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Optimization of ultrasound-assisted extraction of sheep abomasum protein concentrates by response surface methodology and evaluation of their properties

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Abstract

The aim of this study was to extract sheep abomasum protein concentrates (SAPC) by ultrasound-assisted extraction (UAE) and to investigate the properties of SAPC. Response surface methodology and Box-Behnken design were applied to determine the optimal parameters for UAE. The maximum water-soluble protein concentration was 320.5 mg protein/g dry raw material under the optimal conditions with ultrasonic treatment time of 28 min, ultrasonic power of 450 W, liquid/solid ratio of 25 mL/g, and pH of 10. Compare with conventional extraction method (CEM), the UAE not only provided the higher protein concentration and yield but also required much shorter extraction times. Additionally, the SAPC obtained by UAE demonstrated better solubility, emulsifying properties, foaming properties and oil holding capacity as compared to CEM. However, use of the UAE method did not significantly increase the water holding capacity of SAPC.

Keywords: sheep abomasum; protein concentrates; ultrasound-assisted extraction; response surface methodology; functional properties.

Practical Application: Based on its functional properties, SAPC obtained using UAE may have potential uses as pharmaceutical or nutritional ingredients in various food or medicines.

1 Introduction

Large amounts of sheep abomasum (SA) are generated as a by-product of the sheep meat industry (Toldrá et al., 2012; Martínez-Alvarez et al., 2015). Efforts have been made to convert those by-products to a new source of functional ingredients or novel products due to their high nutritional value and good bioavailability (Mora et al., 2014; Queiroz et al., 2017). Several earlier studies have reported that SA is rich in proteins and numerous other nutrients (Li & Chen, 1987; Chen, 1982; Zhao, 2011). Therefore, it is assumed that sheep abomasum protein concentrates (SAPC) are valuable animal proteins that can be applied for functional or pharmaceutical products. Freeze-melting and stirring (Zhang, 2003; Zhao, 2011) are conventional extraction methods used for SAPC extraction, but these methods are time-consuming and can adversely affect bioactivity or pharmaceutical values (Jodayree et al., 2012; Tang et al., 2015). Therefore, to gain SAPC, a more environmentally friendly and highly efficient extraction process should be applied.

Recent years, ultrasonic-assisted extraction (UAE) has been used to extract proteins from various sources including rice bran (Chittapalo & Noomhorm, 2009), rapeseed protein (Dong et al., 2011), porcine placenta (Tang et al., 2015), and chicken egg shell membrane (Jain & Anal, 2016). Compared with conventional methods, the UAE approach is time-reducing, and allows easy operation, high extraction yield, and low energy consumption (Rocha et al., 2017; Li et al., 2017). However, the application of UAE for the extraction of protein concentrates from sheep abomasum has not been reported.

In this study, response surface methodology (RSM) and a four-variable, three-level Box-Behnken design (BBD) were used for the optimization of four parameters of UAE including extraction time, ultrasound power, liquid/solid ratio, and pH for the highest water-soluble protein concentration (WSPC). Additionally, the yield, chemical composition, and functional properties of SAPC obtained by conventional extraction method (CEM) and UAE were determined and compared. This is the first study to determine the appropriate protocol to prepare SAPC, and the results should facilitate utilization of these industrial by-products.

2 Materials and methods

2.1 Materials and reagents

Fresh SA was obtained from Urumqi slaughter house (Xinjiang, China), and rinsed into cold water to remove connective tissue and the residue in stomach, then cut into pieces and immediately frozen at -80 °C for 24 h. The frozen sample was lyophilized (FDU-2100, EYELA), crushed, defatted with ligarine, and stored in polyethylene bags at -20 °C until use. All reagents were of

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analytical grade. The WSPC was measured by Pierce® BCA Protein Assay Kit (Thermo Scientific).

