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WATER EXTRACTION OF L-DOPA FROM *MUCUNA* BEAN

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SUMMARY

Water extraction studies were carried out on *Mucuna* beans (*Mucuna pruriens*) to determine the extraction rate of L-dopa as a function of bean particle size in Phase I, and water circulation, temperature and pH in Phase II. In Phase I, samples consisting of whole intact beans, shelled whole beans, and chopped bean particles of 8, 6, 4, 3, 2, and 1 mm particle size were wrapped in small sachets of porous fabric resembling mosquito netting and placed in a bath with ample water. Sachets of each size were removed after 1, 2, 4, 8, and 24 hours of soaking. Phase II extraction studies were carried out exclusively with 1 mm particle-size in water adjusted to temperatures of 40 ^{o}C and 66 ^{o}C and pH of 3.0, 5.0, 9.0 and 11.0. Samples from which L-dopa had been successfully extracted were also assayed for total protein content.

Extraction with room temperature tap water was feasible only with the smallest 1 mm particle size. At this size, a safe level (0.1%) of L-dopa could be reached within approximately 55 hours (2.5 days) of soaking in a minimum of 40/1 parts water/bean. Extraction rates increased dramatically with increased water temperature, allowing safe levels to be reached within 13 h at 40 ^{o}C and 3 h at 66 ^{o}C. If extrapolated, only 40 min would be required in boiling water. Extraction rates increased further with decreasing pH at any temperature. Acidifying the water at room temperature to pH 3.0 allowed extraction to safe levels in less than 8 h. However, increasing water pH into the alkaline range appeared to induce chemical conversion of L-dopa to melanin, and is not recommended before melanin levels in the bean flour have been determined. No significant loss of protein was found in any of the samples assayed. It appeared that optimum conditions for water extraction of L-dopa from *Mucuna* bean are similar to those required for brewing of coffee. Beans must first be ground to small particle size, then extracted with boiling water.

Key words: *Mucuna*, L-dopa, water extraction, particle size, velvet bean, green manure, cover crop.

INTRODUCTION

The *Mucuna* bean (*Mucuna pruriens)*, also known as velvet bean, has great potential for use as a green manure/cover crop (GMCC) by smallholders in tropical regions of the world. It also has potentially important nutritional value as a rich source of protein (23-35%) as reported by Bressani (2002). However, its use as food or feed has been limited by the presence of anti-nutritional and toxic compounds. Daxenbichler *et al*. (1971) reported significant levels of L-dopa in *Mucuna* bean (3-7% dry weight basis), as well as other toxic compounds in smaller amounts that are present in nearly all commonly eaten beans (Bressani, 2002). One practical approach to defining safe target levels for L-dopa is to adopt the levels of L-dopa found in the faba bean or broad bean (*Vicia faba*). These beans also contain L-dopa, but to a lesser extent than *Mucuna* beans, i.e., in the order of 0.2 - 0.5% dry weight (dw) basis. Cooked faba beans and processed foods made from them have been safely consumed by a large number of people in many parts of the world over generations. On this basis, a concentration of L-dopa at 0.1% dry solids was adopted as a level which processing should reach. This means that any effective extraction process must be capable of achieving approximately 99% reduction in initial L-dopa. Removal of L-dopa by water extraction has been frequently attempted over the years but with limited success, and has often relied on trial and error to improve extraction efficiency. Scientific knowledge and understanding of basic principles of extraction have not been sufficiently brought to bear on the problem. Yet, processing of *Mucuna* beans by simple extraction with water may be a promising short-term solution for decreasing the content of L-dopa to safe levels prior to consumption.

Solvent extraction is widely used in the food, pharmaceutical and chemical industries to extract a soluble constituent from a solid by means of intimate contact with a liquid solvent. In the food process engineering literature, this process is often referred to as leaching, steeping, brewing, or diffusion, such as in the brewing of coffee, steeping of tea, and diffusion of

sugar from sugar beets. Factors important in affecting the efficacy and efficiency of solvent extraction processes include particle size, relative fluid velocity at solid/solvent interface, and solubility of solute in solvent, as well as temperature and chemical/physical properties of the solvent (Charm, 1981; Heldman and Singh, 1981; Balaban and Teixeira, 2002).

Particle size of the solid material significantly influences the rate of extraction in a number of ways. The smaller the size, the greater the surface area is at the solid-liquid interface, and therefore, the greater the rate of transfer of solute at the surface. The smaller size also reduces the distance to be traveled by the solute molecules within the particle interior as they migrate toward the surface (diffusion). However, when taken to extremes, such as with very fine powders, the circulation of liquid solvent around the particles can be impeded, thus compromising the benefit of greater surface area.

Concentration of solute in solvent should be as low as possible because the concentration gradient between the solvent and the substance is an important driving force of the extraction process. Generally, a relatively pure solvent is used initially, but as extraction proceeds, the concentration of solute in the solvent will increase in a closed batch process, and the rate of extraction will progressively decrease in an exponential decay. Agitation of the solvent increases the rate of transfer of solute from the particle surface to the bulk solution. At the same time, this rapid removal of solute from the surface maintains the maximum concentration gradient needed between surface and interior to maximize the internal rate of solute diffusion from the particle interior to the surface. Temperature can affect the rate of extraction significantly. In many cases, the solubility of the material being extracted into the solvent will increase with temperature to give a higher extraction rate. For this reason, heat is often added to maintain elevated temperatures during solvent extraction processes.

