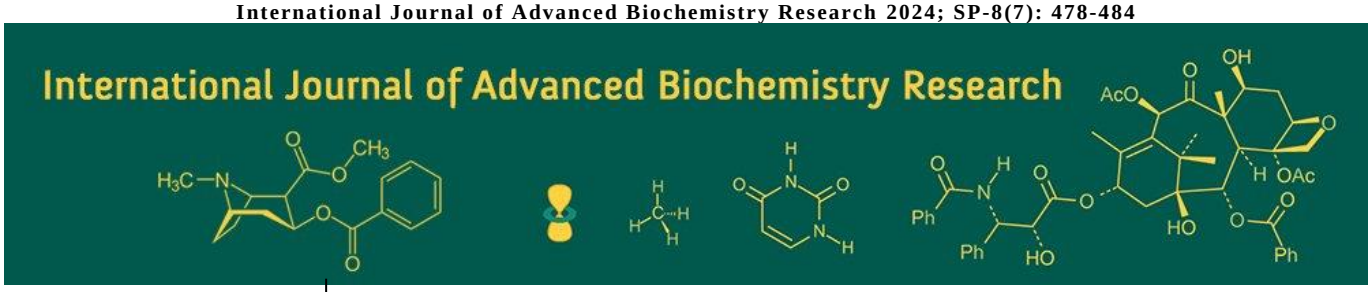

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Reduction of anti-nutrients factors and study of *in-vitro* protein digestibility, fractionation, SDS-PAGE analysis and thin layer chromatography of wild apricot kernel press cake protein isolate

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Abstract
The effect of different treatments (boiling, autoclaving, microwave cooking and Sodium carbonate treatment) on the levels of certain anti-nutritional factors (phytic acid, HCN andtannins) and *in vitro* protein digestibility (IVPD) of wild apricot kernel press cake protein isolate were investigated. All heat treatments significantly reduced the levels of all the investigated antinutrients and improved the IVPD of protein isolate. Apricot kernel press cake protein isolate was observed free from hydrogen cyanide (HCN). Phytic acid and tannins were reduced with the heating at 100 °C for 30 minutes. The digestibility of proteins was an important parameter to evaluate their nutritional quality. In-vitro protein digestibility of protein isolate was 80.58 per cent. The fractionation of PI revealed globulin fraction (433.10 g/kg), albumin fraction (286.20 g/kg), glutelin fraction (198.50 g/kg), and prolamin fraction (81.16 g/kg). Sodium dodecyl-sulphate poly-acrylamide gel electrophoresis was done with standard molecular weight markers (13 to 100 kDa). Further, TLC profiling of the PI from APC revealed the presence of 9 essential amino acids.

Keywords: Anti-nutritional factors, *in vitro* protein digestibility, protein isolate, TLC, SDS-PAGE

Introduction
Anti-nutritional factors are compounds found in most of the food substances in varying amount which are poisonous to human or in some ways limiting the nutrient availability to the body. Most of these anti-nutritional factors are present in foods of plant origin. Thus, the presence of cyanogenic glycosides, tannins, alkaloids, and phytic acid in foods may induce undesirable effects in humans if their consumption exceeds an upper limit. These anti-nutritional factors must be inactivated or removed to maintain the values of food substances (Ogbadoyi *et al.* 2011) [41]. Hydrogen cyanide is a volatile compound and evaporates rapidly in the air at temperatures over 28 °C and dissolves rapidly in water. It may easily be lost during transport, storage and analysis of samples. It is a chemical compound released from cyanogenic glycosides, natural constituents present in bitter almonds, sorghum, cassava, lima beans, stone fruits, apricot press cake and bamboo shoots. The hydrogen cyanide may be toxic to humans, animals and the severity of the toxicity depends on the quantity consumed. Phytate also known as Inositol hexakisphosphate is the salt form of phytic acid, found in plants. Phytic acid causes the decrease in bioavailability of essential minerals and turn into insoluble compounds those absorption and digestion is less in the small intestine (Desphande and Cheryan, 1984) [14]. Phytic acid is reported to chelate metal ions such as calcium, magnesium, zinc, copper, iron and molybdenum to form insoluble complexes those are not readily absorbed from gastrointestinal tract. Phytic acid also inhibits the action of gastrointestinal tyrosinase, trypsin, pepsin, lipase and amylase (Erdman, 1979) [20]. The anti-nutritional properties of tannins depend upon their chemical structure and doses. These are heat stable and decreased protein digestibility in animals and humans, probably by either making protein partially unavailable or inhibiting digestive enzymes and increasing fecal nitrogen.

