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Effect of Alkaline Agent and pH on the Composition of Freeze-Dried Proteins Extracted from Castor Bean (*Ricinus communis* L.) Cake

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Castor-bean cake, a by-product of biodiesel industry, has been used as fertilizer and as parasitic control in soil. However, its protein could be extracted and used to produce biodegradable materials, enhancing the value of this raw material. The most common process for protein extraction uses alkaline solubilisation, which is also the main treatment against ricin, the toxic protein of castor-bean cake. Thus, the aim of this research was to study the effect of alkaline agents (NaOH or KOH) and pH (10, 11 or 12) on the protein extract composition. Dry matter, proteins, ash, lipid and non-protein nitrogen and also macro minerals and trace elements were determined. In general, the increase in pH do not influenced neither the dry matter content (~93.3 %) nor the non-protein nitrogen content, but influenced the protein content as consequence of the pH increasing. Similar behaviour was observed for ash content due to increasing the amount of both alkaline. The freeze-dried protein extracts presented lower lipids content (~0.8 %), regardless of both alkaline agent and pH values. The nitrogen was the most abundant mineral (10 %) in the protein extracts for all the extraction conditions, while the other macro minerals contents were lower than 1.0 %. With respect to the trace elements, iron was the most abundant independently of the alkaline agent used. We can concluded that the protein extracts are protein concentrates, and the protein content increased with increasing extraction pH. The alkaline agent NaOH was slightly more effective for protein extraction.

1. Introduction

The increase in research on biofuels placed castor bean *(Ricinus communis* L.) in evidence in the agribusiness scenario because it can be considered a good source of oil for biodiesel production (Lacerda, 2012). The oil content in seeds varies from 25 to 55 % (Ogunniyi, 2006). But, its chemical composition changes according to the variety, climate and growing region. Overall, the castor bean is constituted by 75 % almond and 25 % shell. Usually, the oil extraction is performed by pressing the castor bean seeds at high temperature.

The extraction process of castor oil produces a cake rich in nitrogen, phosphorous and potassium, but also, containing ricin, a toxic protein. This cake is rich in proteins and is produced in the proportion of 1.2 tons per ton of extracted oil (Makishi et al., 2013). It has been used in soil to protect plants against soil nematodes, insects, and parasites (Ramachandran et al., 2007). Moreover, considering this by-product is rich in protein, it can be used to restore eroded soils or as animal feed, if detoxified (Ogunniyi, 2006). Thus, considering that the valorization of this by-product could contribute to the castor bean biodiesel production chain, news utilizations for this cake as raw material for production of biodegradable material could be interesting.

We know that it is possible to extract proteins from cakes using alkaline solutions prepared with agents such as sodium hydroxide. In these cases, the solubilized protein can be separated from the no solubilized

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residue by centrifugation. However, the composition of the extract (solubilized matter) may vary with solution pH and the alkaline agent used (Makishi et al, 2013).
The objective of this research was to study the effect of solution pH and alkaline agent used for proteins extraction from castor bean (*Ricinus communis* L.) cake in the composition of the freeze-dried extracts.

2. Material and methods

2.1 Extracting proteins from castor bean cake

Initially, castor bean cake was milled in a rotor grinder coupled to a cold bath (10 °C), and subsequently passed through a 20 mesh ABNT sieve for separation of large-sized particles (Figure 1, left). The sieved material was used for the protein extraction, which was carried out in a 4.5 L extractor Tec-Bio V (TECNAL, São Paulo, Brazil) with automatic temperature control and stirring. This process was carried out by dispersing 20 % milled cake in alkaline medium (at pH 10, 11 and 12, adjusted with 10 % NaOH solution) at 50 °C under constant stirring (400 rpm) for 30 min. Then, the dispersions were centrifuged at 4000 rpm for 20 min in a refrigerated centrifuge at 20 °C (Thermo IEC Model Centra GP & R 31250525, USA) to separate the solubilized proteins (supernatant) from non solubilized residues (Makishi et al., 2013). After centrifugation and separation, the supernatant was freeze-dried and submitted to characterizations analyses (Marquié et al.,1995). This freeze-dried protein extract was analysed according to the methods described above.