According to the Merck Index (1983), L-dopa has only limited solubility in water (66 mg in 40 mL). Assuming an initial content of L-dopa in *Mucuna* beans to be at typical maximum levels of 6-7% dry weight, this translates into the need for 40 parts of water to one part seed by weight (forty liters of water for each kilogram of beans) in a batch extraction process in order not to reach the solubility limit in water. In contrast to water, L-dopa is readily soluble in dilute solutions of hydrochloric acid as reported in the Merck index. However, no information was found in published literature concerning solubility of L-dopa in alkaline solutions. Diallo *et al*. (2002) reported that a calcium hydroxide solution appeared to outperform pure water for reducing L-dopa in *Mucuna* bean.

Objectives

The above discussion would suggest that if ample water were used along with sufficiently small particle size, extraction rates in water could improve, and perhaps even more so at higher water temperature and lower pH. Therefore, the purpose of this work was to test this hypothesis by attempting to meet the following specific objectives:

- Determine water extraction rates for L-dopa removal from *Mucuna* bean as a function of bean particle size, water circulation rate, and water temperature and pH.
- Identify optimum conditions for extracting L-dopa from *Mucuna* beans using water as the primary solvent.
- Develop a set of guidelines for effective water extraction of L-dopa from *Mucuna* beans using appropriate technology available at the smallholder or village level.

MATERIALS AND METHODS

Scope of work

Water extraction studies were carried out on *Mucuna* beans in 2 phases of work. The goal of Phase I was to determine the extraction rate of L-dopa as a function of bean particle size using room temperature tap water as a solvent. The goal of Phase II was to determine extraction rates as a function of water temperature and pH. Selected sister samples experiencing successful extraction conditions were also assayed for total protein content for comparison with initial total protein in baseline controls. Results were tabulated and presented graphically as semi-log plots of L-dopa concentration versus time for each set of extraction conditions.

Sample preparation

The *Mucuna* beans used in this study were originally grown and harvested in Mexico, but were obtained from the Animal Science Department at the University of Vermont. Approximately 3 kg of beans were available for the entire study. Moisture content of the beans was determined by measuring weight loss after 72 h of oven drying at 103 ^{o}C, and found to be approximately 10% dw. The beans were slightly kidney-shaped with major, minor and intermediate diameters of approximately 15, 11 and 8 mm, respectively (Teixeira and Rich, 2002). Beans were stored in an air-conditioned laboratory to maintain constant moisture content throughout the course of the study. The bean particle sizes studied during Phase I consisted of whole intact beans, shelled whole beans,

and chopped bean particles of 8, 6, 4, 3, 2, and 1 mm nominal diameters.

Twenty-five grams each of whole and hand-shelled beans were set aside to serve as the largest particle size with mean diameter of 11 mm. Whole beans were shelled by applying a cigar cutter to the bean midsection with just sufficient pressure to crack the shell for easy removal. Bean particles of different sizes were obtained by first cutting whole beans completely in half with the cigar cutter to produce shelled bean halves, followed by chopping shelled bean halves in a small coffee grinder for various pulses of operation. This resulted in chopped samples of wide particle size distribution. A set of standard sieves in the appropriate size range was used in a Ro-tap® machine to segregate the mixture of particles into each particle size category. Each particle size was determined on the basis of being retained by the sieve with openings for that nominal size while having passed through the openings of the next larger size sieve above it. The particle size specified for each category was a nominal particle size rounded to the nearest whole mm. The precise dimensions of screen openings in the sieve used for each nominal particle size are given in Table 1. Upon removal from the bath, samples were rinsed in tap water, patted dry with toweling, then sealed in airtight plastic bags and kept frozen until determination of residual L-dopa

In Phase II only 1 mm particle size samples were used (this particle size had exhibited the most effective extraction of L-Dopa). Sachets containing five grams each were prepared in the same manner as in Phase I, except that additional sachets were prepared for removal from the water bath at each extraction time in order to have library sister samples available for protein assays and any other repeat analyses that might have been needed. When the sachets were removed from the bath and rinsed, they were oven dried overnight at 65 ^{o}C to reduce moisture content prior to freezing. The oven-drying step was introduced in Phase II to facilitate sample preparation for laboratory analysis that would otherwise be hampered by the high moisture content experienced in Phase I samples.

Table 1. Sieve openings for Phase I experiments.

Nominal particle size (mm)	Actual sieve opening (mm)	ASTM E-11* specification
8	8.00	--
6	5.6	3.5
4	4.00	5
3	3.35	6
2	2.38	8
1	1.00	18

*American Society for Testing of Materials, Published Standard E-11

Phase I

Five-gram samples from each particle size were wrapped in tulle circles(i.e. swatches of fine net fabric similar to mosquito netting that are typically used to make party favors) purchased from a local arts and crafts shop. They were used to form porous sachets similar to teabags by twist-tying them closed with pipe cleaners. Five sachets were made for each of the eight particle sizes plus a duplicate set for replication at the 4mm particle size for a total of 45 samples. All 45 sachets were lowered at the same time into a water bath (ISOTEMP® model 1028P from Fisher Scientific) containing 28 L of tap water at 20 ^{o}C with no pH adjustment circulating at a rate of 15 L min^{-1}. This resulted in a water-to-bean ratio of approximately 120 (i.e. three times the minimum required). Note also that normal operation of the bath included circulation of the water. Thus, all Phase I experiments were carried out with circulating bath water. Sachets of each particle size were removed from the water bath after 1, 2, 4, 8, and 24 h.