Tannins are known to be present in food products and to inhibit the activities of trypsin, chymotrypsin, amylase, lipase and decrease the protein quality of foods and interfere with dietary iron absorption (Liener, 1980) [32].

Materials and Methods

Procurement of raw materials

Wild apricot kernel press cake was procured from the agro processing center on oil processing under AICRP unit, Department of Food Science and Technology, Dr YS Parmar, UHF, Nauni, Solan. Whereas, chemical standards were purchased from certified agencies and brought to the laboratory of Department of Food Science and Technology for conducting further studies. Protein isolate was extracted by the standard methods described by Thakur *et al*., 2021.

Removal of anti-nutritional factors

Different combinations/treatments were tried for removal of anti nutritional factors *viz*. HCN, phytic acid and tannin as per following treatments: PT_1 (Control), PT_2 (Heating in water at 100 °C for 10 min), PT_3 (Heating in water at 100 °C for 20 min), PT_4 (Heating in water at 100 °C for 30 min), PT_5 (Heating in 0.1% sodium bicarbonate at 100 °C for 10 min), PT_6 (Heating in 0.1% sodium bicarbonate at 100 °C for 20 min), PT_7 (Heating in 0.1% sodium bicarbonate at 100 °C for 30 min), PT_8 (Autoclaving at 121 °C for 10 min), PT_9 (Autoclaving at 121 °C for 20 min), PT_{10} (Autoclaving at 121 °C for 30 min), PT_{11} (Microwave heating for 3 min), PT_{12} (Microwave heating for 6 min) and PT_{13} (Microwave heating for 9 min).

Analysis

HCN Content

Qualitative method (Prussian blue test)

To preweighed sample (0.5-10 g), 2 ml freshly prepared 3 per cent $FeSO_4.7H_2O$ solution was added followed by addition of single drop of 1 per cent $FeCl_3.6H_2O$ solution. After proper mixing, 10 per cent NaOH solution was added drop wise until no further precipitate formed followed by dissolving of precipitates by adding dilute H_2SO_4 (1+9) solution (AOAC, 1995) [1]. The development of prussian blue colour indicated the presence of HCN (Figure 1).

Quantitative method (Alkaline-titration method)

Alkaline-titration method was followed for quantitative estimation of hydro- cyanic acid. 0.5-20 g sample was mixed with 200 ml water and allowed to stand for 2 hours for complete hydrolysis of amygdalin to HCN. The mixture was then steam distilled to collect 150-160 ml of distillate in NaOH solution (0.5 g in 20 ml H_2O) followed by its dilution to 250 ml. 100 ml aliquat containing 8 ml 6N NH_4OH and 2 ml 5% KI solution was then titrated against 0.02N $AgNO_3$ to a faint permanent turbid colour (AOAC, 1995) [1]. The HCN content (mg/100 g) in the sample was then calculated using following expression:

$$\text{HCN (mg/100g)} = \frac{\text{Titre x 1.08 x Volume made up x Aliquat taken}}{\text{Sample taken x Distillate taken}}$$

Phytic acid content

Phytic acid was determined by extracting 0.5 g of sample from raw material with 0.2 M HCl for 3h followed by centrifugation at 3000 rpm for30 min. The extract (0.5 ml) was mixed with 1 ml of ferric ammonium sulphate solution (0.2 g of$NH_4Fe(SO_4)_2{\cdot}12H_2O$ in 100 ml 2 M HCl and made up to 1000 ml), incubated in a boiling water bath for 30min and then cooled in an ice water bath until reached room temperature. After the cooling 2 ml of 2,2 bipyridine solution was added. The absorbance was measured immediately at 519 nm and phytic acid content was calculated using the calibration curve (Ranganna, 2009) [8-9]. Calibration was set up with the reference solutions prepared by diluting the stock solution (1.3 mg/ml phytic acid).