2.2 Preparation of sheep abomasum protein concentrates

SAPC samples were prepared by two different methods, the CEM and UAE methods. For UAE, this method was performed in an ultrasonic generator (JY98-IIIN Ningbo Scientz Biotechnology Co. Ltd., China). In short, defatted SA were extracted with alkaline solution at the established pH, ultrasound power and time. Then, the mixture was centrifuged at 12,000 r/min for 10 min at 4 °C. The pellet was discarded and the supernatant was collected, dialyzed and the WSPC was determined. Ammonium sulfate was added to the supernatant to reach 60% saturation and stirring for 3 h, then centrifuged at 10,000 r/min for 20 min. The precipitate was washed by de-ionized water, re-dissolved and dialyzed for 24 h against de-ionized water. The solution was lyophilized and kept in a polyethylene bag at -20 °C until use.

As a comparison, CEM method was performed. Defatted SA (1.0 g) were homogenized with alkaline solution (pH = 10, liquid/solid ratio = 25) and then stirred under ice bath for 240 min. Then the mixture was processed with the same protocol as UAE.

2.3 Experimental design and statistical analysis

RSM and BBD with four variables and three levels were used to optimize UAE parameters. The extraction time (X_1), ultrasound power (X_2), liquid/solid ratio (X_3) and pH (X_4) were chosen as the independent variables. The WSPC was determined as the response of the design experiments (Y). Based on the single-factor experiments (data not shown), X_1 (20, 30 and 40 min), X_2 (200, 400 and 600 W), X_3 (20, 25 and 30 mL/g) and X_4 (8, 9 and 10) were determined as critical levels with significant effect on protein extraction. Each independent variable and relative levels are given in Table 1. Twenty-four factorial points and five replicates of central point in the total 29 experiments were performed. The experimental results were analyzed using RSM algorithm and were fitted to the following predictive quadratic polynomial Equation 1:

$$Y = \beta_0 + \sum_{i=1}^{4}\beta_i X_i + \sum_{i=1}^{4}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{4}\beta_{ij} X_i X_j \quad (1)$$

Where Y_i is the response variable, β_0 is the model constant, β_i is the linear terms, β_{ii} are the squared terms, β_{ij} is the interaction terms, and X_i and X_j are independent variables.

Table 1. Box-Behnken design with experimental responses for water-soluble protein concentration (WSPC).

Run	X_1:Extraction time (min)	X_2:Ultrasonic power (W)	X_3:Liquid/solid ratio(mL/g)	X_4:pH	WSPC* (mg/g)
1	40	600	25	9	286.1 ± 13.7
2	20	400	25	8	235.2 ± 9.2
3	20	400	20	9	259.6 ± 7.8
4	30	600	20	9	276.7 ± 11.6
5	30	200	30	9	246.4 ± 11.9
6	30	400	20	8	256.1 ± 6.8
7	30	400	20	10	312.5 ± 13.9
8	30	400	25	9	304.5 ± 9.0
9	30	400	30	10	321.4 ± 8.9
10	30	400	30	8	264.2 ± 10.3
11	30	200	20	9	269.2 ± 18.2
12	30	600	30	9	289.7 ± 11.5
13	30	600	25	8	278.6 ± 10.0
14	30	200	25	8	253.6 ± 12.2
15	20	400	30	9	250.3 ± 8.0
16	30	400	25	9	298.8 ± 9.3
17	30	400	25	9	298.4 ± 14.6
18	40	400	25	8	276.5 ± 9.2
19	40	400	30	9	278.3 ± 16.9
20	30	600	25	10	326.5 ± 9.2
21	20	600	25	9	276.9 ± 10.0
22	20	400	25	10	320.8 ± 11.8
23	40	200	25	9	266.8 ± 8.7
24	40	400	20	9	270.5 ± 10.4
25	30	400	25	9	298.6 ± 14.0
26	30	200	25	10	300.2 ± 10.4
27	40	400	25	10	307.4 ± 6.9
28	30	400	25	9	302.2 ± 11.3
29	20	200	25	9	244.8 ± 11.0

*Values are expressed as mean ± SD (n = 3).

