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ORIGINAL

Amino Acid Profiles and Compositions of Different Cultivars of *Panicum miliaceum* L.

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Abstract

Amino acids are valuable nutrients, responsible for a variety of tasks in the human body. A favourable amino acid profile in gluten-free crops, such as millet, can thus be beneficial for human health, which is why 35 proso millet (*Panicum miliaceum* L.) samples, comprising 23 whole and 12 dehulled, were investigated regarding their amino acid profiles and compositions using acidic hydrolysis and ion-exchange chromatography with ninhydrin derivatization and subsequent detection with photometry. Results for amino acid compositions were compared with gluten-containing wheat and other gluten-free cereals. Furthermore, gained values were put in contrast to estimated essential amino acid requirements for adult humans. The study was able to show that cultivars of proso millet differ and that dehulling does not significantly influence the amino acid compositions. Furthermore, the results display that *Panicum miliaceum* L. holds more essential amino acids than other gluten-free grains and exhibits high amounts of leucine and alanine. The methionine content differs greatly between samples, which means that choosing certain cultivars is important to ensure a high content. The most abundant amino acids in proso millet grains are glutamic acid/glutamine (2.13 ± 0.34 g per 100 g), alanine (1.06 ± 0.18 g per 100 g) and leucine (1.36 ± 0.24 g per 100 g).

Keywords Ion-exchange chromatography · Proso millet · Amino acid composition · Nutrition · Cultivars

Introduction

Proso millet is an ancient crop and has been part of the human diet since the Neolithic times [1]. It is a gluten-free cereal, which is drought-resistant and can grow on soils which are low in nutrients [2–4]. Millet may prove to be an alternative for coeliacs, people with non-coeliac gluten sensitivity (NCGS) and food style adapts [5]. Coeliac disease is a chronic auto-immune disorder, where inflammations in the small intestine occur through exposure to gluten. If patients consume gluten on a regular basis, severe ablation of the intestinal villi can be observed [6]. The prevalence of coeliac disease is about 1–3% in Europe and the USA [7]. The disease is often diagnosed at a young age and the only cure is to follow a strict gluten-free diet for life [7, 8]. Lately, also people without health conditions try to reduce gluten in their diets or avoid it completely. This stems from the general notion that gluten-free food is healthier [9]. Reducing or avoiding gluten without the advice of a nutritionist or doctor can however lead to malnutrition due to a less balanced diet. A gluten-free diet often contains fewer carbohydrates and more salt and fat than recommended. Furthermore, gluten-free crops often have a lower protein content and less dietary fibre than gluten-containing cereals [10–16]. Nevertheless, gluten-free crops like proso millet can help to diversify diets and eat less wheat-based products. This is

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why the implementation of gluten-free and ancient crops in diets is encouraged.

Amino acids are an important class of nutrients. The human body uses them for protein synthesis, cell signalling, synthesis of low-molecular weight nitrogenous substances as well as substrates in certain metabolisms [17, 18]. Amino acids can be grouped into essential (EAA) and non-essential amino acids (N-EAA). The latter can be synthesised in the human body, whereas the former need to be added through diet. Threonine, valine, methionine, isoleucine, leucine, phenylalanine, histidine and lysine belong to the group of essential amino acids, whereas aspartic acid, asparagine, serine, glutamic acid, glutamine, proline, glycine, alanine, tyrosine and arginine belong to the group of non-essential amino acids. Histidine is actually a semi-essential amino acid, which means that it is partially synthesised in the human body. In the interest of simplicity, it is grouped with the essential amino acids in this work.

This study deals with the amino acid profiles and compositions of different cultivars of *Panicum miliaceum* L. (namely cv. AUT-, cv. AUT-2, cv. Early Bird, cv. Gierczyckie, cv. GLRH16106, cv. Horizon, cv. Huntsman, cv. ITA-4, ITA-5, cv. Jagna, cv. Kornberger, cv. Lisa, cv. Quartett, cv. Sunrise and cv. Tiroler) and investigates the variation between them. All grains were sown and harvested in Austria and Italy. Whole and dehulled grains were also examined regarding their differences. The latter were produced using a conventional dehuller (Yamomoto, FC2K-Y, Yamagata, Japan) before analysis. Proso millet grains are often dehulled before food processing to reduce storage volume and improve sensory and edible quality [19]. Proso millet flours, however, can be purchased from whole or dehulled grains, consequently both types need to be investigated. By understanding the possible differences between cultivars and the effect of dehulling on the grains, it will be possible to identify favourable samples for food consumption and breeding.

Methods and Materials

Sample Management

A set of 35 proso millet (*Panicum miliaceum* L.) grains whole and dehulled were analysed for their amino acid profile. All samples were dried whole in an infrared chamber at 35 °C for three days after harvest. Then, some samples were divided into two parts and one part was dehulled, while the other was not. For dehulling a conventional dehuller was used to remove the hull before analysis. All samples whole and dehulled were stored in a freezer at -20 °C until analysis. The final moisture content was 9.4 ± 0.4 g per 100 g for all samples after thawing and was analysed using a standard method [20].