Phase II

The goal of Phase II was to determine extraction rates as a function of water temperature and pH with the optimum particle size found from Phase I. Therefore, only 1mm particle size samples were used throughout Phase II of the study. A series of extractions were carried out with the water bath adjusted to different temperatures and pH following the experimental design described in Table 2. Note that an additional extraction was carried out at pH 7.0 and 20 ºC with stagnant water to determine the importance of circulation (relative fluid velocity). With exception of this one experiment, all extractions in this study were carried out with circulation in the bath.

Adjustment and control of water bath temperature was accomplished through the electronically controlled heating and cooling systems that were an integral component of the ISOTEMP® water bath. The pH was adjusted downward to nominal target levels of 5.0 and 3.0 (such as found in most citrus fruit juices) by addition of 17.4 N acetic acid to the bath water. The pH was adjusted upward to nominal target levels of 9.0 and 11 by addition of 1 N sodium hydroxide solution. After the sachets containing bean particles were immersed in the bath, the water pH was measured periodically since it tended to drift over the extraction time. The pH reported as established in Table 3 for each set of experimental conditions was the average found over the time of extraction. However, the intended pH continued to be used for the purpose of identifying the experimental conditions in reporting results.

Table 2. Phase II design of experiments showing time (in hours) at which samples were removed during water extraction at various temperatures and pH.

			pH		
Temperature (^oC)	3.0	5.0	7.0	9.0	11.0
20	1, 4, 8, 24	4, 8, 24, 48	8, 24, 36, 48, 72	2, 4, 18, 24	1, 2, 5, 8
20 (stagnant)			24, 48, 72		
40		1, 2, 4, 8	2, 4, 6, 25		
66			1, 2, 4, 8		

Table 3. Intended pH and actual pH (average of periodic measurements) at different bath water temperatures.

Temperature (^oC)	Intended pH	Actual pH
20	3.0	3.1
20	5.0	5.3
40	5.0	5.0
20 (Phase I)	7.0	6.0
20 (Phase II)	7.0	5.8
20 (Phase II, stagnant)	7.0	6.5
40	7.0	7.1
66	7.0	6.8
20	9.0	8.3
20	11.0	10.4

Analyses conducted

L-Dopa was extracted from dried powdered *Mucuna* seed using a modification of the St. Laurent *et al*. (2002) sonication method. Approximately 0.2 g of dried seed sample was weighed into a 20-mL vial, shaken to mix with 10 mL of water, and sonicated for 5 min. After allowing the residue to settle, the supernatant was transferred to a graduated tube. The residue was extracted a second time using a fresh aliquot of water with the supernatants being collected together. The combined extract was then taken to a known volume in water, usually 10 or 20 mL, and passed through a 0.45 µm PTFE syringe filter.

L-Dopa content was determined using a Hewlett-Packard HP1100 high-performance liquid chromatography system (Hewlett-Packard Company, Wilmington, DE) with autosampler, degasser, binary pump modules, and variable wavelength UV detector. Fifty microliters of each extract were injected onto an Adsorbosil C18 column (10 µm; 350 x 4.6 mm; Alltech, Deerfield, IL) held at 25 C. Separation was isocratic at 1 mL min^{-1} in 975.5:19.5:1 water:methanol:phosphoric acid at pH 2.0 (v/v/v; Siddhuraju and Becker, 2001). The analyte was detected at 280 nm quantified against a five-point standard curve (R^2 ≥0.997) composed of L-Dopa (Sigma Chemical, St. Louis, MO) diluted in mobile phase. All samples were extracted and analyzed in triplicate. All solvents used during the extractions were of reagent grade and during analysis, of HPLC grade. All water was distilled and de-ionized. L-dopa analyses were performed at the Analytical Toxicology Core Laboratory, University of Florida (Gainesville, FL).

Total protein content for several *Mucuna* bean treatments was assayed using a modification of the AOAC 984.13 method (AOAC, 2000), which uses the Kjeldahl approach briefly described in the following. Each powdered bean sample was weighed into a Kjeldahl digestion flask and digested in 15 mL of concentrated sulfuric acid with a catalyst (K_2SO_4 and $CuSO_4$ in Kjeldahl tablets; Fisher Scientific, Pittsburgh, PA) at 400–450 ^oC for 50-60 min. The samples were cooled for 10 min, and diluted in 50 mL of water with mixing to ensure dissolution of any precipitate. After loading the diluted digests into the protein/nitrogen analyzer (Tecator-Kjeltec Auto, Model 1035/38; Foss Tecator, Foss North America, Inc., Eden Prairie, MN), each sample was made alkaline by the addition of 40% w/v sodium hydroxide, and steam-distilled to free ammonia. The ammonia was trapped in a receiver solution containing boric acid (Kjel-Sorb Solution; Fisher Scientific), which was then titrated with 0.2 N hydrochloric acid (Technical Kjeldahl grade) to give a value for the amount of nitrogen released. Total protein content was calculated from the nitrogen value. Unless specified, all materials were reagent grade. Protein analyses were performed at the ABC Research Corporation (Gainesville, FL).