2.4 Electrophoretic analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of SAPC samples were carried out according to reported procedures with slight modifications (Laemmli, 1970) using 12% separating gel and 4% stacking gel. The electrophoresis was run at 75 V in the stacking gel and 150 V in the separating gel until the tracking dye reached the bottom of the gel. Then, the gels were stained with Coomassie Brilliant Blue G 250.

2.5 Yield and proximate composition

Protein, moisture, fat and ash contents of samples were determined according to the AOAC standard procedures (Association of Analytical Communities, 2007). The contents were expressed in g/100 g. Yield of samples were estimated as Equation 2:

$$Yield\,(\%) = \left(\frac{m_d}{m_{de}}\right) \times 100\% \quad (2)$$

Where m_d represented as the weight of extracted SAPC (g); m_{de} represented as the weight of defatted SA (g).

2.6 Functional properties

Protein solubility

The assay was determined according to the reported procedures with slight modifications (Mao & Hua, 2012). Samples containing two milliliters of aqueous solution (0.1% w/v) were adjusted to different pH values ranging from 2.0 to 12.0 using 0.1mol/L HCl or 0.1mol/L NaOH, and then stirred and vortexed for 30 min. The mixtures were then centrifuged at 10000 r/min for 10 min. The supernatants were collected and the WSPC was measured. The solubility was calculated as Equation 3:

$$\text{Solubility}\,(\%) = \left(\frac{m}{m_T}\right) \times 100\% \quad (3)$$

Where m represented as WSPC in supernatant (mg/g); m_T represented as the content of total protein in SA (mg/g).

Emulsifying properties

Evaluation of emulsifying activity index (EAI) and emulsion stability index (ESI) were performed based on the reported procedure (Pearce & Kinsella, 1978). Samples containing thirty milliliters of solution (10 g/L) were mixed with 10 mL of soybean oil, and were homogenized with an ultrasonic disperser for 2 min at 200 W. An aliquot of the emulsion (50 μL) was immediately pipetted out after homogenization from the bottom at 0 and 10 min and diluted with 5 mL 0.1% (w/v) sodium dodecyl sulfate (SDS) solution. The absorbance of the diluted emulsion was measured at 500 nm using UV/VIS spectrophotometer (China, WFZ 754, CO, LTD). EAI and ESI were calculated as Equation 4 and Equation 5 respectively:

$$EAI\left(m^2/g\right) = \left(\frac{2 \times 2.303 \times A_0 \times dilution\ factor}{C \times \varphi \times 10000}\right) \quad (4)$$

$$ESI\,(\min) = \left(\frac{A_0}{A_0 - A_{10}}\right) \times \Delta t \quad (5)$$

Where A_0 and A_{10} are the absorbance of analyzes at 0 min and 10 min respectively, dilution factor is 100, C is the initial concentration of protein (g/mL), φ is equal to 0.25 (oil volumetric fraction), $\triangle A = A_0 - A_{10}$ and $\triangle t$ is 10 min.

Foaming properties

Foaming capacity (FC) and foam stability (FS) of samples were determined following a previous method (Turan et al., 2015) with minor modifications. Samples containing solution (10 g/L, 25 mL) were whipped for 10 min with an ultrasonic disperser for 2 min at 200 W. The whipped samples were immediately transferred into a cylinder (50 mL). The volumes before and after whipping were recorded. The total volume of foam remaining was recorded after 30 min quiescent period at room temperature. All experiments were conducted with triplicate samples. FC and FS were calculated as Equation 6 and Equation 7 respectively:

$$FC\,(\%) = \left(\frac{V_1 - V_2}{V_2}\right) \times 100 \quad (6)$$

$$FS\,(\%) = \frac{V_3 - V_2}{V_2} \times 100 \quad (7)$$

Where V_1 is the volume after whipping, V_2 is the volume before whipping, V_3 is the volume after standing.