Tannin content

Tannin was determined by measuring 0.2 g of finely ground samples into a 50 ml beaker. 20 ml of 50 per cent methanol was added and covered, and placed in a water bath at 79ºC for1hour and stirred with a glass rod to prevent lumping. The extract was quantitatively filtered using a double layered Whatman No.1 filter paper into a 100 ml volumetric flask using 50 per cent methanol to rinse. This was made up to mark with distilled water and thoroughly mixed. 1 ml of sample extract was pipetted into 50 ml volumetric flask, and 20 ml distilled water, 2.5 ml Folin-Denis reagent and 10 ml of 17 per cent Na_2C0_3 were added and mixed properly. The mixture was made up to mark with distilled water, mixed well and allowed to stand for 20minutes when a bluish-green colour developed. Standard Tannic Acid solutions of range 0-10 ppm were treated similarly as 1 ml of sample above. The absorbance of the Tannic Acid Standard solutions as well as samples were read after colour development on Spectrophotometer at a wavelength of 760 nm (Ranganna, 2009) [8-9].

In-vitro protein dig esbility

In vitro protein digestibility includes two steps of proteolysis by pepsin and pancreatin enzymes. Ground sample (0.5 g) was mixed with 50 ml of pepsin solution (5 g pepsin in one litre of 0.1 N HCL) in 250 ml conical flask and incubated at 37 °C for 2 hours. The solution was neutralized with 30 ml of 0.2 N NaOH. About 50 ml of pancreatin solution (4 g pancreatin in one litre of phosphate buffer) were added and the mixture was incubated at 37 °C for 2 hours. Few drops of toluene were added to the mixture to maintain aseptic environment. The mixture was centrifuged at 3000 rpm for 20 min and filtered through Whatman No 42 filter paper. The residue was analyzed for N content by macro-kjeldahl method. Digestibility was calculated by subtracting the residue protein from the initial protein on the basis of 100 g of sample.

Fractionation of Protein isolate

Fractionation of protein isolate was estimated by a method described by Dey, and Sinhababu (2018) [14] with the slight modification. In this method four solvents were used, consecutively, to extract the protein fractions from protein isolate. Initially about 10 g sample of PI was mixed with distilled water (1:10, w/v) and stirred with a magnetic stirrer (Gallenhamp-model SS-615) at 20 °C temperature for 30 min, followed by centrifugation at 10,000 rpm for 30 min to obtain the albumin fraction. The resulted residue was used to extract the protein by stirring for 4 hr with 250 ml of 10% NaCl (1:10, w/v) at 4°C using magnetic stirrer and followed by centrifugation at 10,000 rpm for 30 min. The recovered supernatant was filtered to obtain the globulin fraction and again the residue was extracted with 250 ml of 70% aqueous

2-propanol (1:10, w/v) for 4 hr followed by centrifugation at 10,000 rpm for 30 min to obtain the prolamin fraction. At the end, residue left was extracted with 250 ml of 0.1 N NaOH for 4 hr to obtain glutelin fraction. All the fractions were obtained by precipitating the respective supernatant with 1 N trichloroacetic acid at pH 4 and each precipitate was washed (three times) and dialyzed (48 hr at 4 °C) with distilled water. Each protein fractions (albumins, globulins, prolamins, and glutelins) were freeze dried and kept for further analysis.

Sodium dodecyl-sulphate poly-acrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE studies of protein isolate fractions were estimated by the method described by Shevkani, Singh, Kaur, and Rana, (2015) [55] with slight modification. About 10% of gel (Biorad) was used for molecular mass and purity analysis of protein fractions. Protein sample of 20 µg was loaded to the lane of the gel at a steady voltage of 150 V in 10% Sodium dodecyl-sulphaterunning buffer followed by staining with 0.006% coomassie blue dye in 10% (v/v) acetic acid and 90% (v/v) double-distilled water solution for 30 min and destained by 45% (v/v) methanol and 10% (v/v) acetic acid aqueous solution on a rocker at room temperature. The resulted bands were compared with standard proteins bands of range molecular weight, Bio-Rad Hercules, 13-100 kDa) and followed by visualized with Coomassie Brilliant Blue dyeR-250 for 1 hr.