RESULTS AND DISCUSSION

Phase I: Particle size study

Residual L-dopa levels from the particle size study in Phase I are presented in Table 4. Note that even after 24 hours of water extraction, the larger bean particles show little more than 50% reduction, which occurs within the first 2 hours and tends to level off. Only the very smallest 1 mm particle size samples show a decline reaching a level of 0.25% (90% reduction) in 24 h. The standard deviations reported reflect the

variability found from performing assays in triplicate on each sample. The variability inherent with replication of experiments can be seen by comparing results from the two replications with 4 mm particle size. This variability accounts for why some data points show a slight increase in residual levels with additional extraction time. In reality such increase is not likely to occur, but does indicate that no significant extraction has occurred over the additional time interval.

Table 4. Particle size effect on residual L-Dopa content in *Mucuna* bean (%Wt, dw) after selected times of extraction with tap water at room temperature (pH 7.0, 20 ^{o}C).

	Extraction time (h)					
	0	1	2	4	8	24
Particle size	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)
Whole, Intact bean	4.39 (.25)	4.22 (.44)	3.89 (.57)	2.51 (.31)	2.49 (.14)	2.80 (0.11)
Whole, shelled bean	4.23 (.13)	4.13 (.30)	2.26 (.12)	2.28 (.23)	2.00 (.05)	2.39 (0.09)
8 mm	--*	3.89 (.03)	2.16 (.13)	1.68 (.04)	1.83 (.08)	2.16 (0.03)
6 mm	--	3.05 (.02)	2.03 (.15)	1.90 (.05)	1.90 (.07)	2.21 (0.06)
4 mm (a)	--	2.47 (.06)	2.17 (.05)	2.05 (.09)	1.73 (.06)	1.80 (0.12)
4 mm (b)	--	3.24 (.09)	1.73 (.17)	1.96 (.07)	1.76 (.14)	1.47 (0.10)
3 mm	--	3.68 (.06)	2.21 (.25)	1.80 (.09)	1.60 (.05)	1.45 (0.05)
2 mm	--	2.95 (.08)	1.76 (.07)	1.31 (.04)	1.41 (.12)	1.05 (0.03)
1 mm	--	1.54 (.05)	0.81 (.15)	0.65 (.04)	0.81 (.01)	0.25 (0.01)

*Baseline values were only tested for whole intact beans and whole shelled beans. It was assumed that further chopping of the beans would not alter the baseline L-dopa values.

The trend of these data to exhibit a relatively rapid rate of decline early in the extraction process that continually diminishes with time indicates that the extraction may be following a pattern of exponential decay. This is to be expected because as extraction proceeds, the concentration of solute (L-dopa) in the solvent (water) will increase, thus reducing the concentration gradient (driving force) causing the rate of extraction to progressively decrease.

Mathematically, an exponential decay is depicted as a straight line on a semi-log plot. Thus, the rate of extraction can best be quantified by plotting the data from Table 4 on a semi-logarithmic plot using log-linear regression analysis (Figure 1). The two log cycles along the ordinate axis represent a 99% reduction in initial concentration that is more than sufficient to reach the desired safe target level of 0.1%. Some of the intermediate particle sizes were omitted from the graph to allow for visual clarity. The regression analysis revealed that the extraction process seemed to be biphasic, i.e. having two phases, each with its own rate of exponential decay (as indicated by the two different slopes in the graph). This could suggest that L-dopa molecules near the particle surfaces are more easily extracted (early phase) than the L-dopa molecules trapped deep within the particle interior that must diffuse through the solid matrix of the bean structure in order to reach the surface once the surface regions become depleted (predominant phase). Linear extrapolation of the predominant phase curves can be used to predict the time required to reach the safe target level for any particle size (Table 5).

Table 5. Extrapolated times needed to reach 0.1% residual L-Dopa in *Mucuna* bean (%Wt, dw) for various particle sizes exposed to extraction with tap water at room temperature (pH 7.0, 20 ^{o}C).

Particle size (mm)	Required extraction time
3	306 h (12-13 d)
2	204 h (8-9 d)
1	55 h (2-3 d)

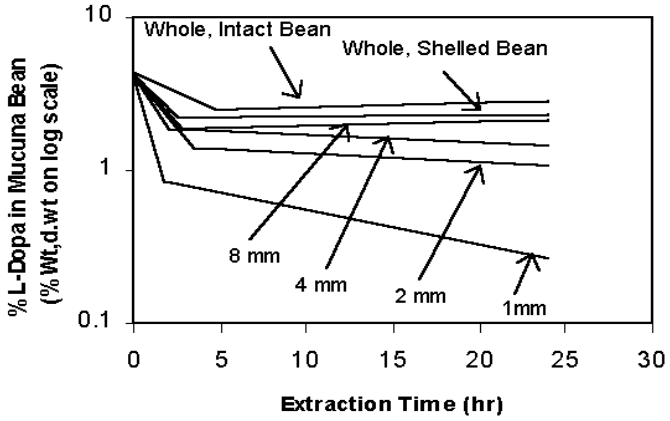

Figure 1. Particle size effect on water extraction rates of L-Dopa from *Mucuna* bean with tap water at room temperature (pH 7.0, 20 ^{o}C).

