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Production of leaf protein concentrates from cassava: Protein distribution and anti-nutritional factors in biorefining fractions

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ABSTRACT

Nowadays, cassava leaves are mostly treated as a byproduct of cassava root production, yet this readily available biomass is rich in protein with a balanced content of amino acids. Cassava leaves therefore represent a promising, underutilized biomass for extraction of proteins. The purpose of this study was to provide updated information on the feasibility of producing cassava leaf protein concentrate for use in feed and food. In this context, protein concentrates were refined from cassava leaves using different precipitation methods and the refining process evaluated with focus on protein, amino acids and selected antinutritional factors. Crude protein was mainly distributed to the press cake and protein concentrates during the two processing steps, i.e., pressing and precipitation, and between 21% and 26% (w/w) of leaf crude protein was recovered in the concentrates. After drying, these contained 40–45% crude protein with an amino acid profile comparable to soybean and tolerable levels of tannins (>1% of TS) for feed purposes. However, the refining process did not significantly reduce the cyanogenic potential, i.e., the total amount of releasable HCN, which accumulated in the dried protein product to around 150–250 ppm. This lies significantly above the 10–50 ppm deemed safe for food and feedstuff by several food safety authorities. Based on these results, extraction of leaf protein from cassava appears promising, but additional research is required to evaluate its full potential, especially in relation to its use in food products.

1. Introduction

The cassava plant (*Manihot esculenta*) is extensively grown and a highly important crop in many African, Asian, and Latin American countries. It is primarily cultivated for its tuberous root with an estimated 275.7 million tons fresh weight produced globally in 2017 (FAO, 2018). The tuberous root is a compact source of carbohydrates and a regular part of the daily diet for millions of people worldwide. However, they contain only little protein, a macronutrient that is often limited in many areas of production. In contrast, cassava leaves are rich in protein, and typically contain 20–40% crude protein on dry matter basis with an essential amino acid content comparable to that of hen's egg (Awoyinka et al., 1995; Lancaster and Brooks, 1983). They also contain large amounts of vitamins such as vitamin B_1/B_2 and minerals like magnesium and calcium (Ravindran and Ravindran, 1988). The leaves of the cassava plant are processed and consumed regularly as a source of protein in several Asian and African countries (Burns et al., 2012a; Hidayat et al., 2002). An example is Ntoba Mbodi, a traditional food from central Africa obtained through alkaline, semi-solid fermentation of cassava leaves (Kobawila et al., 2005). However, the leaf biomass is still largely considered a byproduct of the cassava tuberous root production, being either burnt or left to decompose in the field. This can be attributed to their high content of cyanogenic glucosides, mainly linamarin and lotaustralin, that upon cellular disruption, are converted to hydrogen cyanide. This cyanide potential (total cyanide content) can range from 80 to 1860 mg/kg fresh leaves (Lancaster and Brooks, 1983), which if not processed correctly, can cause severe cyanide poisoning in humans and animals (Montagnac et al., 2009). In addition, the leaves contain high amounts of lignocellulose and polyphenols (tannins), which can reduce nutrient availability and digestibility (Chung et al., 1998; Ravindran and Ravindran, 1988).

Nonetheless, due to its high content of crude protein and suitable amino acid profile, cassava leaves represent a promising biomass for extraction of proteins into so-called CLPC; cassava leaf protein

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concentrate (Latif and Müller, 2015). CLPC is typically produced through a two-step process with an initial mechanical pressing resulting in a liquid fraction from which proteins can then be extracted through thermocoagulation or pH-precipitation, amongst other methods (Coldebella et al., 2013; Latif and Müller, 2015; Santamaría-Fernández and Lübeck, 2020). Furthermore, when extracting proteins into CLPC, issues related to cyanide toxicity should be reduced through partial conversion and/or removal of cyanogenic glucosides and degradation products (Balasundaram et al., 1976; Fafunso and Bassir, 1976). The yield of fresh cassava leaves varies considerably, depending upon factors such as cultivar, planting density and the harvesting scheme, but can be like that of the roots (~10 tons fresh weight/ha) and even higher (>20 tons fresh weight/ha) if produced under optimal conditions (Lancaster and Brooks, 1983; Müller, 1977). Thus, if managed correctly CLPC could represent a broadly available and valuable addition to the production of tuberous root-based products.

CLPC has been produced by several research groups with varying results. In one study, leaf protein extracted from 15 different cassava varieties using pressing and heat precipitation displayed a relatively stable total nitrogen extractability of 56–61%. The produced CLPC also displayed similar *in vitro* protein digestibility of 53–61% across the cultivars (Fafunso and Oke, 1976). In another study, CLPC obtained through blending and acid precipitation contained 42–51% crude protein, with an essential amino acid profile comparable to FAO references, except for methionine (Tupynambá and Vieira, 1979). The same study also demonstrated very low protein efficiency ratio (PER) values for rats fed a diet with the CLPC as the protein source, even when supplemented with methionine. Studies with poultry, however, indicate that CLPC could be used as protein source in broiler starter, replacing up to 60% of fish meal with no deleterious effects (Fasuyi and Aletor, 2005a; Ravindran, 1993). More recently, a study combining pulping, grinding and heat precipitation produced dried CLPC from different varieties, containing up to 48.8% crude protein and non-toxic levels (<10 ppm) of cyanide when using variety TME 419 (Oresegun et al., 2016).

Despite a large initial interest in CLPC during the 1970's, no examples of commercial production could be found during this study. As many studies on the concept are now either quite dated (Balasundaram et al., 1976; Castellanos et al., 1994; Fafunso and Oke, 1976; Fafunso and Bassir, 1976; Tupynambá and Vieira, 1979) or published in low accessibility journals (Coldebella et al., 2013; Modesti et al., 2007; Oresegun et al., 2016), there is a need for updated information to reliably evaluate the feasibility of producing CLPC for use in feed and food. Therefore, this project sought to explore the production of CLPC using different protein precipitation methods and evaluating the content of important feed- and foodstuff components in the biorefinery fractions. To evaluate the efficacy of the refining process, mass distributions based on total solids and crude protein were constructed and final recovery levels determined for each precipitation method. Furthermore, by analyzing the amino acid composition, cyanogenic potential, and polyphenol/tannin content in all refinery fractions, both the suitability of the produced CLPC as feed and/or food was assessed as well as the distribution of these components throughout the biorefinery process.

2. Materials & methods

2.1. Cassava leaf material

Leaf biomass from cassava cultivar *TME12* was used in this experiment. The cultivar was grown in green house facilities belonging to the University of Copenhagen in Taastrup, DK. The plants were vegetatively grown from stakes and have been growing for 5–6 years with cut down twice a year. Due to this continued growth scheme, the exact age of the processed biomass was unknown. The leaf biomass was provided as larger stems with top shoot and 10–20 mature leaves. The material was processed shortly after harvest (max. 2 h).

2.2. Mechanical separation

Initially, the top shoot and larger leaves were separated by hand from the main stem. The separated leaf biomass was then processed into two fractions (green juice and press cake) through screw pressing using a twin gear Angelia 8500S juicer (Angel Juicer Co., AU). The produced green juice was immediately used for protein precipitation.