Detection of amino acids on Thin Layer Chromatography (TLC) plates

Thin Layer Chromatography was done according to the modified method of Sen *et al*, (2012) [53]. Standard solutions (1 mg/ml) of amino acids were prepared in 0.01 M phosphate buffer (pH 8.0) and spotted (1 µL) on the TLC plates. Plates were air dried and subjected to TLC using solvent [Butanol, acetic acid, water in 8:2:2 (v/v)] as mobile phase. After the development of spots, plates were dried and sprayed with 0.25% ninhydrin reagent followed by drying in air and colors were noted. The plates were then heated at 110 °C for 10 min in an oven and the colors were recorded again. Then plate was exposed to vapors of concentrated NH_4OH which helps in the stabilization of colour. The R_f values were calculated and comparison was made between standard and unknown sample by using the following formula:

$$R_f = \frac{\text{Substance Distance}}{\text{Solvent Distance}}$$

Statistical analysis

All the analytical parameters were recorded in triplicates and the mean value of each parameter was described. The data of quantitative estimation of biochemical characteristics were assessed by factorial CRD using two factors analysis of variance (ANOVA) with the help of OPSTAT software (Cochran and Cox, 1967) and also followed by Duncan's multiple range test at P = 0.05 to compare means of variables using SPSS statistical package (16.0 version).

Results and Discussion

Removal/Reduction of hydrogen cynide (HCN) content

The qualitative test (Prussian blue colour test) for the presence of HCN content in apricot kernel press cake protein isolate has been presented in Fig 2-c. It is clear from that there was no indication of Prussian blue color which was the end point of the test. It indicates that the protein isolate contained no HCN content. Our results are similar with the findings of Sharma *et al*. (2010) [54], who reported that apricot kernel press cake protein isolate had nil HCN content.

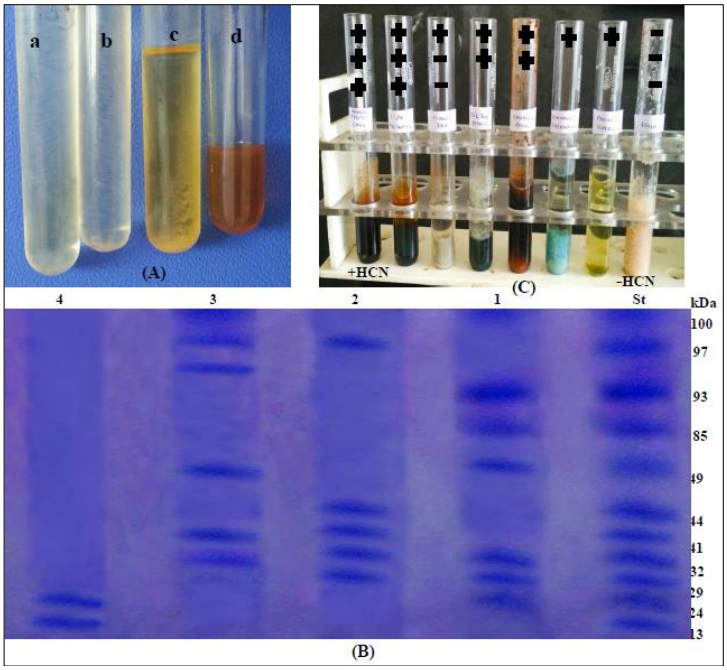

Fig 2: (A) Protein Fractionation
(B) SDS-PAGE profiles of protein fractions from protein isolates
Lane St-Standard protein markers with molecular weights of 13-100 kDa
Lane 1. Albumin traction; Lane 2, Globulin fraction
Lane 3, Glutelin fraction; Lane 4. Prolamin fraction.
(C) Prussian blue colour test for qualitative estimation of hydrogen cynide (HCN)