Phase II

Circulation effect

Recall that all experiments in Phase I and all but one in Phase II were carried out with circulation of bath water at a rate of 15 L min^{-1}. In order to study the effect of circulation, two additional experiments were carried out with 1 mm particles using room temperature tap water (pH 7.0, 20 ^{o}C), but with and without circulation (i.e., stagnant water). The extraction experiment with 1 mm particles using room temperature tap water in Phase 1 was also considered a replicate of the circulation experiment in Phase 2. Results from these three experiments are presented in Table 6, and shown graphically in Figure 2.

These data show that the circulation rate used in this study had no effect on extraction rate. Use of water bath circulation in this study was originally done to achieve uniform temperature distribution in the water bath. It was also expected to induce relative fluid velocity at the water-particle surface interface, but the rate was evidently much too slow to induce any significant relative velocity at the particle interface. Therefore, experiments designed with significant relative velocity should be planned for future study. Note also the wide variability in results from replicate experiments with circulating bath water. One experiment resulted in nearly twice as much residual L-dopa as the other after 24 h. Part of this variability could stem from the difference in initial baseline levels reported for Phase I and Phase II, which could be a reflection of the relatively wide standard deviation reported for some of the assay results shown in Table 4. Another contributing factor could be the difficulty in achieving precise control of particle size distribution. Within the 1 mm particle size category, particles vary in size between 1 and 2 mm, and samples with greater proportions of any one size within this range will exhibit different extraction rates from those exhibited by replicate samples prepared at another time. Across these studies, the time to reach safe target level is a range from 42 to 70 h (Figure 2). Averaging together all the data points yielded the "lumped" curve in Figure 2 as the basis for suggesting 55 h as a likely extraction time.

Temperature effect

Residual L-dopa levels from the temperature effect study in Phase II are listed in Table 7, and plotted on a semi-log graph in Figure 3. Clearly, raising the water temperature improves the extraction rate dramatically. The extraction curves shown in Figure 3 suggest that the safe target level with 1 mm particles can be reached within 13 h at 40 ^{o}C and 3 h at 66 ^{o}C using neutral pH tap water. At the highest temperature, the bath water color turned black during the extraction, which did not occur at lower temperatures. The extraction rate at each temperature can be quantified mathematically by a rate constant that can be estimated from the slopes of each curve on the semi-log plot. Moreover, by knowing the rate constant at three different temperatures, a mathematical expression can be used to predict the extraction time required to reach safe target levels for other water temperatures that were not tested, such as at boiling (Table 8).

Table 6. Residual levels of L-dopa (%Wt, dw) in 1 mm *Mucuna* bean particles after various times of extraction with tap water at room temperature (pH 7.0, 20 °C) under circulating and stagnant water bath conditions.

	Extraction time (h)					
	0	8	24	36	48	72
	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)
Phase 1 stirred	4.23 (.13)	0.81 (.01)	0.25 (.01)	--	--	--
Phase 2 stirred	3.54 (.11)	0.79 (.03)	0.43 (.02)	0.25 (<.01)	0.16 (<.01)	0.12 (<.01)
Phase 2 stagnant	3.54 (.11)	--	0.22 (.01)	--	0.07 (<.01)	0.03 (<.01)

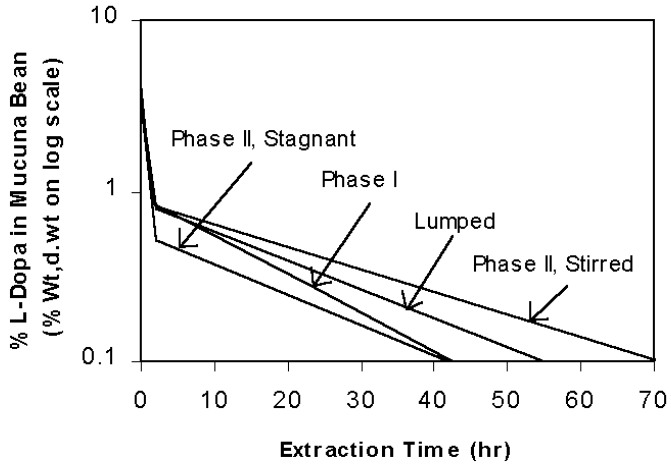

Figure 2. Effect of stirred versus stagnant water bath on extraction rates of L-dopa (%Wt, dw) from 1 mm particle size *Mucuna* bean with tap water at room temperature (pH 7.0, 20 °C).

Table 7. Residual L-dopa content (%Wt, dw) in 1 mm particles of *Mucuna* bean after selected times of extraction with tap water (pH 7.0) at different temperatures.