2.3. Protein precipitation methods and oven drying

Three methods of protein precipitation were utilized: heat precipitation (based on thermocoagulation), acid precipitation (based on isoelectric precipitation) and spontaneous fermentation (based on isoelectric precipitation). The latter relied on the ability of the leaf's native microbiome to lower the solution pH during consumption of free sugar in the juice. The methods were each applied to 300 mL of fresh green juice. For the spontaneous fermentation, the juice was transferred to a clean 0.5 L blue cap bottle and left to ferment overnight (~20 h) at 38 °C with shaking (150 RPM). For heat precipitation, the juice was pumped through a heated glass coil with 20 s transfer time and an outgoing temperature of 80 °C. For acid precipitation, the juice was slowly titrated with 6 M HCl until the pH reached 4.00. All treatments ended with the processed juice being centrifuged (5000 g, 15min, 20 °C) and the solid fraction (protein concentrate, LPC) isolated. The residual juice is called brown juice, due to its color after protein and chlorophyl removal.

With the aim of mimicking a drying method feasible in areas of cassava production, part of the isolated protein concentrate was dried overnight (20 h) at 45 °C in a forced-air oven to produce dry protein powder. The remaining LPC, together with samples of the other refinery fractions, were stored at −20 °C until further analysis.

2.4. Analysis of total solids

Total solids (TS) were determined using standard methodology. Briefly, biomass was added to porcelain crucibles and subjected to drying at 105 °C (typically overnight) until reaching a constant weight. Weight measurements before and after drying were then used to calculate the percentage of TS in the fractions. All samples were analyzed in triplicates.

2.5. Biomass powder preparation

For most of the subsequent composition analyses dried powder was required. Subsamples from all refinery fractions (except the oven dried LPC) were freeze-dried using a Telstar LyoQuest −55 (Azbil Co., JP). Dried samples (including the oven dried LPC) were then ground through ball milling (10 × 30 s at 600 RPM) with a Pulverisette 6 ball mill (Fritsch, DE). Powder samples were stored within airtight containers in the dark at room temperature.

2.6. Crude protein content

Total nitrogen was determined using a FlashSmart Elemental Analyzer (Thermofisher, Waltham, MA, USA) according to the manufacturer's instructions. Nitrogen measurements were carried out in technical triplicates, using a standard curve based on methionine and urea as control samples (~46% N). Total nitrogen was then converted to crude protein (CP) using a conversion factor of 6.25 for mass-N to mass-protein.

2.7. TS- and CP distributions and recovery levels

Distribution of total solids and crude protein was determined for the whole biorefinery process, assuming steady-state operation at every separation step. Calculations were based on composition analysis (%TS

& %CP) and the wet weight (%WW) of each fraction:

$\mathbf{TS_D}$: $(\%TS_{fraction} * \%WW_{fraction}) / \%TS_{input}$

$\mathbf{CP_D}$: $(\%TS_{fraction} * \%CP_{fraction} * \%WW_{fraction}) / (\%TS_{input} * \%CP_{input})$

Where TS_D: total solids distribution and CP_D: crude protein distribution. Input refers to the fresh leaf material before pressing and green juice prior to protein precipitation, respectively.

Mass distributions were used to calculate the final TS- and CP-recovery in the LPCs:

$\mathbf{TS_R}$: $TS_{D\ (GJ)} * TS_{D\ (LPC)}$ $\mathbf{CP_R}$: $CP_{D\ (GJ)} * CP_{D\ (LPC)}$

Where TS_R: total solids recovery, CP_R: crude protein recovery, and GJ: green juice.

2.8. Amino acid analysis and amino nitrogen

The amino acid profile of the different fractions wase determined using the method described in Dahl-Lassen et al. (2018) and La Cour et al. (2019) and reported on a DM basis.

In short, three hydrolysis were performed: 1) An acid hydrolysis was performed using 6 M HCl with 0.1% w/v phenol at 110 °C for 24 h. After hydrolysis, the samples were neutralized with 6 M NaOH. 2) An oxidative hydrolysis for analysis of the sulfur-containing amino acids; oxidation with performic acid for 1 h at room temperature was performed prior to hydrolysis. Solid sodium metabisulfite was added to quench the reaction. The hydrolysis then proceeded as described above. 3) An alkaline hydrolysis for analysis of tryptophan; 20 mg of sample was mixed with 50 mg ascorbic acid, and 3 mL 4 M LiOH, followed by hydrolysis at 110 °C for 20 h. The samples were then neutralized with 2 mL 6 M HCl. Derivatization of amino acids was done using the analytical grade AccQ-Tag kit (Waters, Millford, MA, USA). Pierce Amino Acid standard H (Waters, Millford, MA, USA) supplemented with cysteic acid, methionine sulfone, and hydroxyproline was used as the standard. Cell-free 13C–15N-labeled amino acid mixture (Sigma-Aldrich, St. Louis, MO, USA) was added as the internal standard. Separation was performed on a Waters UPLC system (Waters, Millford, MA, USA) using a Waters Cortecs UPLC C18 (1.6 μm particle size, 2.1 × 150 mm) column with a VanGuard Cortecs UPLC C18 (1.6 μm particle size 2.1 × 5 mm) guard column. Quantification was done on a Waters QDa single quadrupole mass detector.

AA-measurements were carried out in technical duplicates. True protein content was estimated based on the total AA-content and a conversion factor of 0.862 to adjust for added water during hydrolysis (Feng et al., 2016). The percentage of amino nitrogen was then calculated as true protein divided by total (crude) protein.

2.9. Cyanogenic potential

To be able to evaluate the potential release of cyanide from the different fractions, the cyanogenic potential was investigated using a modified version of (Halkier and Møller, 1989). Analysis of cyanogenic potential was performed on all fractions. Immediately after producing a fraction, three times 10 mg were sampled and 180 μL tricine buffer (50 mM, pH 7.9) was added to sample and then boiled for 10 min to avoid any interfering proteins/enzymes. The samples were then flash frozen in liquid nitrogen and stored at −80 °C for further analysis. Subsequently, the samples were thawed, and determination of cyanogenic potential performed according to (Jørgensen et al., 2005). In brief, purified linamarase, prepared according to Jørgensen et al., (2005), was added to the frozen samples to degrade any present cyanogenic glucosides. After 20 min the samples were thawed, and the cyanide release was determined by adding glacial HOAc followed by the addition of reagent A (50 mg succinimide and 125 mg N-chlorosuccinimed in 50 mL water) and then adding reagent B (3 g barbituric acid and 15 mL pyridine in 35 mL water). The samples were thoroughly mixed, and the cyanide content was then determined spectrophotometrically (580 nm–750 nm scan) after 5 min incubation.

2.10. Total polyphenol and tannin content

The total polyphenol content (TPP) was determined based on the Folin-Ciocalteu method described by (Singleton and Rossi, 1965) with slight modifications. In brief, 200 mg dried and milled sample was mixed with 10 mL acetone:water (70:30 v/v) for 40 min in a Vevor ultrasonic cleaner bath (Vevor, US). After centrifuging, 100 μL extract was mixed with 1.9 mL MQ, 0.5 mL Folin-Ciocalteu reagent, and 2.5 mL of $NaCO_3$ 7% (v:w). After 40 min, absorbance was measured at 755 nm using an uniSPEC 2 UV/VIS spectrophotometer (LLG, DE). An additional 100 μL of the extract was mixed with 50 mg of the insoluble polymer polyvinylpolypyrrolidone (PVPP) and then 1.9 mL MQ, 0.5 mL Folin-Ciocalteu reagent, and 2.5 mL of $NaCO_3$ 7% (v:w) (Makkar et al., 1993). This was done to precipitate tannins out of the solution. Samples were vortexed and after 40 min, absorbance was again measured at 755 nm. The total tannin content (tannic acid equivalents) was then determined as the difference between the two absorbance readings, converted using a standard curve with tannic acid (0–10 μg/mL). All measurements were done in triplicates.