Removal/Reduction of Phytic acid content

The effect of different heating methods on phytic acid content of apricot kernel press cake protein isolate has been observed by using Duncans's multiple range test (Table 1). The protein isolate was observed with phytic acid content (0.35%) and reduce significantly with application of different treatments. The treatment PT_4 (Heating in water at 100 °C for 30 min), PT_7 (Heating in 0.1% sodium bicarbonate at 100 °C for 30 min)and PT_{10} (autoclaving at 121 °C for 30 min) significantly reduced the phytic acid content to 0.10 per cent. However the effect of microwave heating on phytic acid content was found to reduced 0.16 per cent after 9 min of application. Our results are conformity with the finding of Idoko *et al*. (2014) [26] in water melon protein isolate reported that phytic acid content reduced 0.29 to 0.21 per cent by boiling and 0.29 to 0.23 per cent by autoclaving. Similar results for maximum reduction in phytic acid content (6.15 to 5.90 mg/g) by autoclaving was reported by Kadam *et al*. (1987) [29] in winged bean.

Table 1: Optimization of process for removal/reduction of phytic acid from protein isolates

Treatments	Phytic acid (%)
PT_1 (Control)	0.35^{a}
PT_2 (Heating in water at 100 °C for 10 min)	0.25^{bc}
PT_3 (Heating in water at 100 °C for 20 min)	0.15^{ef}
PT_4 (Heating in water at 100 °C for 30 min)	0.10^{f}
PT_5 (Heating in 0.1% sodium bicarbonate at 100 °C for 10 min)	0.22^{bcd}
PT_6 (Heating in 0.1% sodium bicarbonate at 100 °C for 20 min)	0.15^{ef}
PT_7 (Heating in 0.1% sodium bicarbonate at 100 °C for 30 min)	0.10^{f}
PT_8 (Autoclaving at 121 °C for 10 min)	0.20^{cde}
PT_9 (Autoclaving at 121 °C for 20 min)	0.17^{de}
PT_{10} (Autoclaving at 121 °C for 30 min)	0.10^{f}
PT_{11} (Microwave heating for 3 min)	0.28^{b}
PT_{12} (Microwave heating for 6 min)	0.20^{cde}
PT_{13} (Microwave heating for 9 min)	0.16^{def}

*Mean values (n=3) followed by different lower case letters are significantly different at $p \leq 0.05$ according to Duncans's multiple range test

Removal/Reduction of Tannins content

The protein isolate was observed with tannin content (0.40%) and reduce significantly with application of different treatments (Table 2). The treatment TT_4 (Heating in water at 100 °C for 30 min), TT_7 (Heating in 0.1% sodium bicarbonate at 100 °C for 30 min), TT_{10} (autoclaving at 121 °C for 30 min) and TT_{13} (Microwave heating for 9 min) significantly reduced the tannin content to 0.15 per cent, 0.20 per cent, 0.15 per cent and 0.25 per cent, respectively. Similar results for reduction of tannin content were reported by Maidala *et al*. (2013) [33] in Cowpea from 8.07 to 5.93 mg/100 g during cooking. Whereas, Rahman *et al*. (2015) [45] had reported in apricot meal that different cooking methods (heating, microwave heating and autoclaving) had significant effect in the reduction of tannin content. Embaby (2011) [18] had studied the effect of boiling, microwave and autoclaving on tannin content in peanut seed and observed that tannin content reduced from 8.90 to 5.20 per cent by boiling for 20 min to 40 min, 8.90 to 7.30 per cent by microwave heating for 6 to 12 min and 8.90 to 2.80 per cent by autoclaving for 10 to 20 min.