		Extraction time (h)						
		0	1	2	4	6.5	8	24
Temperature (°C)	pH	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)
20	7.0	3.54 (0.11)	1.54 (0.05)	0.81 (0.15)	0.65 (0.04)	--	0.81 (0.01)	0.25 (0.01)
40	7.0	3.54 (0.11)	--	0.58 (0.02)	0.37 (<0.01)	0.17 (<0.01)	--	<0.02
40	5.0	3.54 (0.11)	0.92 (0.03)	0.56 (0.01)	0.32 (<0.01)	--	0.09 (<.01)	--
66	7.0	3.54 (0.11)	0.45 (0.03)	0.11 (0.01)	0.03 (0.01)	--	0.003 (<.001)	--

Table 8. Extrapolated times needed to reach 0.1% residual L-dopa in *Mucuna* bean (%Wt, dw) for 1 mm bean particles exposed to extraction with tap water (pH 7.0) at different temperatures.

Temperature (^oC)	Required extraction time (h)
20	55
40	13
66	3
100	<1 (40 min)

Acid range pH effect

Residual L-Dopa levels in response to adjusting pH in the acid range with water at room temperature are listed in Table 9, and plotted on a semi-log graph in Figure 4. These results also show dramatic increase in extraction rates with 1 mm particles in acid solution at pH 3, similar to increasing temperature to 66^{o}C with pH neutral tap water. Extrapolated times needed to reach safe target level for selected conditions of pH and temperature in the acid range are given in Table 10. Note that even at just pH 5.0, safe target levels can be reached within 8 hours if water is heated to only 40 ^{o}C.

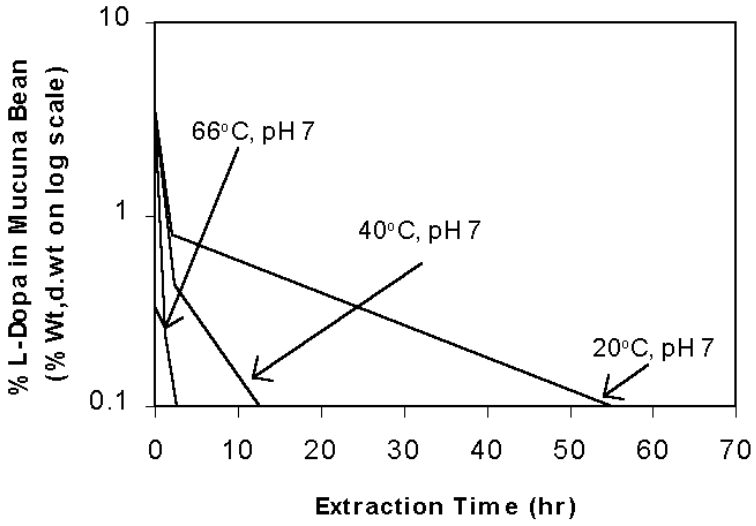

Figure 3. Water extraction rates of L-dopa from 1 mm particle size *Mucuna* bean using tap water (pH 7.0) at different temperatures.

Table 9. Residual L-dopa content in 1 mm *Mucuna* bean particles (%Wt, dw) after selected times of water extraction at 20 ^{o}C (pH 3.0, 5.0, and 7.0), and at 40 C (pH 5.0 only).

		Extraction time (h)											
		0		1		2		4		8		24	
pH	Temperature (C)	%	(SD)	%	(SD)	%	(SD)	%	(SD)	%	(SD)	%	(SD)
3.0	20	3.54	(0.11)	0.40	(0.01)	--*		0.23	(0.01)	0.04	(<0.01)	0.01	(<0.01)
5.0	20	3.54	(0.11)	--*		--*		0.65	(0.02)	0.49	(0.01)	0.20	(0.01)
5.0	40	3.54	(0.11)	0.92	(0.03)	0.56	(0.01)	0.32	(<0.01)	0.09	(<0.01)	--*	
7.0	20	3.54	(0.11)	1.54	(0.05)	0.81	(0.15)	0.65	(0.04)	0.81	(0.01)	0.25	(0.01)

* No samples taken for judicious laboratory cost savings

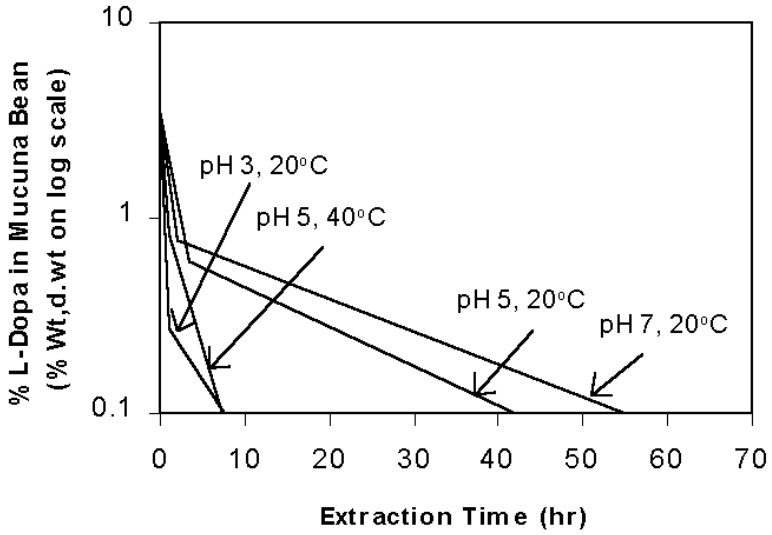

Figure 4. Water extraction rates of L-dopa from 1 mm particles *Mucuna* bean after selected times of water extraction at 20 ºC (at pH 3.0, 5.0, and 7.0), and at 40 ºC (at pH 5.0 only).

