*). It is also possible that steric repulsion between the particles was reduced by the binding of cations to the wattle seed proteins on the droplet surfaces. Brooksbank et al. (*17*) has shown that monovalent cations, such as Na^+, adsorbed to protein surfaces led to a reduction in the steric repulsion between latex particles.

Retorting the emulsions caused a slight increase in the d_{43} average size of both sets of emulsions, but the emulsions formed at pH 4 remained very stable throughout the observation period of 7 days (**Figure 5C**). Conversely, emulsions at pH 5 were stable only within the first 2 days, forming a significant cream layer afterward. It is significant to note that retorting did not cause immediate separation at either pH level and suggests that the wattle proteins may be heat-labile after adsorption onto the oil surface. It is also possible that the carbohydrates in the serum layer acted as stabilizers for the emulsion system.

Our study has shown that water-soluble extracts from wattle seed contain predominantly proteins and carbohydrates, mainly within the cotyledons. These extracts have also been shown to be an excellent emulsifier/stabilizer for the oil-in-water emulsion

system even at relatively high oil content and very low protein levels. It is, however, important to understand the nature of these proteins, especially the possibility that some may possess antinutritional factors, and how processing for this could affect the use of the extracts in the food industry. Research also continues on how to elucidate the relative roles of the two major components of wattle seed extracts, proteins and soluble carbohydrates, in the stabilization of oil-in-water emulsions.

ABBREVIATIONS USED

CE, capillary electrophoresis; kDa, kilodalton; MPa, megapascal; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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