Table 2: Optimization of process for removal/reduction of tannin content from protein isolates

Treatments	Tannin (%)
TT_1 (Control)	0.40^{a}
TT_2 (Heating in water at 100 °C for 10 min)	0.30^{bc}
TT_3 (Heating in water at 100 °C for 20 min)	0.20^{cd}
TT_4 (Heating in water at 100 °C for 30 min)	0.15^{d}
TT_5 (Heating in 0.1% sodium bicarbonate at 100 °C for 10 min)	0.32^{ab}
TT_6 (Heating in 0.1% sodium bicarbonate at 100 °C for 20 min)	0.22^{cd}
TT_7 (Heating in 0.1% sodium bicarbonate at 100 °C for 30 min)	0.20^{cd}
TT_8 (Autoclaving at 121 °C for 10 min)	0.25^{bcd}
TT_9 (Autoclaving at 121 °C for 20 min)	0.15^{d}
TT_{10} (Autoclaving at 121 °C for 30 min)	0.15^{d}
TT_{11} (Microwave heating for 3 min)	0.30^{bc}
TT_{12} (Microwave heating for 6 min)	0.25^{bcd}
TT_{13} (Microwave heating for 9 min)	0.25^{bcd}

*Mean values (n=3) followed by different lower case letters are significantly different at $p \leq 0.05$ according to Duncans's multiple range test

***In vitro* protein digestibility**

The digestibility of proteins is an important parameter to evaluate their nutritional quality. *In vitro* protein digestibility of protein isolate was (80.58%). Where as, *In vitro* digestibility of beach pea protein isolate with pepsin-trypsin and pepsin-pancreatin were evaluated and observed that protein isolate (NaOH extracted) had digestibility values with pepsin-trypsin of 80.60 to 82.60 per cent and with pepsin-pancreatin of 78.60-79.20 per cent (Chavan *et al*. 2001) [10]. Nicanor *et al*. (2014) [38-39] had studied the *in vitro* digestibility of mamey sapote defatted meal (MSDM) and mamey sapote protein isolate (MSPI) and observed that the digestibility of the MSPI (73.6 ± 0.1%) was higher than that of the MSDM (68.71 ± 0.2%). This difference was most likely caused by the isolation process because this process increased the protein concentration, denatured the proteins,

and destroyed protease inhibitors, thereby increased protein availability. The relatively high digestibility obtained suggests MSPI as a promising food ingredient (Bernardino *et al*. 2001) [7].

Fractionation of protein isolate

The Osborne solubility based protein fractionation were famed into four different fractions (Fig 1&2) which are albumin (water-soluble), globulin (NaCl-soluble), prolamins (aqueous 2-propanol-soluble) and glutelins (NaOH-soluble). Among different fractions, globulin fraction was the dominant 433.10 ± 1.25 g/kg protein, followed by albumin fraction 286.20 ± 1.12 g/kg protein, glutelin fraction 198.50 ± 0.80 g/kg protein and prolamin fraction 81.16± 0.22 g/kg protein (Fig 3). The findings of our study are also in line with the results reported by Tounkara *et al*, (2013) [61] in defatted Roselle seed. They reported globulin fraction (31.18%), albumin fraction (16.47%), glutelin fraction (10.20%) and prolamin fraction (5.57%). Amza *et al*, (2015) [5] had also reported glutelin, albumin, globulin and prolamin fractions of ginger-bread plum seeds were 406 g/kg protein, 276 g/kg protein,258 g/kg protein and 64.8 g/kg protein, respectively.