Water and oil holding capacity

Water holding capacity (WHC) and oil holding capacity (OHC) of samples were determined according to the previous method (Jain & Anal, 2016). Approximately 25 mL of distilled water or refined soybean oil were added into pre-weighed centrifuge tubes containing 1 g of dry sample and subjected to stirring and mixing for 30 min. After being centrifuged at 2000 r/min for 30 min, the supernatant was discarded and the residue was drained for 15 min at room temperature and then weighted. WHC and OHC were calculated as the weight of water and oil adsorbed by 1 g sample.

2.7 Statistical analysis

Design-Expert 8.06(State-Ease Inc., Minneapolis, USA) was used for experimental design and data analyses of RSM. Other experiments were performed with SPSS Version 19.0 (Chicago, IL, USA) using ANOVA and Tukey analysis. The statistical differences between samples were measured by the least significant difference (LSD) test. $P < 0.05$ was considered to be significant. All experiments were performed in triplicate and SD was calculated.

3 Results and discussion

3.1 Optimization of the extraction parameters by RSM

Model fitting

The fitted quadratic model for WSPC is: Y = 300.50 + 8.17 X1 + 12.79 X2 + 0.48 X3 + 27.06 X4 - 3.20 X1 X2 + 4.28 X1 X3 -13.68 X1 X4 + 8.95 X2 X3 + 0.32 X2 X4 + 0.17 X3 X4 - 18.94 X12 - 13.65 X22 - 16.24 X32 + 3.52X42. Model validity was confirmed by using the lack of fit test, as summarized in Table 2. In the regression model, Y was highly significant ($P < 0.01$), and ANOVA for the lack of fit was insignificant ($P = 0.1896$). This result indicated that this model well fit the experimental data. The coefficient of determination ($R2 = 0.9874$) obtained for the predicted model indicated a quadratic relationship between Y and extraction parameters with a good regression coefficient. The most prominent factor affecting WSPC was pH ($X_{4)}$, followed by ultrasonic power (X_2), extraction time (X_1), and liquid/solid ratio (X_3). The quadratic term (X_{14}, X_{12}, X_{22}, and X_{32}) was highly significant ($P < 0.01$), and X_1X_3, X_2X_3, and X_{42} terms were moderately significant ($P < 0.05$). However, other interaction terms were insignificant ($P > 0.05$).

Analysis of response surface

A series of three-dimensional (3D) response surface graphs were generated and are presented in Figure 1 a-f, which shows the relationship between WSPC and extraction parameters. According to Figure 1 c, e, f, WSPC increased significantly with the increase of pH from 8 to 10. This result was consistent with previous findings that alkaline solvents are the most effective solvent to extract SAPC (Zhang, 2003). As shown in Figure 1 a, d, e, the WSPC of SA increased when ultrasonic power increased from 200 W to 400 W, which may be attributed to the further breaking of SA cells, leading to the release of more proteins into the liquid/solid system (Tang et al., 2015). However, when the ultrasonic power was further increased from 400 W to 600 W, the WSPC slowly decreased because the higher ultrasound power generated high-pressure conditions that reduced the solubility of the protein (Zhu et al., 2009). As presented in Figure 1 a, b, c, WSPC increased rapidly from 20 min to 30 min. During this period, the cell walls of SA broke gradually, which accelerated the release of proteins. However, with additional time, no obvious increase of WSPC was observed. Similarly, as shown in Figure 1 b, d, f, WSPC increased with the increase of the liquid/solid ratio, and then decreased.