Since the husk of proso millets is often removed before food processing, the set included 23 whole and 12 dehulled samples. Furthermore, the sample set comprised 15 unique cultivars, but some samples were sowed and harvested in two consecutive years. All cultivars were harvested from trial fields in Austria and Italy. Supplementary Table 1 shows the acronyms of the samples and their year of harvest.

Chemicals

For the hydrolysis of the samples 6 mol L^{-1} hydrochloric acid, which was produced by mixing fuming HCl (11.6 mol L^{-1}, Carl Roth, Karlsruhe, Germany) with deionized water which passed a reverse osmose system twice (18.2 MΩ cm), was used. The samples were then mixed with Lithium Citrate Loading buffer (pH = 2.2), internal standard (*S*-2-aminoethyl-L-cystein-hydrochloride, 2.5 µmol L^{-1}), 50 g per 100 mL sulfosalicylic acid and 1 mol L^{-1} sodium hydroxide. The amino acid analyser used five different Lithium Citrate buffers between pH 2.8 and 3.55 as a mobile phase. Furthermore, a Lithium Regeneration buffer with pH over 13 was used to regenerate the column after each run. All lithium-containing buffers and ninhydrin were purchased from Biochrom (Berlin, Germany). The external standard calibration was measured using the physiological standard mix from Laborservice Onken (Gründau, Germany), which contains 42 different amino acids and their derivatives, each with a concentration of 1 µmol mL^{-1}.

Determination of Amino Acid Profile

Sample Preparation

To investigate amino acids, all proteins in the samples have first to be split into their building amino acids. Thus, all samples were first subjected to acid hydrolysis according to an international standard method [21, 22]. Therefore, 50 mL of 6 mol L^{-1} hydrochloric acid were added to 0.5 g of ground samples and stirred under reflux for 24 h at 110 °C without purging out air. Afterwards, the hydrolysates were filtered through a fritted glass with a porosity of 4 to remove solid particles and then hydrochloric acid was removed by using a rotary evaporator (Büchi, Flawil, Switzerland). The hydrolysate was evaporated at 60 °C and 40 bar until an oily residue, which could not further be evaporated, was yielded. The residue was washed three times using 20 mL of deionized water. Lastly, 25 mL of deionized water from a MilliQ device (18.2 MΩ cm) (Merck, Darmstadt, Germany) were added to the residue.

Next, the samples were filtered again using a 0.2 µm sterile non-pyrogenic filter with a polyethersulfone (PES)

membrane. Then, 500 µL of samples were diluted with 1000 µL Lithium Citrate Loading buffer (pH 2.2). Next, 500 µL of this solution were mixed with 50 µL of internal standard and 50 µL 50% sulfosalicylic acid. The mixture was vortexed and then centrifuged at 10,700 rpm (10,880*g*) for 5 min. 300 µL of the supernatant were mixed with 300 µL of Lithium Citrate Loading buffer and finally 30 µL of 1 mol L^{-1} sodium hydroxide solution were added. The mix was then stored at -20 °C until analysis. Each sample was prepared for analysis three times.

Analysis of Amino Acid Composition with Icon-Exchange Chromatography

The amino acid profile was determined using an amino acid analyser (Biochrom 30 +, Biochrom, Cambridge, UK), which is based on ion-exchange chromatography [23]. It was calibrated using an external standard and a response factor. Furthermore, an internal standard was used to control the separation and the derivatisation. The Biochrom 30+ separated the amino acids using a high-pressure PEEK column packed with Ultropac 8 cation exchange resin. The mobile phase comprised buffers of varying pH. After column separation, the eluent was mixed with a ninhydrin reagent at 135 °C and passed through a high-temperature reaction compartment with 10 m length and 0.3 mm diameter with 20 mL h^{-1}. Ninhydrin reacted with the amino acids and yielded a coloured complex, which was used for detection with a photometer. The absorbance was measured at two wavelengths: 570 and 440 nm. The column was then cleaned and regenerated using the Lithium Citrate Regeneration buffer and the Lithium Citrate Loading buffer [24]. The concentrations of the amino acids were then calculated using external standards and their respective response factors.

For sample measurements, the temperature was adjusted during the run. In the first 36 min the column was heated to 30 °C, then the temperature was changed to 43 °C for 15 min followed by a change to 50 °C for 3.5 min. Next, the temperature was set to 70 °C for 21 min and then to 76 °C for 46 min. After that, the column was cooled to 70 °C for 20 min and finally the column was cooled to 33 °C for 14 min. The flow rate was kept constant at 25.0 mL h^{-1}. The mobile phase followed a pH-gradient, which was created by lithium citrate buffers with varying pH values. For the first 6 min, a buffer with pH 2.8 was used, followed by a Lithium Citrate buffer with pH 3.0 for 30 min. Next, the mobile phase was changed to a buffer with pH 3.15 for 18.5 min and then to a buffer with pH 3.5 for 21 min. Then, a mobile phase with pH 3.55 was used for 32 min followed by the Lithium Citrate Regeneration buffer with pH > 13, which was used for 6 min. For the remaining run time, the first buffer with pH 2.8 was used. In total the run time was 119.5 min.