3. Results

3.1. Total solids and crude protein composition

The composition of the different fractions obtained during the biorefinery process in terms of total solids and crude protein is presented in Table 1. Crude protein was measured by an elemental analyzer and calculated on the basis of total N. The used leaf material contained 21.3% total solids with 30.1% being crude protein. After screw pressing, the total solids content increased with roughly 80% in the remaining press cake, whilst it was halved in the produced green juice. The content of crude protein (in the solids) remained stable for the three fractions (30–32%). Subsequent precipitation and centrifugation of the green juice resulted in leaf protein concentrates (wet) with a total solids content like the leaf material (19–25% TS). In contrast, the crude protein content (in the solids) ranged from 42 to 47% in the concentrates, which was a 40–57% increase compared to the starting leaf material. After 20 h of oven drying at 45 °C (to mimic a locally feasible drying method), the leaf protein concentrates (dry) contained roughly 93% total solids with

Table 1
Total solids (TS) and crude protein (CP) in the biorefinery fractions. Standard deviations are shown in brackets. LM is leaf material, PC is press cake, GJ is green juice, BJ is brown juice, and LPC is leaf protein concentrate. The abbreviations Ferm., Acid and Heat represent the protein precipitation methods using fermentation, acid or heat treatment.

Fraction		TS in fraction (%)	CP in TS (%)
LM		21.3 (±0.6)	30.1 (±0.1)
PC		38.9 (±0.4)	31.4 (±0.2)
GJ		10.3 (±0.1)	30.6 (±0.1)
BJ	Ferm.	6.81 (±0.1)	25.5 (±0.1)
	Acid	6.22 (±0.1)	17.1 (±0.2)
	Heat	6.58 (±0.1)	18.1 (±0.1)
LPC (wet)	Ferm.	21.2 (±0.9)	41.9 (±0.2)
	Acid	25.1 (±0.1)	46.9 (±0.1)
	Heat	19.6 (±0.4)	43.0 (±0.1)
LPC (dry)	Ferm.	92.6 (±0.3)	40.4 (±0.2)
	Acid	93.5 (±0.1)	45.1 (±0.2)
	Heat	93.8 (±0.2)	42.2 (±0.2)

slightly reduced crude protein levels (Table 1).

3.2. Distributions and final recovery

Table 2 shows the distribution of total solids and crude protein throughout the refinery process. These distributions were based on measured wet weight recoveries during fractionation, which was 62% for the green juice and 22–30% for the protein concentrates (data not shown). During screw pressing, most of the total solids and crude protein present in the leaf material remained in the solid fraction, i.e., the press cake. At the protein precipitation step, total solids were distributed more evenly between the two produced fractions, while most of the crude protein available in the green juice (approx. 68–85%) was recovered in the solid fraction, i.e., the wet protein concentrates (LPCs).

The final total solids- and crude protein-recovery levels using the different precipitation methods are presented in Fig. 1. Heat and acid precipitation resulted in very similar recovery levels, while the spontaneous fermentation resulted in slightly lower recovery of both total solids and crude protein. However, no significant change in neither pH nor the free glucose content of the green juice was detected during the incubation period, indicating limited or no microbial growth (data not shown).

3.3. Amino acid profiles

Amino acid profiles, i.e., the content of 18 amino acids in g/kg total solids, for the produced fractions are presented in Table 3. The total content of amino acids (AA-total) and essential amino acids (EAA-total) is also shown, with the latter being the sum of the amino acids His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val. The amino acid profile was very similar for the starting leaf material and the press cake, with both AA- and EAA-totals remaining stable. For most amino acids, except aspartic- and glutamic acid, the content was slightly lower in the produced green juice. All amino acids were further reduced in content in the brown juice fractions after precipitation (Table 3). In contrast, the content of all amino acids increased in the protein concentrates relative to the leaf material, with acid precipitation resulting in the highest AA- and EAA-totals (approx. 421 and 196 g/kg TS). Of the three used precipitation methods, the spontaneous fermentation resulted in the protein concentrate with the lowest content of amino acids, again indicating an insufficient precipitation process. Profiles of the oven dried LPCs were like the wet LPCs, albeit with slightly lower content for most amino acids. The AA-profiles of the LPCs from acid precipitation and heat coagulation (both wet and dry) were comparable to the soybean reference, with the content of some essential amino acids, e.g., methionine, leucine, and valine, being higher (Table 3). The AA-totals and EAA-totals of these two LPCs (both wet and dry) were also on level with or higher than the estimated total content for the soybean reference (not including tryptophan).

3.4. Amino nitrogen content

Amino nitrogen, i.e., the percentage of nitrogen originating from amino acids, in the biorefinery fractions is presented in Fig. 2. For the leaf material and press cake, amino nitrogen constituted roughly 79% of total nitrogen. This level was reduced with 13.5% in the green juice and dropped even further in the brown juices. In the wet LPC, amino nitrogen was at a similar level to that of the leaf material, ranging from roughly 72%–80%. The percentage of amino nitrogen was slightly reduced in the dried LPCs, indicating a loss of AA's during the drying process (Fig. 2). Of the three used precipitation methods, the heat coagulation resulted in the dry protein concentrate with the highest percentage of amino nitrogen, again at a comparable level with the soybean reference.

Table 2
Total solids (TS) and crude protein (CP) distribution in the biorefinery process steps using the thre different precipitation methods. The percentage of error in the mass balance caused by process loss and/or technical variation in analytical procedures is also shown.

Process step	TS-distribution (%)			CP-distribution (%)		
	Liquid	*Solid*	*Error*	*Liquid*	*Solid*	*Error*
Screw pressing	30.1	65.3	−4.54	30.6	68.2	−1.15
Fermentation	49.9	49.6	−0.47	41.6	67.9	9.48
Acid precipitation	47.8	54.9	2.72	26.8	84.3	11.1
Heat coagulation	43.7	57.5	1.19	25.9	81.0	6.85

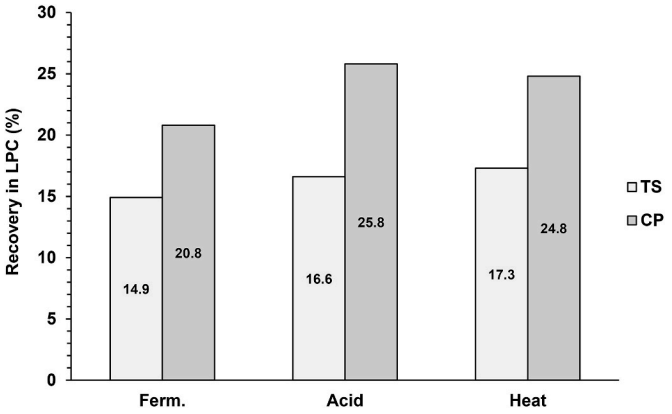

Fig. 1. Final total solids (TS)- and crude protein (CP)-recovery levels in the produced LPC fractions. The abbreviations Ferm., Acid and Heat represent the protein precipitation methods, using fermentation, acid or heat treatment.