Validation of the model

The optimum UAE conditions were obtained using Design Expert 8.06 software, and determined as a practical optimum: extraction time of 28 min, ultrasonic power of 450 W, liquid/solid ratio of 25 mL/g, and pH of 10. Verification experiments were performed under optimal conditions (n = 3), to further validate the reliability of the theoretical model prediction. The results showed that experimental results for WSPC were very close to the predicted values (320.5 ± 23.6 and 331 mg/g, respectively), values were not significantly different ($P > 0.05$). Thus, it could be concluded that the established model in this study was appropriate and valid.

3.2 Comparison of UAE method with CEM

CEM was performed and compared to UAE to evaluate the extraction efficiency. The extraction time of UAE (28 min) was less significantly than CEM (240 min). The WSPC of extract

Table 2. ANOVA results of water-soluble protein concentration (WSPC).

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*P*-value
Model	17324.04	14	1237.43	78.68	< 0.0001
X_1 (Extraction time)	800.33	1	800.33	50.89	< 0.0001
X_2 (Ultrasonic power)	1963.52	1	1963.52	124.84	< 0.0001
X_3 (Liquid/solid ratio)	2.80	1	2.80	0.18	0.6793
X_4 (pH)	8785.84	1	8785.84	558.61	< 0.0001
$X_1 X_2$	40.96	1	40.96	2.60	0.1289
$X_1 X_3$	73.10	1	73.10	4.65	0.0490
$X_1 X_4$	748.02	1	748.02	47.56	< 0.0001
$X_2 X_3$	320.41	1	320.41	20.37	0.0005
$X_2 X_4$	0.42	1	0.42	0.03	0.8722
$X_3 X_4$	0.12	1	0.12	0.01	0.9309
X_1^2	2327.27	1	2327.27	147.97	< 0.0001
X_2^2	1209.32	1	1209.32	76.89	< 0.0001
X_3^2	1711.08	1	1711.08	108.79	< 0.0001
X_4^2	80.41	1	80.41	5.11	0.0402
Residual	220.19	14	15.73		
Lack of fit	190.39	10	19.04	2.56	0.1896
Pure error	29.80	4	7.45		
Total	17544.23	28		$R^2 = 0.9874$	