2.2 Chemical composition of the freeze-dried protein extract

The freeze-dried protein extract was submitted to chemical analysis to determine dry matter according to the classical gravimetric method (Silva and Queiroz, 2002). The protein content was determined based on the content of total nitrogen, according to the Kjeldahl method, using a conversion factor of 6.25 (Silva and Queiroz, 2002). The lipids were determined by extraction using petroleum ether with a Soxhlet-type extractor apparatus (Silva and Queiroz, 2002). The ash content was determined by incineration of the material at 550 °C in a muffle furnace until complete combustion of organic matter (Silva and Queiroz, 2002). And, the nitrogen-free extract was calculated by difference (Silva and Queiroz, 2002).
The crude fiber, which is the portion of total carbohydrates that resists to acid and alkaline hydrolysis was determined using a Sebelin apparatus. After digestion, the material was dried at 105 ºC in an oven, and burned at 550 ºC in a muffle furnace. Thus, the crude fiber was determined by weight difference (Silva and Queiroz, 2002). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) constituted the residue of the sample after the dissolution of proteins, fats and carbohydrates soluble both in neutral detergent and acid detergent, respectively. The hemicellulose (Hem) was calculated by difference between NDF and ADF, and cellulose was calculated by difference after acid hydrolysis of ADF. The acid detergent lignin (ADL) was quantified by digestion with 72 % sulfuric acid (Goering and Van Soest, 1970).
The minerals calcium (Ca), phosphorus (P), magnesium (Mg), zinc (Zn), manganese (Mn), copper (Cu) and iron (Fe) were determined by atomic absorption spectrometry; the non-protein nitrogen (NPN), sodium (Na) and potassium (K) were determined by flame photometry (AOAC, 1995).

3. Results and discussion

The freeze-dried protein extract was observed using a scanning electronic microscopy (SEM TM 3000, Hitachi, Japan), presenting a typical morphology of freeze-dried material with well structured walls (Figure 1, right).
In general, increasing the pH of extraction solutions resulted in higher ash and crude protein contents. It is suggested that increasing pH may have contributed to better solubilization of proteins, thus facilitating its extraction. The protein content varied from 64 to 68% and the alkaline agent sodium hydroxide was able to extract a greater amount of protein. Thus, it can be considered that the freeze-dried protein extracts produced in this study consisted of protein concentrates. Contrarily, several authors produced protein isolates, which consists of precipitation of protein by lowering the pH to the isoelectric point (pI), thus obtaining a material having a protein content of around 90 % (Makishi et al., 2013). Ayhllon-Meixueiro et al. (2000) studied the extraction of proteins from sunflower cake (*Helianthus annuus*), and they produced an isolate with 90% of proteins. . Orliac et al. (2002) worked with the same material (sunflower cake) with the same results (90% of proteins). The same result was observed later by Orliac et al. (2003). Lestari et al. (2011) studied the *Jatropha curcas* and they obtained an isolate with 98% of proteins. But, the production of protein concentrates may be a good alternative, since it is possible to obtain a significant amount of protein with fewer operations in comparison with the isolate protein process. When compared to

other protein concentrates, the castor bean freeze-dried protein extracts showed protein content very similar to that found, ca 66%, for liquid protein extract from lupin cake (*Lupinus albus*) (Neves et al., 2001).

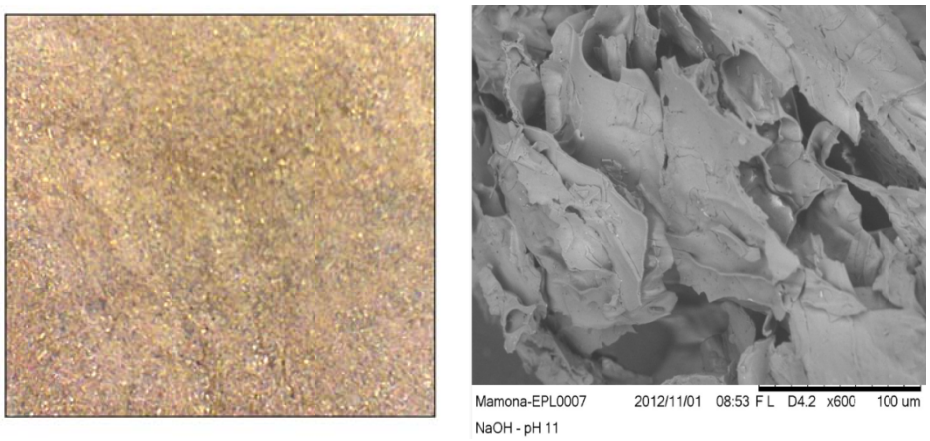

Figure 1. Single photography of the milled castor bean cake (left), and micrograph of freeze-dried protein extracted (pH = 11 using NaOH) (right).