3.5. Cyanogenic potential

Table 4 shows the cyanogenic potential of all the biorefinery fractions, expressed as CN-equivalents in parts per million (ppm) on weight basis. The starting leaf material contained 42.6 ppm, which increased slightly in the produced press cake and green juice fractions. After heat- and acid precipitation, there was a small decrease in the cyanogenic potential of the wet LPCs, whilst the fermentation resulted in wet LPC with a 40% higher potential compared to the green juice. Of the three used precipitation methods, the acid precipitation resulted in wet LPC with the lowest cyanogenic potential. After oven drying, the cyanogenic potential of the LPCs (on weight basis) increased significantly, ranging from roughly 150 ppm to 260 ppm. In contrast, when viewing the cyanogenic potential on a total solids' basis, there was a small decrease in the LPCs potential (10–15%) after oven drying (Table 4).

3.6. Total polyphenols & tannins

The total polyphenol and tannin content (as percentage of total solids) in the different biorefinery fractions is presented in Fig. 3. Brown juice samples were excluded from the analysis due to technical reasons. The starting leaf material contained roughly 1.63% polyphenols and 0.34% tannins, and the produced green juice had a similar polyphenol content, but with roughly half the tannin content. In the wet LPCs, the total polyphenol content was reduced compared with the green juice, while the total tannin content increased with 70–150%. Similar to the cyanogenic potential, the total polyphenol and tannin content increased in the oven dried LPC (Fig. 3).

4. Discussion

4.1. Composition of biorefined fractions

The measured total solids (TS) and crude protein (CP) in the fresh

Table 3
Amino acid profiles of the biorefinery fractions. LM is leaf material, PC is press cake, GJ is green juice, BJ is brown juice, and LPC is leaf protein concentrate. The abbreviations Ferm., Acid and Heat represent the protein precipitation methods, using fermentation, acid or heat treatment. Content of 18 amino acids in g/kg TS for the different fractions in comparison to soybean. The total content of amino acids (AA total) and essential amino acids (EAA total), defined as the sum of His, Ile, Leu, Lys, Met, Phe, Thr, Trp, and Val, are also shown. Trp value not included in soybean totals. Soybean reference is from Steenfeldt and Hammershøj (2015).

Amino acids	LM	PC	GJ	BJ			LPC (wet)			LPC (dry)			Soybean
(g/kg TS)				Ferm.	Acid	Heat	Ferm.	Acid	Heat	Ferm.	Acid	Heat	
Alanine	17.4	17.4	15.2	9.5	4.6	4.9	21.8	26.0	24.8	20.6	23.2	22.7	16.9
Arginine	17.0	16.8	14.5	7.6	2.9	2.9	22.0	26.8	25.7	20.8	22.8	23.1	31.4
Aspartic acid	25.7	24.5	27.5	20.8	16	16.7	32.4	37.4	36.8	30.5	34.9	34.3	43.8
Cysteine	4.7	4.0	4.9	2.6	2.7	3.0	4.1	6.2	4.9	4.0	5.1	4.1	5.8
Glutamic acid	30.5	28.4	31.9	23.3	17.8	18.6	37.2	44.0	42.7	34.4	39	39.4	69.3
Glycine	15.9	16.4	13.9	7.2	3.4	4.0	20.8	24.4	22.7	19.1	21.1	20.7	16.6
Histidine	6.8	6.4	6.0	2.2	1.2	1.0	6.7	11.2	9.6	6.5	9.5	8.1	10.1
Isoleucine	14.4	15.2	12.5	5.6	2.2	2.1	19.8	23.5	21.2	18.4	19.7	19.5	18.5
Leucine	27.2	28.3	22.1	9.9	3.3	3.2	36.4	41.1	39.6	34.1	36.6	35.5	29.3
Lysine	17.7	17.7	14.5	8.5	3.2	3.2	20.4	27.9	25.6	18.1	23.7	21.7	26.2
Methionine	5.8	6.2	5.0	1.9	0.5	0.7	8.2	8.9	8.4	7.5	8.3	7.8	5.2
Phenylalanine	18.0	18.2	14.9	6.6	2.9	2.6	24.1	27.3	25.6	22.6	24.0	23.6	19.7
Proline	13.5	14.9	11.0	5.7	2.2	2.3	16.8	20.9	20.0	15.8	18.3	17.1	18.3
Serine	13.5	14.2	12.1	8.0	4.5	4.9	16.7	19.6	18.8	15.8	17.6	17.7	20.9
Threonine	14.1	14.0	12.9	9.0	5.5	5.5	17.1	20.7	20.1	16.4	18.3	18.7	15.6
Tryptophan	5.5	5.8	5.1	2.4	1.3	1.2	7.9	8.5	8.0	7.4	7.4	7.7	n.a
Tyrosine	12.4	12.4	10.9	5.4	2.0	2.0	17.1	19.9	19.4	16.1	17.7	17.2	14.4
Valine	17.1	17.8	14.8	7.6	2.9	3.0	22.1	26.8	24.9	20.8	23.3	22.5	17.9
AA total	**277.2**	**278.6**	**249.7**	**143.8**	**79.1**	**81.8**	**351.6**	**421.1**	**398.8**	**328.9**	**370.5**	**361.4**	**379.9**
EAA total	**126.6**	**129.6**	**107.8**	**53.7**	**23.0**	**22.5**	**162.7**	**195.9**	**183.0**	**151.8**	**170.8**	**165.1**	**142.5**

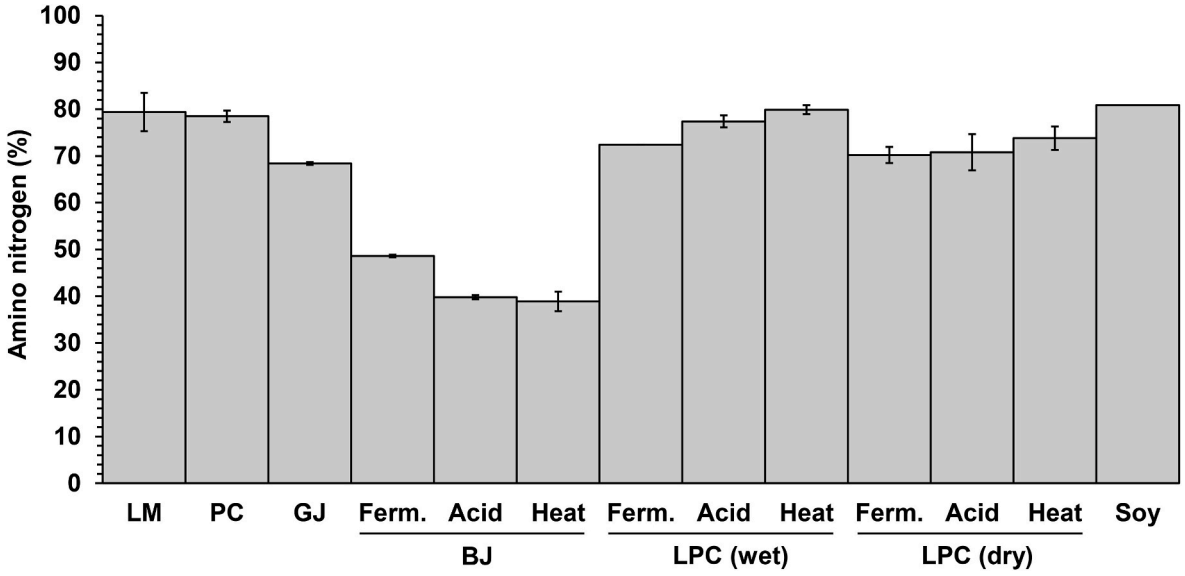

Fig. 2. Amino nitrogen percentage in the different biorefinery fractions. LM is leaf material, PC is press cake, GJ is green juice, BJ is brown juice, and LPC is leaf protein concentrate. The abbreviations Ferm., Acid and Heat represent the protein precipitation methods, using fermentation, acid or heat treatment. Soybean reference calculated using values from Steenfeldt and Hammershøj (2015). Error bars show the standard deviation of the mean.