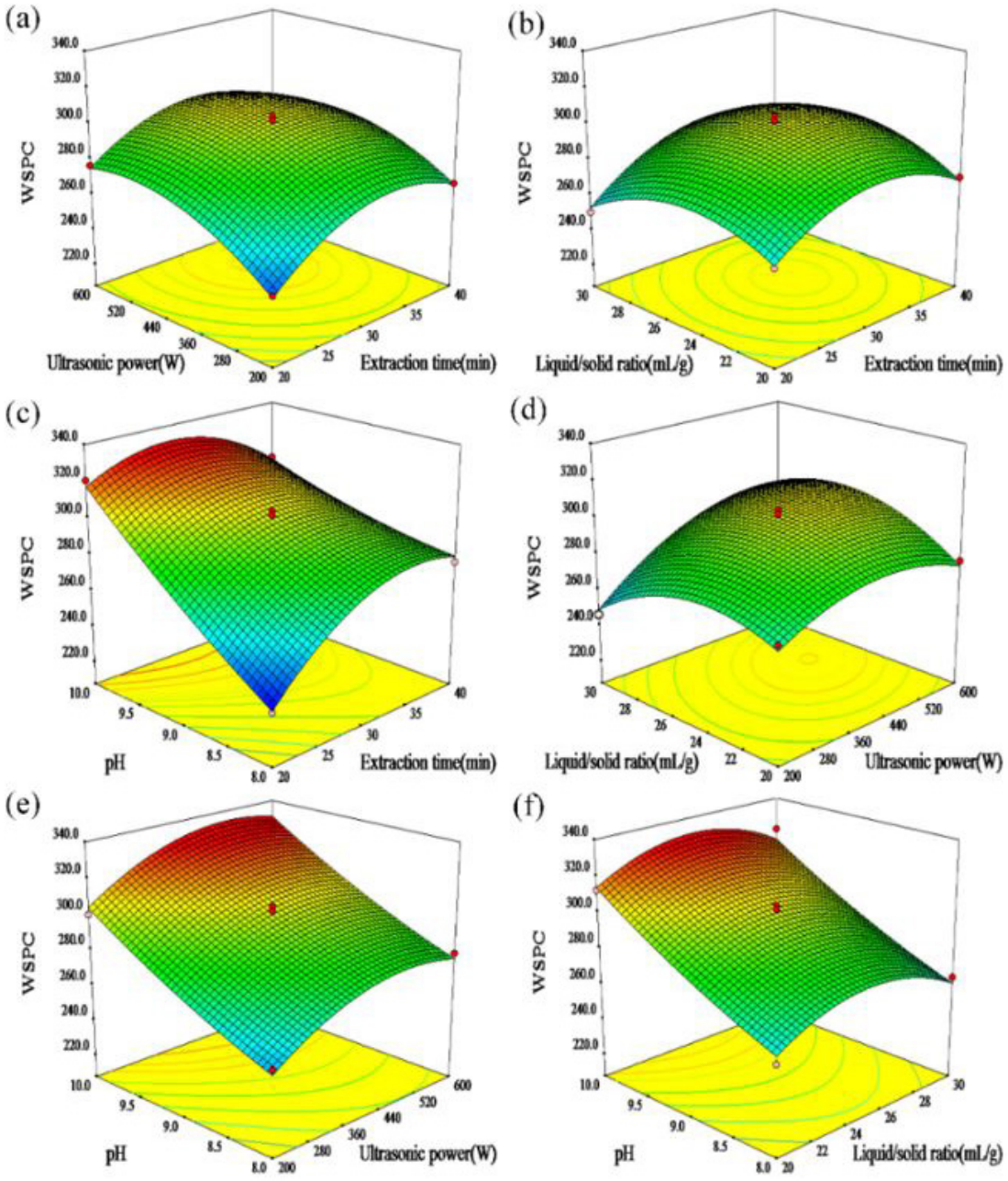

Figure 1. Response surface plots (a, b, c, d, e, and f) showing the interactive effects of extraction time (X_1), ultrasonic power (X_2), liquid/solid ratio (X_3), and pH (X_4) on water-soluble protein concentration (WSPC) using ultrasound-assisted extraction.

obtained by UAE was higher than for CEM (320.5 ± 23.6 mg/g, 306.2 ± 18.9 mg/g respectively). The better performance with UAE may be due to ultrasound enhanced mass transfer and particle diffusion within the liquid/solid system, as CEM agitation only enhances external mass transfer (Tang et al., 2015).

As shown in Figure 2, the protein species and the molecular weight of proteins in the SAPC samples were determined by SDS-PAGE. The SAPC samples obtained by UAE and CEM contain a variety of protein bands of similar molecular weight (MW) between 10 and 130 kDa, based on comparison to a standard composed of a mixture of proteins of known MW. Further analysis of the composition of the proteins in SAPC and evaluation of the biological activities of SAPC is warranted.

3.3 Yield and proximate composition

The yield and proximate composition of defatted SA and SAPC samples were measured and are shown in Table 3. Significant ($P < 0.05$) differences were found in yield, fat, ash, protein, and moisture contents of all samples. The highest yield and protein values were observed in SAPC obtained by the UAE method, and were 24.41 ± 0.71% and 92.36 ± 3.80 g/100g, respectively. These findings indicate that UAE was the most effective method

Table 3. Yield and proximate compositions of sheep abomasum protein concentrates (SAPC).

Properties	Defatted sheep abomasum	SAPC	
		Ultrasound-assisted method	Conventional method
Yield (g/100g)		24.41 ± 0.71[a]	22.46 ± 0.90[b]
Protein (g/100g)	36.74 ± 1.65[c]	92.36 ± 3.80[a]	86.52 ± 2.74[b]
Fat (g/100g)	1.12 ± 0.04[a]	0.81 ± 0.04[c]	0.93 ± 0.05[b]
Moisture (g/100g)	5.73 ± 0.26[a]	1.02 ± 0.10[c]	1.31 ± 0.05[b]
Ash (g/100g)	9.63 ± 0.98[a]	4.24 ± 0.09[c]	6.15 ± 0.06[b]

Values are expressed as mean ± SD (n = 3).