Moreover, the protein content of the extracts obtained in this study were higher than that (31.2 %) obtained by Carvalho et al. (2009) for cupuassu seed extracts (*Theobroma grandiflorum*). This suggests that not only the type of alkaline agent can influence the level of protein extracted, but also the botanical origin of the cake from which the protein is extracted. The content of protein in the freeze-dried protein extracts suggests that this product can be a good raw material to produce biodegradable films (Makishi et al., 2013).

Moreover, we can see that increasing the amount of alkali used to increase pH values may have contributed to the increase of ash content (Table 1). The ash content of the freeze-dried protein extract varied from 12 to 18 % for all treatments, and the treatment using the alkaline agent NaOH at pH 11 and 12 presented the highest ash content (Table 1). These data were higher than those found for sunflower protein isolates (7.6 % - Ayhllon-Meixueiro et al, 2000), rape (7.3 % - Barbin et al, 2011), and lupin protein concentrate (2.4 % - Wäsche et al, 1998). The different ash contents between the protein isolates from others authors and the freeze-dried protein extracts of this study can be justified by the different pH value for the protein extraction.

Table 1. Chemical composition of freeze-dried protein extracts of castor bean cake obtained with alkaline agents KOH and NaOH at pH 10, 11 and 12.

Alkaline Agent /pH	Dry matter (DM)	Ash (AS)	Lipid (L)	Protein (P)[1]	Non protein nitrogen (NPN)[2]
KOH/10	92.4±0.4^{b}	12.9±1.6^{dc}	1.4±0.2^{ba}	65.3±0.5^{c}	1.1±0.0^{bc}
KOH/11	92.4±0.3^{b}	15.2±0.5^{bc}	1.8±0.2^{a}	67.4±0.2^{ba}	1.1±0.0^{bc}
KOH/12	93.7±0.2^{ba}	16.6±0.3^{ba}	0.5±0.1^{c}	66.6±0.1^{b}	1.0±0.0^{c}
NaOH/10	93.5±0.4^{ba}	10.6±0.0^{d}	1.0±0.2^{bac}	64.4±0.6^{c}	1.7±0.2^{a}
NaOH/11	94.6±1.0^{a}	18.6±0.4^{a}	1.7±0.4^{a}	68.2±0.1^{a}	1.0±0.0^{c}
NaOH/12	93.5±0.2^{ba}	18.4±0.2^{a}	0.8±0.1^{bc}	67.8±0.3^{ba}	1.4±0.1^{ba}

[a,b,c,d] different letters in the same column indicate different values ($p < 0.05$); (1) conversion factor of 6.25; (2) % based on Protein content.

The dry matter (DM) ranged from 92 to 94 %, with no differences ($p > 0.05$) among the samples, being similar to that obtained for sunflower protein isolate (DM = 94 %) (Ayhllon-Meixueiro et al., 2000). With respect to the lipid content (L), a significant difference ($p < 0.05$) was observed only at pH 12, with values of 0.5 and 0.8 % for KOH and NaOH extraction, respectively, indicating that freeze-dried protein extracts did not have high lipids content.

The lipid content of the freeze-dried protein extracts of this study was close to the lipid content found for sunflower protein isolate (0.6 % - Orliac et al., 2003), but was lower than that observed for the cupuassu seed protein concentrate (3.8 % - Carvalho et al., 2009). The sunflower cake and castor bean cake were derived from industrial processes of oil extraction, while the cupuassu cake was produced in laboratory scale, i.e., the oil extraction was performed following a less aggressive protocol.

The non-protein nitrogen (NPN) in the freeze-dried protein extracts (Table 1) was associated to the protein denaturation due to the high pH of extraction, whose values ranged from 1.0 to 1.7 %, without much variation between treatments.

The total fiber, ADF and NDF results determined in the freeze-dried extracts from the castor bean cake were less than 1 %, which can be attributed to the partial solubilization of hemicellulose, cellulose and lignin from the cellular wall (Van Soest et al., 1984).