leaf material was comparable to values reported in previous CLPC studies, e.g., 28% TS with 27.7% CP in Coldebella et al. (2013) and 22.6% TS with 33.6% CP in Ayele et al. (2021). However, as both components are highly dependent on the variety and age/maturity of the used leaf biomass (Fafunso and Bassir, 1976; Ravindran and Ravindran, 1988), some variation is expected between studies. After mechanical pressing, the CP content in the green juice solids remained the same as in the leaf material, in line with observations from previous biorefinery studies showing a stable or slightly increased level in GJ from cassava leaves and other leaf biomasses (Ayele et al., 2021; Santamaría-Fernández et al., 2018).

From the green juice, LPC was produced using different precipitation methods resulting in concentrates with a TS content around 20–25% with CP accounting for 42–47% of the TS. In terms of CP content, this lies in range with values reported in previous studies on production of CLPC. In Tupynambá and Vieira (1979), several experiments with extraction using blending and acid precipitation resulted in CLPC with TS containing 42–51% crude protein. Comparable CP levels were also obtained in CLPC produced in Castellanos et al. (1994), where the use of both heat precipitation and ultrafiltration of juice from finely crushed cassava leaves resulted in concentrates with TS containing 42.9% CP and 43.9% CP, respectively. Similarly, a process combining pulping, pressing, and heat precipitation resulted in CLPC with TS containing 42–50% CP, using leaf biomass from four different cassava varieties (Fasuyi and Aletor, 2005b). These comparable values indicate that the CP content in CLPC produced by one step precipitation lies relatively stable around 40–50% of TS, despite differences in the used variety and the exact combination of process steps. Furthermore, this overall CP level is

Table 4
Cyanogenic (CN) potential throughout the biorefining process. LM is leaf material, PC is press cake, GJ is green juice, BJ is brown juice, and LPC is leaf protein concentrate. Left column shows CN-equivalents (ppm) in all fractions on weight basis. Right column shows CN-equivalents (ppm) in the solids of the wet and oven dried LPC. Error bars show the standard deviation of the mean. The abbreviations Ferm., Acid and Heat represent the protein precipitation methods, using fermentation, acid or heat treatment.

		CN-equivalents	
Fraction		ppm in fraction	ppm in solids
LM		42.6 (±7.7)	ND
PC		46.4 (±11.7)	ND
GJ		50.5 (±8.8)	ND
BJ	Ferm.	41.8 (±2.3)	ND
	Acid	47.5 (±0.7)	ND
	Heat	50.7 (±2.6)	ND
LPC (wet)	Ferm.	70.9 (±12.4)	335 (±58.3)
	Acid	44.7 (±1.0)	178 (±3.91)
	Heat	46.0 (±4.4)	183 (±17.6)
LPC (dry)	Ferm.	260 (±23.2)	280 (±25.0)
	Acid	151 (±21.0)	161 (±22.4)
	Heat	145 (±35.3)	154 (±37.7)

comparable to LPC produced from more traditional biorefinery crops such as ryegrass, alfalfa, and grass-clover mixtures (Corona et al., 2018; Santamaría-Fernández et al., 2017).

After oven drying, the CP percentages in the produced concentrates were reduced with 2.0–3.5% compared to when analyzing lyophilized powder. It is well acknowledged that lyophilization is a gentler drying method and the decrease in the % CP could be a result of increased volatilization of non-amino N compounds such as urea and ammonia during oven drying (Morris et al., 2019). Despite this reduction, the CP content of the acid precipitated LPC remained at 45% of TS, which is only slightly lower than that of average soybean meal (~50% of TS), the most used protein source in animal feed (Ibáñez et al., 2020).

4.2. Distribution levels and crude protein recovery

The percentage distribution of TS and CP during the two processing steps (screw pressing and precipitation) was estimated using measured WW, TS, and CP values. During screw pressing roughly 60% of the initial wet weight was channeled into the green juice fraction. This is comparable with a previous CLPC study by Ayele et al. (2021) that achieved 69.2% transfer using a comparable screw pressing scheme. Despite most mass going into the liquid fraction during screw pressing, the majority of TS and CP present in the leaf material remained in the press cake fraction (~70%). This is in line with observations from a previous study by Santamaría-Fernández et al. (2017) that also found 65–75% of TS and CP remaining in the press cake when using an identical twin screw press for extraction of leaf protein from clover grass, alfalfa, and oilseed radish. These relatively poor extraction levels indicate a large potential for improvement which could be achieved through multiple pressings and/or combining the pressing with a pretreatment step such as chopping or pulping. Alternatively, the pressing step itself could be optimized as was done by Colas et al. (2013) that were able to recover 58% of alfalfa proteins in the green juice after testing different screw profiles of

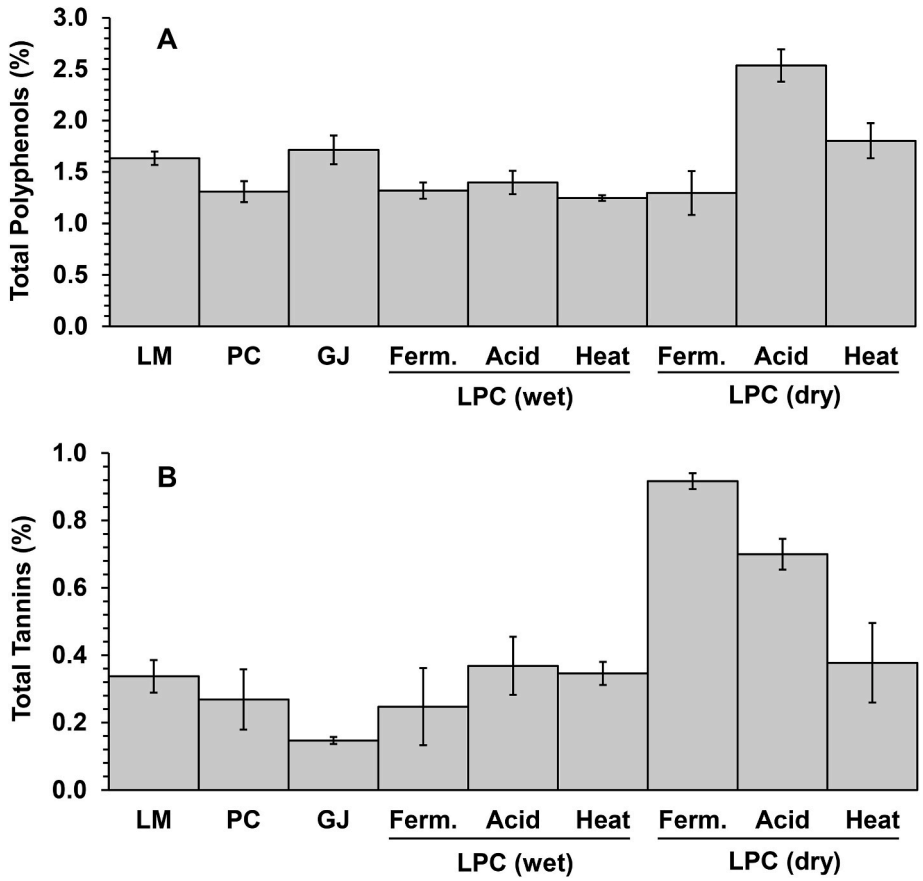

Fig. 3. Total polyphenols and tannins throughout the biorefining process. The abbreviations Ferm., Acid and Heat represent the protein precipitation methods, using fermentation, acid or heat treatment. (**A**) Total polyphenols (% of TS) in the different fractions. (**B**) Total tannins (% of TS) in the different fractions. Brown juice samples were excluded due to technical reasons. Error bars show the standard deviation of the mean.