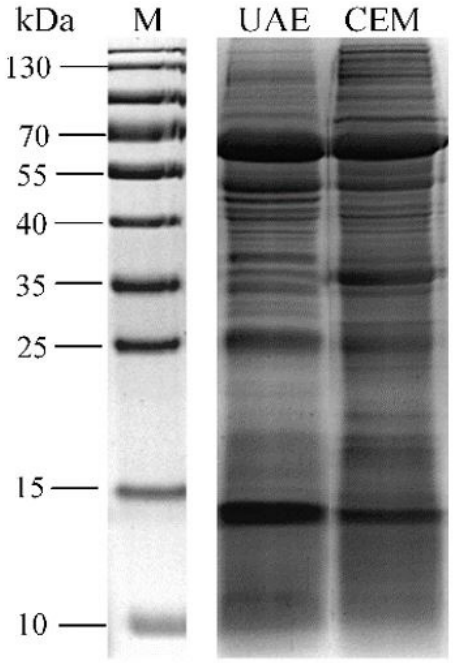

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of sheep abomasum protein concentrates (SAPC) obtained by ultrasound-assisted extraction (UAE) and conventional extraction method (CEM) using 12% separating gel. M, protein standard.

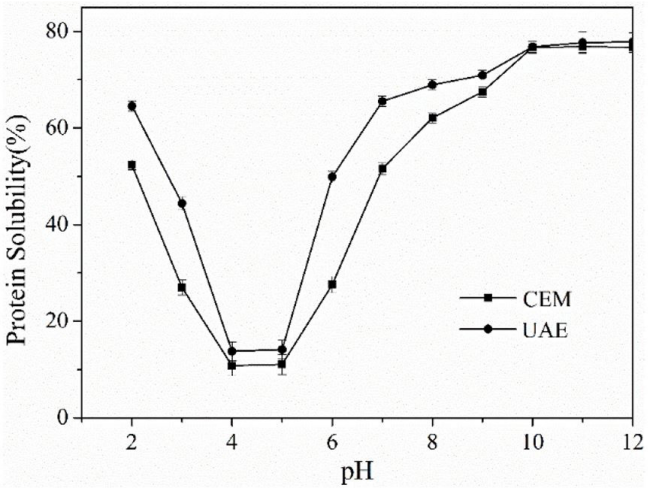

Figure 3. Protein solubility of sheep abomasum protein concentrates (SAPC) at varying pH. CEM, conventional extraction method. UAE, ultrasound-assisted extraction.

for extraction of SAPC due to the higher WSPC value and better extraction yield within the shortest time.

Means with different letters within the same row indicate significant differences ($P \leq 0.05$).

3.4 Functional properties of SAPC

The protein solubility profile of SAPC over a wide range of pH values (2.0-12.0) was determined and is shown in Figure 3. There was an observed difference in the solubility of SAPC prepared by UAE and CEM ($P < 0.05$). The poorest solubility of both SAPC samples was observed at pH = 4 (isoelectric point), but below and above this pH, the solubility increased, consistent with the report of Gbogouri et al. (2004). At the isoelectric pH, the net charge of the proteins will be small or zero, and the reduced electrostatic repulsive forces of proteins cause protein aggregation and precipitation that decreases solubility (Wu et al., 2009). At pH 2, the solubility of the SAPC sample prepared using the UAE method was about 64.5% and that of the conventional method was about 52.4%. With the increase of pH, the solubility of both methods gradually increased, and the solubility of the UAE was always higher than that of CEM, consistent with the report of Jain & Anal, (2016). The maximum solubilities of SAPC samples were observed at pH 12 with 76.8% for CEM and 77.8% for the UAE method. The results indicated that SAPC obtained by UAE method exhibited excellent solubility at alkaline pH, an important qualification for food or medicine formulation.