Regarding the contents of macro minerals present in the freeze-dried extracts (Table 2), it was observed that the nitrogen [N] was the most abundant (10 %) for all treatments. Phosphorus [P] showed values ranging from 0.7 to 0.2 %, lower than those (0.8 %) determined by Oliveira et al. (2010) for protein concentrate extracted from castor bean cake using calcium hydroxide. However, these differences can be attributed to several factors, including the different alkaline agent used, the extraction process itself, and even the variability of the cultivar. The mineral K ranged from 1.0 to 4.4 %. Similar values to those obtained for the NaOH extraction [pH 12] (1.0 %) were found by Oliveira et al. (2010) (Table 2). The Mg content varied from 0.1 to 0.2 %, with higher values for NaOH extraction at pH 12.

Table 2. Macrominerals of freeze-dried protein extracts from castor bean cake.

	Components (%DM)					
Alkaline Agent /pH	Sodium (Na)	Magnesium (Mg)	Nitrogen (N)	phosphorus (P)	Potassium (K)	Calcium (Ca)
KOH/10	0.1±0.0^{d}	0.2±0.0^{c}	10.4±0.1^{c}	0.2±0.0^{bc}	3.1±0.03^{c}	0.4±0.0^{cb}
KOH/11	0.1±0.0^{d}	0.2±0.0^{b}	10.8±0.0^{a}	0.2±0.0^{ba}	3.9±0.00^{b}	0.3±0.0^{cd}
KOH/12	1.0±0.0^{c}	0.2±0.0^{c}	10.7±0.0^{b}	0.2±0.0^{a}	4.4±0.00^{a}	0.3±0.0^{d}
NaOH/10	2.4±0.2^{b}	0.2±0.0^{d}	10.3±0.1^{c}	0.2±0.0^{d}	1.4±0.00^{e}	0.4±0.0^{b}
NaOH/11	2.7±0.0^{b}	0.1±0.0^{e}	10.9±0.0^{a}	0.2±0.0^{ba}	1.6±0.01^{d}	0.3±0.0^{cb}
NaOH/12	6.0±0.0^{a}	0.2±0.0^{a}	10.8±0.1^{a}	0.2±0.0^{c}	1.0±0.07^{f}	0.4±0.0^{a}

Lower Na contents were found for the alkaline agent KOH at pH 10 and 11, with values of 0.1%, which was lower than the other treatments. The highest Na contents were obtained for NaOH extraction, which ranged from 2.4 to 6.0 %. Concerning the Ca content, significant differences ($p < 0.05$) were observed for KOH extraction at pH 12 (0.3 %) that was lower than the other pH values. In contrast, the NaOH pH 12 presented higher Ca content than the others (0.4 %).

With respect to the trace elements, statistical differences were observed for all results (Table 3). Iron was the trace element which played at higher values ranging from 324 to 386 mg / g. The treatment with NaOH at pH 12 showed the highest Fe content (386.6 mg / g) and Zn content (169.8 mg / g). There was no information in the literature on the levels of trace elements in residual cakes.

Table 3. Trace elements of freeze-dried protein extracts from castor bean cake.

Alkaline Agent /pH	Components (mg/g) Iron Fe (mg/g)	Zinc Zn (mg/g)	Manganese Mn (mg/g)	Copper Cu (mg/g)
KOH/10	334.4±04^{d}	74.3±0.2^{e}	17.5±0.0^{f}	99.4±0.2^{a}
KOH/11	366.5±0.1^{c}	121.5±0.4^{b}	33.4±0.1^{c}	81.6±0.2^{c}
KOH/12	377.4±0.6^{b}	95.5±0.3^{c}	43.5±0.3^{a}	37.5±0.1^{d}
NaOH/10	325.0±2.1^{e}	86.8±1.1^{d}	21.4±0.2^{d}	84.1±2.7^{cb}
NaOH/11	338.5±2.1^{d}	65.9±0.5^{f}	19.6±0.3^{e}	86.4±0.5^{b}
NaOH/12	386.6±0.3^{a}	169.8±0.8^{a}	40.2±0.1^{b}	83.0±0.6^{cb}

4. Conclusions

We can conclude that the protein extracts are protein concentrates, in which the protein content increased as consequence of the increased pH for both alkaline agent. This factor may be related to the solubilization of proteins, thus allowing a greater extraction at higher pH values. However, the alkaline agent also influenced the extraction process. When NaOH was used, the protein content was slightly higher than those found when KOH was used. Thus, it is possible to conclude that NaOH is a good alkaline agent to extract some protein fractions from castor bean cake. In general, we can suggest that the freeze-dried protein extracts may be an appropriate raw material to produce biodegradable films.

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