a twin-screw extruder, combined with addition of water to the biomass.

After precipitation and centrifugation, the majority of CP present in the green juice was recovered in the protein concentrates (68–84%), whilst the TS was distributed more evenly between the concentrates (50–57%) and the remaining brown juice fractions (44–50%). As expected, this shows a selectivity of the utilized methods towards precipitation of soluble proteins. Of the three used precipitation methods, acid- and heat precipitation resulted in the highest transfer of CP from the juice into the LPCs (>80%), with very similar distribution levels. This observation was also made by Modesti et al. (2007), that found heating to 80 °C and acid precipitation with HCl to be equally efficient, and with little compositional difference in the final CLPC. The spontaneous fermentation resulted in significantly lower TS and CP transfer to the concentrate. However as mentioned, this could be the result of insufficient microbial growth, as no change in pH or free glucose was observed during the incubation period. It is unknown why the spontaneous fermentation did not occur as expected. The method has been used successfully in a previous CLPC study by Coldebella et al. (2013), and fermentation by indigenous microorganisms is a traditional processing technique for detoxification of both cassava tubers and leaves (Kobawila et al., 2005; Montagnac et al., 2009). However, as the biomass used in this study was grown in controlled greenhouse facilities, it might not have contained the microflora needed to ferment sugars in the green juice under the presence of cyanide.

Based on the distribution levels, final TS and CP recoveries were determined. As expected, the acid- and heat-based precipitation resulted in the highest TS and CP recovery levels, with roughly 25% of the CP available in the leaf material ending up in the concentrates. This recovery level falls in the low end of the values reported by early studies on CLPC production. For example, in Fafunso and Oke (1976) total nitrogen extractability (CP-recovery) in CLPC produced from 15 different cultivars was 56–61%, whilst in Tupynambá and Vieira (1979) recovery levels ranged from 30 to 65% across a number of extraction experiments with locally harvested cassava leaves. However, both studies used mechanical separation schemes differing from the one in this study, making direct comparison difficult. In addition, the studies fail to present stepwise distribution levels and calculation principles, making it difficult to assess the reliability of the reported values. More recently, Ayele et al. (2021) were able to obtain 24% CP recovery by ultrafiltration of centrifuged green juice made through screw pressing of chopped leaves. The previously reported recovery levels for CLPC thus span a broad range, something which is also seen in studies working with production of LPC from alfalfa, grasses, and clovers (Santamaría-Fernández and Lübeck, 2020). This is because the recovery efficiency is not only affected by the specifics of the used extraction procedure, but also by several agronomic factors such as the type of soil, climate, and maturity/age of the biomass (Arkcoll and Festenstein, 1971; Solati et al., 2017), that can be very difficult to standardize.

Despite the heat and acid-based precipitation resulting in the recovery of 19–24% extra CP compared to the attempted fermentation, it is uncertain whether these extraction methods would be advantageous from an economic and environmental point of view. As green juice consists mainly of water (approximately 90% in this study), large amounts of liquid will have to be processed in up-scaled production, requiring either significant energy or acid usage. Alternatively, induced fermentation with for example *Lactobacillus salivarius* could be applied to ensure a more reliable fermentation. This approach showed promising results in previous work with production of CLPC (unpublished data) and is a well-established precipitation method for extraction of leaf protein from alfalfa, grasses, and clover (Lübeck and Lübeck, 2019; Santamaría-Fernández et al., 2018). However, as induced fermentation takes a minimum of 8–10 h (per batch), it will prolong the processing time considerably compared to heat- and acid precipitation, which might not be cost-effective at large-scale.

4.3. Amino acid composition and suitability as feed/food

Although analysis of nitrogen (crude protein) is the golden standard for estimation of protein in feed- and foodstuff, the method does not provide any information about the quality and suitability of that protein. For this purpose, amino acid analysis is often applied. In this study, amino acid analysis revealed that the leaf material contained 277.2 g/kg TS amino acids, which is substantially higher than the 150–190 g/kg TS measured in the leaves of more traditional protein refinery crops such as clovers, alfalfa, and oilseed radish (Santamaría-Fernández et al., 2017). This was somewhat expected, considering that the crude protein content was 30.1% of TS, which is also significantly higher than the 15–20% typically found in these biomasses (Corona et al., 2018; Santamaría-Fernández et al., 2019). After pressing, a similar content of amino acids remained in the solids of the press cake, suggesting a potential for further protein extraction. Alternatively, the press cake could also be used as ruminant feed, which has been demonstrated to have comparable *in situ* rumen degradation and intestinal protein digestibility to that of the leaf material from alfalfa, clovers, and grasses (Damborg et al., 2018). The amino acid content in the green juice was only slightly lower than the starting biomass, but this was significantly reduced in the brown juices after precipitation. Approximately 80 g/kg TS remained after heat- and acid precipitation, indicating successful extraction of soluble proteins.

Overall, the amino acids contained in the fresh material were recovered in the wet LPCs, and the amino acid content was increased by a factor of 1.4 and 1.5 for the heat- and acid precipitation, respectively. This is slightly lower than the upconcentration factors achieved with red clover (2.3) and alfalfa (1.8) in a comparable protein refining setup (Santamaría-Fernández et al., 2017). After oven drying the total content of amino acids in the LPCs was reduced with between 6.5% (fermentation) and 12% (acid precipitation). The exact cause of this loss is uncertain but could be the result of microbial protein degradation and catabolism during the low temperature drying. For example, in lactic acid bacteria, the metabolism of amino acids includes both deamination and decarboxylation for production of amines and carboxylic acids (Wang et al., 2021). If so, this effect could be reduced by drying at temperatures above the tolerance of common microorganisms, although too high temperatures might also result in protein denaturation.