Table 10. Extrapolated times needed to reach 0.1% residual L-dopa content (%Wt, dw) in 1 mm particles of *Mucuna* bean exposed to water extraction at 20 °C (at pH 3.0, 5.0, and 7.0) and at 40 °C (at pH 5.0 only).

pH	Temperature (°C)	Required extraction time (h)
3.0	20	8
5.0	20	42 (1-2 d)
5.0	40	8
7.0	20	55 (2-3 d)

Alkaline range pH effect

Residual L-Dopa levels in response to adjusting pH in the alkaline range with water at room temperature are listed in Table 11, and plotted on a semi-log graph in Figure 5. Increasing alkalinity of the water to pH 11.0 also showed equally dramatic decline in residual L-dopa. However, the bath water immediately turned totally black during these extraction experiments at high pH. This near instantaneous color change suggests that the decline in residual L-dopa levels observed at high pH may be the result of a chemical conversion of L-dopa to melanin, rather than actual extraction into the water. Samples of water extract at pH 7.0 and pH 11.0 were assayed for residual L-dopa. Results showed expected concentration levels at pH 7.0 but none detected at pH 11.0, which further suggests chemical conversion to melanin. No attempt was made to detect melanin within the bean particles. Therefore, it is unknown whether or not the beans may contain any residual melanin. This should also be a topic for further study.

Table 11. Residual L-dopa content in 1 mm particles of *Mucuna* bean (%Wt, dw) after selected times of water soaking at 20 °C at different alkaline pH levels.

	Extraction time (h)							
	0	1	2	4	5	8	18	24
pH	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)	% (SD)
7.0	3.54 (0.11)	1.54 (0.05)	0.81 (0.15)	0.65 (0.04)	--*	0.81 (0.01)	--*	0.25 (0.01)
9.0	3.54 (0.11)	--*	1.07 (0.06)	0.74 (0.05)	--*	--*	0.41 (0.01)	0.29 (0.01)
11.0	3.54 (0.11)	1.49 (0.02)	0.98 (0.09)	--*	0.59 (0.01)	0.11 (0.01)	--*	--*

* No samples taken for judicious laboratory cost savings

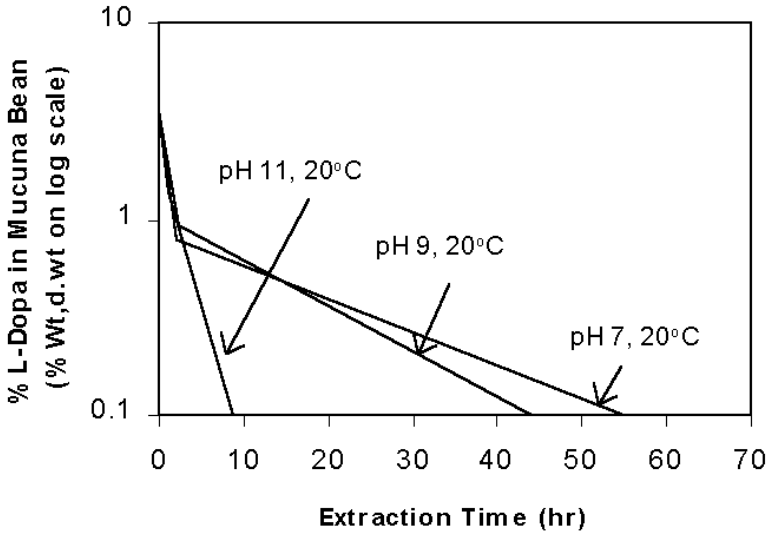

Figure 5. Reduction rate of L-dopa from 1 mm particles of *Mucuna* bean soaked in water at 20 °C at different alkaline pH levels.

Protein retention

Results from determination of residual total protein in selected sister samples from Phase II are presented in Table 12. These results reveal that very little, if any, loss of total protein occurred under any of the extraction conditions studied. The small differences reported among extraction conditions fall within the standard deviation of the protein analysis. The standard deviation is larger than normal for total protein assays because the sample sizes that were available and submitted for protein analysis were too small for good reproducibility (5 g instead of 10 g normally required). Results from a subsequent appropriate baseline sample size indicated initial protein content of 22.3%. The minimal loss of protein is surprising because some leaching of protein was expected to occur, particularly at the higher temperatures and pH. Myhrman (2002) reported about 50% loss of protein from *Mucuna* bean flour (very small particle size) after several successive soakings in room temperature tap water, but less than 10% loss from larger bean fragments (4-5 per bean) even after boiling water extraction. One possible explanation was that, even at 1 mm particle size, the mean distance of migration for large protein molecules was still too great to accomplish significant leaching. To test this hypothesis, protein assays on samples of fine powder made from the *Mucuna* beans used in this study were made and revealed only 11% loss of protein after soaking 44 hours in tap water at room temperature (Table 13). Because of these surprising results, an attempt should be made to duplicate this work by others for confirmation.