All other measured functional properties of SAPC are shown in Table 4. In the preliminary experiments, the emulsifying properties and foaming properties could not be reliably determined at pH 2.0 and 5.0, which might be due to the poor solubility of the protein (Chove et al., 2001). Instead, those properties were determined at pH 8.0. The emulsifying properties play an important role in food and medical material processing. EAI is defined as the ability of a protein to form an emulsion by adsorbing oil at the water-oil interface. ESI is defined as the ability to stabilize an emulsion without coalescence or flocculation over a period of time (Jain & Anal, 2016). The EAI and ESI values of the SAPC sample extracted using UAE method were higher than the values for the sample prepared using CEM ($P < 0.05$). The maximum EAI and ESI values were 42.26 ± 0.18 and 82.05

Table 4. Functional properties of sheep abomasum protein concentrates (SAPC).

Properties	SAPC	
	Ultrasound-assisted method	Conventional method
Emulsifying activity index (m^2/g)	42.26 ± 0.18[a]	38.53 ± 0.14[b]
Emulsion stability index (min)	82.05 ± 1.97[a]	60.17 ± 0.96[b]
Foaming capacity (%)	23.19 ± 0.48[a]	23.16 ± 0.08[a]
Foam stability (%)	16.44 ± 1.70[a]	12.13 ± 1.45[b]
Water holding capacity (g/g)	4.03 ± 0.05[b]	6.43 ± 0.19[a]
Oil holding capacity (g/g)	21.20 ± 1.10[a]	16.26 ± 0.93[b]

Values are expressed as mean ± SD (n = 3). Means with different letters within the same row indicate significant differences ($P \leq 0.05$).

± 1.97 respectively. Therefore, SAPC obtained by UAE has better emulsifying properties that should facilitate use in food or medicine applications.

The foaming properties are also important properties in manufacturing food or medicine formulations. FS of SAPC prepared using the UAE method was higher than that using CEM, 16.44 ± 1.70 and 12.14 ± 1.45% respectively ($P < 0.05$). This difference might be due to the higher protein solubility of the SAPC sample prepared using the UAE method compared to that of the sample prepared by CEM (Suppavorasatit et al., 2011). However, FC of SAPC samples were not significantly different ($P > 0.05$), 23.19 ± 0.48 and 23.16 ± 0.08% respectively. Similarly, previous study also showed that foam properties are typically influenced by factors affecting solubility during modification processes (Hou et al., 2017; Zou et al., 2017).

WHC and OHC are important properties that determine the shelf life of products (Wu et al., 2009; Jain & Anal, 2016). The values of WHC and OHC were significantly different ($P < 0.05$) for the different SAPC samples. SAPC prepared by the UAE method had higher OHC values (21.20 ± 1.10 g/g), but lower WHC values (6.43 ± 0.19 g/g) than those of the SAPC prepared by CEM. These results indicate that the SAPC sample prepared by UAE retained more oil than water, and was also more lipophilic than the material prepared using CEM.

4 Conclusion

This is the first report of testing UAE for SAPC extraction. The effects of extraction time, ultrasonic power, liquid/solid ratio, and pH were investigated using RSM to maximize the WSPC of the extract. The results indicated that UAE technology increased the protein contents and yield but also shortened the extraction time. The SAPC obtained by UAE method showed enhanced functional properties, such as solubility, emulsifying properties, foaming properties, and oil holding capacity as compared to the properties of the extract prepared by CEM. Taken together, the UAE method can be applied as a novel procedure to prepare functional SAPC, a valuable animal protein with potential uses as a pharmaceutical or nutritional ingredients in various food or medicines.

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