Regarding the specific amino acids, focus was on histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine, as they are the shared essential amino acids for monogastrics such as humans, pigs, and poultry. In addition to these, pigs and poultry also require arginine in their diets (Rezaei et al., 2013; Santamaría-Fernández et al., 2017). The combined content of these essential amino acids followed the same development as the total AA-content, staying relatively stable in the fractions during pressing, whereafter they were significantly reduced in the brown juice fractions and up concentrated in the LPCs. Moreover, in the dried LPC from heat- and acid precipitation, the amino acid profile was equal to or even superior to soybeans for some essential amino acids (Steenfeldt and Hammershøj, 2015) and the EAA-total was 15–20% higher. As soybean is considered a complete source of protein for humans and is extensively used in animal feed today, the produced CLPC can, based on amino acid composition, be considered as a source of high-quality protein and amino acids for monogastrics. Interesting is also the high combined content of the sulfur-containing amino acids methionine and cysteine, which are regarded as limiting amino acids in poultry (Santamaría-Fernández et al., 2017). It would be particularly advantageous for CLPC to be suitable for poultry feed as approximately 61% of the livestock population in Africa consists of poultry (MaMo Panel, 2020). Indeed, CLPC has already been tested for use in poultry feed, where it has been demonstrated that it can replace up to 40% of fish meal with no obvious, deleterious effect. Furthermore, these CLPC-containing diets displayed values for weight gain, feed consumption, feed efficiency, and protein efficiency ratio similar or close to that of the control diet (Fasuyi and

Aletor, 2005a). Overall, the amino acid content of the produced CLPC seems appropriate for monogastrics and might become a good protein alternative in the future production of feed and food products.

4.4. Amino nitrogen levels

In addition to providing detailed amino acid profiles, the amino acid analysis also enables calculation of true protein content. True protein has been proposed as a better indicator of the protein content than crude protein, as the latter also includes non-protein N containing compounds (Sriperm et al., 2011). By further dividing true protein with the crude protein content, the percentage of nitrogen originating from amino acids, termed amino nitrogen percentage (%AN), was estimated for the biorefinery fractions. This revealed that amino nitrogen constituted roughly 80% of N in the leaf material, a level similar to that in leaf material used in previous CLPC studies, ranging from 81.5% (Tupynambá and Vieira, 1979) to 83.1% (Ayele et al., 2021). The remaining nitrogen could be a combination of inorganic ions (nitrate and ammonium) and different N-containing metabolites such as urea, chlorophyll, cyanogenic glucosides, and the osmoprotectant glycine betaine (Feng et al., 2019). After pressing, %AN remained unchanged in the press cake while it fell in the green juice fraction, which might be due to the ease of extractability of some non-protein N containing compounds like chlorophylls (Zhang et al., 2014). This level was further reduced in the brown juice fractions because of the protein extraction, while %AN in the wet LPCs (from heat and acid precipitation) was restored to the starting level around 80%. The obtained %AN in these LPCs is on level with that obtained in a previous CLPC study by Castellanos et al. (1994), who achieved 80.4% amino nitrogen in heat precipitated CLPC. The result is also comparable with the values that can be estimated for LPC from grasses, clovers, and alfalfa, based on the presented true- and crude protein content. Here, the amino nitrogen percentage, independent of the precipitation method, seems to lie in the range of 80–90% (Damborg et al., 2015; Santamaría-Fernández et al., 2017). After oven drying, %AN in the LPCs dropped slightly due to the loss of amino acids, resulting in dried powder with a slightly lower level than the soybean reference (Steenfeldt and Hammershøj, 2015).

The amino nitrogen percentages can also be used to estimate the final true protein recovery in the LPCs by multiplying the crude protein recovery with the ratio between %AN in the leaf material and %AN in the LPCs (data not shown). This results in true protein recoveries of 19% (fermentation), 25.3% (acid precipitation), and 24.9% (heat). Thus, true protein recovery was roughly 9% lower for the attempted fermentation, while it was like the crude protein recovery for heat- and acid precipitation, further setting these two methods apart.

4.5. Cyanogenic potentials

Cassava leaves' toxicity is mainly related to their content of cyanogenic glucosides (mostly linamarin) that upon cellular disruption is converted into cyanohydrins and free cyanide (Latif et al., 2019; Montagnac et al., 2009). Cyanogenic potential or total cyanide content of a cassava sample or product is the combined amount of HCN that can be released from a sample during full conversion of the various cyanogen forms (Bradbury et al., 1999). As cyanide inhibits cellular respiration in all aerobic organisms and is known to cause a range of diseases and disorders in both humans and animals (Burns et al., 2012a; Ravindran, 1993), the cyanogenic potential is a highly relevant measure when assessing the safety of using CLPC for feed and food purposes.

Analysis of the cyanogenic potential (total cyanide content) showed that the starting leaf material contained roughly 43 ppm HCN-equivalents on weight basis. This is substantially lower than the starting potential observed in early CLPC studies, for example in Fafunso and Bassir (1976), where the cyanogenic potential of the starting leaf material ranged from 450 to 630 ppm across eight different varieties, harvested locally in Nigeria. However, reported levels of total cyanide in fresh cassava leaves range from 80 to 1860 ppm, although many measurements seem to be above 100 ppm (Burns et al., 2012b; Lancaster and Brooks, 1983). This large variation is seen because the level of cyanogenic glucosides is affected by both location, cultivar, environmental conditions like drought and soil nutrient, and the exact leaf tissue used for analysis (Jørgensen et al., 2005; Lancaster and Brooks, 1983). After pressing, the cyanogenic potential increased slightly in the green juice and this level was maintained in the wet LPCs, ranging from 45 to 71 ppm. This result is contrary to the findings from previous studies looking at the effect on cyanogenic potential during CLPC production (Balasundaram et al., 1976; Castellanos et al., 1994; Fafunso and Bassir, 1976), which all demonstrate a significant reduction from the starting material to wet CLPC. For example, Fafunso and Bassir (1976) reported a 75–90% reduction (compared to the leaf material) in the cyanogenic potential of CLPC produced in similar fashion to the heat precipitated CLPC from this study. Furthermore, they showed that different methods of drying (including oven drying) resulted in a final 95–99% cyanide disappearance in the dry CLPC. Again, this stands in contrast to the results of this study showing an increase in cyanogenic potential (on weight basis) after drying. When looking at the produced LPCs on a total solid basis, the cyanogenic potential was reduced during drying with approximately 10–15%. However, as the percentage of TS increased roughly 4.5 times, this resulted in an upconcentration effect during the drying process. It should also be mentioned that different quantification methods were used in each of these CLPC studies, including this one. As the enzymatic conversion of cyanogenic glucosides occurs rapidly and HCN is a very volatile compound, sampling and handling during analysis can have a significant impact on the results. This complicates direct comparison between studies using different methods, yet the overall observed trends should still be reliable (Kirsten Jørgensen, personal communication, March 23rd, 2022).

Of the three used precipitation methods, the spontaneous fermentation resulted in the LPC (both wet and dry) with the highest cyanogenic potential. This was somewhat unexpected as the fermentation method lasted 20 h and time is known to be an important factor in the disappearance of cyanide during processing of cassava leaves (Kobawila et al., 2005; Latif et al., 2019). In contrast, the two other precipitation methods lasted only 20 min. Furthermore, the green juice was incubated under favorable conditions (>pH 4 & 30 °C) for the degradation of cyanohydrins into volatile cyanide (Montagnac et al., 2009). This result, in combination with the minimal effect of drying, could indicate that the lack of cyanide removal during CLPC production is partially caused by the perseverance of non-converted cyanogenic glucosides residing in the solids distributed throughout the process. A hypothesis, that could be further elucidated by analyzing the content of intact cyanogenic glucosides by LC-MS analysis (Jørgensen et al., 2005).