PRACTICAL GUIDELINES AND RECOMMENDATIONS FOR WATER EXTRACTION

Tap water at room temperature

Results from this work show that residual L-dopa in *Mucuna* bean can be reduced to a safe level by extraction with water, provided beans are shelled and ground to a minimum 1 mm nominal particle size and soaked in ample water (40/1) for sufficient length of time. Although the ratio of water to bean used in this study was three times the minimum required, extraction rates should be no different because excess water beyond the minimum has negligible effect on concentration gradient. Three days extraction time is recommended with water at room temperature, while one hour is sufficient with boiling water. Just as in brewing of coffee, highest extraction rates are achieved with ground beans soaked in near boiling water. Fuel requirements for boiling large quantities of water can be costly, so practical methods for water extraction at room temperature should be considered. Where access to a nearby flowing stream or river is possible, ground beans can be held in a porous sack and submerged into a flowing river or stream for the necessary three days extraction time. This method

brings a constant supply of continuous-flowing pure solvent in contact with the bean particles but requires little energy, labor, and equipment. Alternatively if pressurized tap water is available, ground beans can be packed in a simple adsorption column with a continuous supply of tap water entering at the bottom and overflowing from the top for subsequent use in washing or irrigation. A simple column can be fabricated from a section of PVC pipe with removable screens at the top and bottom for holding the ground beans between end caps equipped with garden hose fittings.

Table 12. Residual total protein and L-Dopa remaining in 1mm *Mucuna* bean particles after water extraction at different temperatures and pH and in stagnant vs. flowing water.

pH	Temperature (°C)	Extraction time (h)	Total protein (%)*	L-dopa (%Wt, DW)
--	--	0 (Baseline)	21.9	3.54
3.0	20	4	20.3	0.23
3.0	20	8	20.0	0.04
5.0	20	49	23.2	0.08
5.0	40	8	23.5	0.09
7.0	20	48	18.5	0.16
7.0	20	72	17.8	0.12
7.0	20 (Stagnant water)	24	18.1	0.22
7.0	20 (Stagnant water)	48	22.0	0.07
7.0	20 (Powder)	44	21.9	0.06
7.0	40	25	22.3	< 0.02
7.0	66	2	19.0	0.11
9.0	20	24	24.1	0.29
11.0	20	8	18.4	0.11

* STD 2.4%

Table 13. Residual total protein remaining in powdered *Mucuna* bean particles (< 1 mm) after water extraction at pH 7.0, 20 ºC.

Extraction Time (h)	Total Protein (%)	L-dopa (%Wt, DW)
0 (Baseline)	24.7	4.10
44	21.9	0.06

Heated tap water

Results from the study on temperature effect showed that raising water temperature could improve extraction rate dramatically with 1 mm particles. The data suggested that the safe target level with 1 mm particles could be reached within 3 h using neutral pH tap water heated to 66 °C. However, the cost of fuel consumption to achieve the heating must be considered, particularly in light of the high water-to-bean ratio required. The increased extraction rate with increased water temperature may also signal the possibility that the solubility of L-dopa in water at higher temperatures may also increase. If so, this could lead to a significant reduction in quantity of water needed to be heated. Solubility of L-dopa in water at high temperatures needs to be known from further study in order to determine the minimum water quantity for extraction at higher temperatures.

Acidification

Results have also shown that dramatic increase in extraction rates with 1 mm particles is possible in an acid solution at pH 3.0, achieving rates similar to increasing temperature to 66 °C with pH neutral tap water. However, bringing the quantity of water needed to this level of acidity with food grade acid ingredients may not be practical. For example, extraction of 1 kg of beans would require approximately 500 mL of lemon juice to be added to 40 L of water (assuming the same solubility limit for L-dopa as in pH neutral water). However, just as with the temperature effect, the solubility of L-dopa in water at lower pH needs to be known from further study in order to determine the minimum quantity of water required for extraction with acid solutions.

Alkalinity

Increasing alkalinity of the water to pH 11.0 also showed dramatic decline in residual L-dopa at room temperature. However, the black color of the extract water suggests that the decline may be the result of chemical conversion of L-dopa to melanin, rather than actual extraction into the water. Samples of water

extract at pH 11.0 were assayed for residual L-dopa, and none was detected. No attempt was made to detect melanin within the bean particles, themselves. Therefore, it is unknown whether or not the beans may contain any residual melanin. This should also be a topic for further study.

CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDY

- Residual L-dopa in *Mucuna* bean can be reduced to a safe level by extraction with water, provided beans are shelled and ground to a minimum 1mm nominal particle size and soaked in sufficient quantity of water (40/1) for sufficient length of time (approximately 55 hours at room temperature, based upon extrapolation data).
- Improved extraction rates can be achieved by either heating the water at the expense of fuel consumption, or carrying out the extraction at room temperature in an acid solution at the expense of fruit consumption (e.g. 10 mL lemon juice per liter tap water to achieve pH 3.0).
- Solubility studies are needed to determine solubility of L-dopa in pH neutral tap water at high temperatures, as well as in room temperature water at lower pH. Findings from these studies will help to determine the minimum water quantity for extraction at these alternate conditions.
- Further study is needed on the fate of L-dopa in *Mucuna* bean when treated with alkaline solutions (at higher pH) in order to determine if the bean particles, themselves, contain any melanin in spite of melanin in the extract water.

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