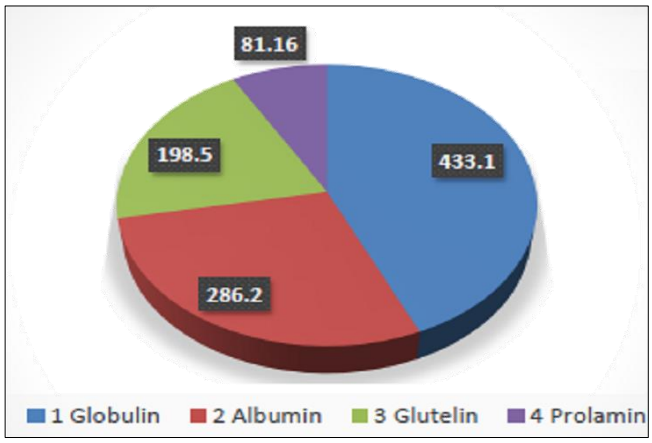

Fig 1: Fractionation of protein isolate

Distribution of molecular weight

Sodium dodecyl-sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) of protein isolate from wild apricot press cake (WAPC) is presented in Fig. 2-B. This SDS-PAGE confirmed the presence of fractions present in PI with different ranges of bands along with standard molecular marker band. PI showed a number of polypeptide subunits of molecular weight (Mw) between 100 and 13 kDa with main subunits of 13, 24, 32, 41, 49 and 93 kDa. The albumin fraction appears as smears of six bands around the 24, 29, 32, 49, 85 and 93 kDa. The polypeptide subunits of 93 were most prominent in albumin fraction. Similar albumin fraction from *Akebia trifoliata* var. Australis seed were reported by Du *et al*, (2012) [15] and displayed eight distinct subunits with major bands at about 49.0, 46.5, 35.6, 30.3, 19.0, 16.5, 13.9 and 12.3 kDa. The globulin fraction showed five subunits at 29, 32, 41, 44 and 97 kDa and glutelin fraction showed three subunits at 32, 41 and 49 kDa, while prolamin fraction showed only two subunits at 13 and 24 kDa. Our results were noteworthy with the earlier findings of Tounkara *et al*, (2013) [61] who reported the SDS-PAGE of roselle seed protein isolate (RSPI) and observed that the molecular weight of all fractions ranged from 55 kDa to 14 kDa. The RSPI possessed ten intense polypeptide bands with the molecular weights of 55, 41, 35, 29, 25, 23,22, 17, 16, and 14 kDa. The globulin fraction showed ten major polypeptide bands with molecular weights of 55, 41, 40, 29, 25, 23, 22, 17, 16 and 14 kDa. Where as, glutelin fractions showed eight polypeptide bands at molecular weight of 55, 41, 35, 28, 23, 22, 20, 19 and 14 kDa. Rui *et al*, (2011) [50] and Ulloa *et al*, (2017) [62] had also reported similar electrophoresis profiles of PI in bean proteins and jackfruit seed flour protein.

Qualitative estimation of essential amino acids

Thin layer chromatography (TLC) was done for qualitative analyses of the presence of amino acids. WAPC protein isolate had all the nine essential amino acids determined on the basis of their retardation factor (R_f) values. The calculated R_f value for methionine, arginine, tryptophan, threonine, valine, isoleucine, phenylanine, lysine and histidine were 0.41, 0.17, 0.56, 0.23, 0.36, 0.45, 0.52, 0.19 and 0.23 respectively (Table 2) and was within the range of R_f values of standard amino acids (Fig. 3). Similar qualitative estimation of amino acids was done by Elijah *et al*, (2016) [16] in soybean products.

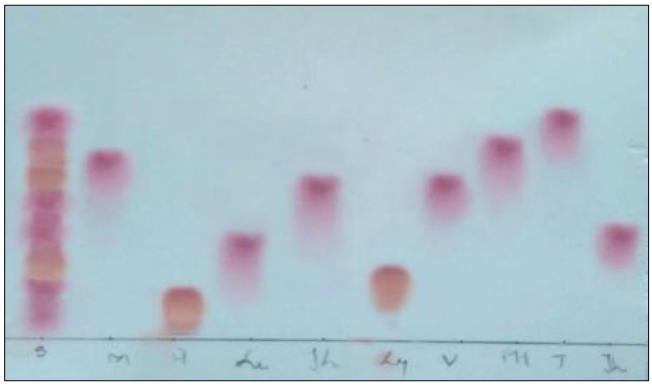

Fig 3: Qualitative analysis of essential amino acids (Protein isolate) using thin layer chromatography

Conclusion

It is clear from the study that different anti-nutritional factors significantly reduced from wild apricot kernel protein isolate. Apricot kernel press cake protein isolate was observed free from hydrogen cyanide (HCN). Phytic acid and tannins were reduced with the heating at 100 °C for 30 minutes. The digestibility of proteins was an important parameter to evaluate their nutritional quality. In-vitro protein digestibility of protein isolate was 80.58 per cent, So it act as good raw material for value added functional food with low cost.

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