The presented cyanogenic potentials can give an indication of whether the produced CLPC is suitable for use in feed and food formulations for monogastrics. Yet, even with these values it is difficult to predict potential adverse effects as the toxicity of cyanide depends on several factors such as body mass, health status, the dose of cyanide ingested and the time duration (Burns et al., 2012a). Furthermore, the toxicity of cyanogenic compounds varies considerably, with cyanohydrin breaking down rapidly to HCN under the neutral pH conditions of the gut, whilst some linamarin may pass through the body unchanged (Bradbury et al., 1999). At current, there is no universal standard for the level of HCN in cassava and related food products, but the World Health Organization (WHO) has set a safe limit of 10 ppm total cyanide for cassava flour (FAO/WHO, 2020), a product extensively used in many African and Asian countries. This maximum limit has also been adopted in Australia for cassava chips by Food Standards Australia and New Zealand (FSANZ), while 40 ppm is the accepted limit in foods sold in Indonesia (Burns et al., 2012a). Regarding animal feed, many countries have yet to adopt recommended safety limits, yet the European Food Safety Authority (EFSA) has set a maximum of 50 ppm total cyanide in feedstuff based on several animal toxicology studies (EFSA CONTAM

Panel, 2019). As storage life and handling improve significantly after drying, CLPC will in most cases be used for feed and food purposes in the form of dried powder. In this study, protein extraction did not reduce the cyanogenic potential and the oven dried CLPC contained 150–260 ppm total cyanide, which lies significantly above the presented safe limits. This result illustrates the importance of evaluating cyanide-based toxicity of CLPC on a batch-to-batch basis to ensure that the tolerated levels in the final products are not overstepped.

4.6. Polyphenols and tannins

Traditionally polyphenols and tannins have been considered as anti-nutritional factors in animal nutrition, due to their ability to bind proteins and reduce their uptake (Hassan et al., 2020). However, more recent studies are challenging this notion, showing that a low content can be of benefit for monogastric animals like broilers and pigs, by reducing zoonotic pathogens and gastro-intestinal parasites (Huang et al., 2018). Similarly, certain levels of tannins can have positive effects in ruminants by increasing protein utilization and reducing the presence of gastro-intestinal parasites (Mueller-Harvey, 2006). Knowing the content of these compounds in CLPC is therefore highly relevant for its potential use in feed and food.

There seems to be no significant differences in the total polyphenolic content of the different fractions after pressing, and similarly there is no difference between the three LPC (wet) fractions. Supplementation of polyphenols to animal diets is something that is gaining increased interest with regards to reducing oxidative stress, and several studies have shown that supplementation of 0.2–2 g/kg feed has positive effects on reducing oxidative stress in chicken and pigs (Serra et al., 2021). The content in the LPCs of 1.2–1.4 g/kg thus indicate that a positive effect on reducing oxidative stress could be expected depending on the polyphenol composition.

When assessing the three LPC (wet) fractions, there is no significant upconcentration of tannins in the protein concentrates compared to the starting material, irrespective of precipitation method. This seems contradictory, given the strong protein binding abilities of tannins, however it may be explained by the differences in water solubility of different types of tannins (Hassan et al., 2020). Condensed tannins are less water soluble than hydrolysable tannins, and for both types of tannins, solubility decreases with increasing degree of polymerization. A tannin profile dominated by condensed tannins or with a high degree of polymerization could be the reason why no upconcentration in the protein concentrate fractions were found. Several authors have stated that feeds with $<$5% tannin on a dry matter basis are safe to use. Tannic acid in feeds, being one of the common hydrolysable tannins, is tolerated at the levels of 10–15 g/kg feed for ruminants, 2–10 g/kg feed for pigs and chicken and less for younger animals (EFSA FEEDAP Panel, 2014). The levels of tannins found in the CLPC from this study are within the tolerated range and potentially beneficial, since polyphenols/tannins exhibit a range of biological activities such as anti-parasitic, antioxidant, anti-inflammatory, and immunomodulating properties (Huang et al., 2018).

There are significant differences between the tannin and total polyphenolic content of the wet LPCs (lyophilized before analysis) and the oven dried LPC fractions. Similar results have been obtained in previous studies (Ferreira et al., 2004) and might be due to non-phenolic/tannic compounds being modified by the higher temperatures during oven drying, making them react as phenolic compounds and hence interfering with the assay. The complex nature of polyphenols/tannins and the large variation between plant material, makes it difficult to generalize and determine what level is “optimal”, since it depends on the composition in the polyphenol/tannin fraction, their content, the diet they are included in, and the animal being fed (Mueller-Harvey, 2006).

4.7. Expanding the refining process

Based solely on protein quality and content of antinutritional compounds, it is possible that CLPC as produced in this study could be used for both feed and food purposes. However, the final protein products also had a very strong and distinct “leafy” smell and taste, which is common for leaf protein concentrate extracted directly from green juice (Fiorentini and Galoppini, 1983). When it comes to using leaf protein in food products, aspects such as color, taste, and odor are essential for consumers (Mittermeier-Kleßinger et al., 2021). Furthermore, as consumer perception of food products is strongly affected by its structural characteristics, leaf protein from cassava would also need good functional properties within solubility, foaming, gelation, emulsification etc. to be successfully integrated into a complex food matrix (Nissen et al., 2021; Nynäs, 2018). These technofunctional properties are highly dependent on the structural status of the extracted protein, which in turn is strongly affected by the used method of extraction. As heat precipitation at 80 °C mainly results in denatured and coagulated protein, it might not produce protein with suitable functionalities for food purposes. Isoelectric precipitation, through addition of acid or fermentation, has been shown to be a less harsh method preserving more native protein, but the solubility of the protein is still often compromised (Damborg et al., 2020; Møller et al., 2021). These issues with perceptual qualities and inadequate technofunctional properties could potentially be reduced or fully resolved by using a multiple extraction process, where two distinct protein fractions are generated exclusively for either feed or food purposes (Møller et al., 2021; Nynäs et al., 2021). This expanded refining process can be set up in multiple ways, but a common approach consists of an initial heat precipitation at 50–60 °C for recovery of feed suitable “green protein”, removing most of the unwanted taste and smell, followed by a secondary extraction of food quality “white protein”, mainly consisting of the protein RuBisCO (Nynäs et al., 2021). In the secondary extraction step, a gentle method such as ultrafiltration can be applied to ensure maximum conservation of the protein’s native structures and physiochemical properties (Martin et al., 2019). Moreover, as the use of a two-step extraction would further reduce the cyanogenic potential of the final protein fraction, it could be a way of better ensuring the safe use of CLPC for food purposes, whilst still producing green protein for feeding monogastric farm animals. However, the two-step extraction process also involves higher cost for equipment and operation, and there is thus a trade-off between quality and cost, and the resulting protein products.

5. Conclusion

Leaf protein concentrate was successfully extracted from cassava leaves using different precipitation methods. Approximately 21–26% of the leaf crude protein was recovered in the LPCs, which after drying contained 40–45% protein with a balanced amino acid profile, comparable to soybean. The spontaneous fermentation did not work but could perhaps be substituted with induced fermentation by lactic acid bacteria. The levels of tannins found in the LPCs lie within the tolerated range for feed, but the measured cyanogenic potentials are cause for concern, especially for use in food. The cyanide disappearance presented in previous studies did not occur here, and further investigations are needed to fully evaluate whether processing can ensure a consistently safe product. Potentially, two-step extraction for production of dedicated feed and food protein could be the way forward.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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