Data Evaluation

For statistical evaluation of the measured amino acid concentrations, the means and standard deviations were calculated. Additionally, the medians of hulled and dehulled samples were calculated, to compare them to their respective means. Statistical differences between hulled and dehulled samples from the 1st and 2nd year of harvest were evaluated using one-way ANOVA ($\alpha = 0.05$). The correlation between each individual amino acid concentration and the sum of amino acid concentrations was determined using the Pearson correlation coefficient.

Results and Discussion

Amino Acid Profiles

The amino acid concentrations were calculated using an external standards and their respective response factors and are given in g per 100 g sample. In this study aspartic acid/asparagine, threonine, serine, glutamic acid/glutamine, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine and proline were investigated. The proline content was determined at 440 nm, all other amino acids were investigated at 570 nm. Additionally, the sum of these amino acids was calculated, to get an idea about the total amount of amino acids in the samples. The crude protein content of all but two samples (15-Jagna-H and 19-Kornberger-H) was measured as well and already published in a previous study [25]. The association between the sum of amino acids and crude protein of the remaining samples was 0.92 according to the Pearson correlation coefficient. In Fig. 1 the sum of amino acid concentrations is listed for each sample.

The samples set holds dehulled and hulled samples. The sum of amino acid concentrations of all samples ranges from 6.91 to 14.30 g per 100 g and has a mean of 10.42 g per 100 g and a median of 10.19 g per 100 g. The standard deviation (SD) of the total amino acid concentrations of all samples is 1.68 g per 100 g and the relative standard deviation (RSD) is 6.22%. Hulled grains range from 6.91 to 14.30 g per 100 g with a mean of 10.59 and a median of 10.36 g per 100 g. SD of samples with husk was 1.89 g per 100 g and RSD was 5.62%. Dehulled samples had a sum of amino acid concentrations between 7.74 and 11.49 g per 100 g with a mean of 10.10 and a median of 10.14 g per 100 g. SD of dehulled grains was 1.19 g per 100 g and RSD was 8.52%.

Whole samples show a broader concentration range regarding the sum of amino acids (6.91–14.30 g per 100 g) than dehulled samples (7.74–11.49 g per 100 g).

Furthermore, the median of dehulled samples is closer to the respective mean compared to the median and mean of hulled samples. The lowest sum of amino acids was found in ITA-5 with husk, the highest in Kornberger with husk. The latter held about double the amount of amino acids than the former. ITA-5 without husk had the lowest amount of amino acids within the dehulled samples. The highest amount of amino acids in dehulled samples was found in ITA-4. Dehulled ITA-5 holds about 1.5 times less amino acids than ITA-4 without husk. Comparing whole and dehulled samples using one-way ANOVA, a p value of 0.42 was found, indicating no significant difference between the means of those two groups. Consequently, it can be postulated that there are hardly any amino acids in the husk. However, looking at each year individually, it is interesting to note that for the second year dehulled sample hold more amino acids, but in the first year the amounts are often quite similar or the dehulled samples have a smaller total sum of amino acids. For the cultivars Early Bird, GLRH16106, Quartett and Sunrise, the differences between samples whole and dehulled are not very pronounced. For some other cultivars, this difference is however very pronounced. Furthermore, the samples from the first year on average exhibit a higher amount of amino acids than samples from the second year. The respective p value yielded with one-way ANOVA was below 0.05, which means that the two years differ significantly from each other.

Of the investigated amino acids eight belong to the group of essential amino acids (phenylalanine, leucine, methionine, lysine, isoleucine, valine, threonine and histidine) for humans. The remaining eight are non-essential amino acids (glutamic acid/glutamine, glycine, alanine, aspartic acid/asparagine, arginine, proline, serine and tyrosine). Tryptophan could not be measured since acid hydrolysis was performed and this amino acid can only be analysed using basic hydrolysis. Cysteine could also not be analysed as it is not stable during acid hydrolysis [26]. Additionally, the amino acids can be group into basic amino acids (lysine, arginine and histidine), polar amino acids (tyrosine, threonine, glycine, serine, aspartic acid/asparagine and glutamic acid/glutamine) and nonpolar amino acids (methionine, alanine, valine, leucine, isoleucine, proline and phenylalanine) In Fig. 2 a box plot with error bars depicts the distribution of the different amino acids in the samples.

It becomes clear that the most abundant amino acids are glutamic acid/glutamine and leucine, whereas the least abundant are methionine and lysine. Additionally, more abundant amino acids differ more between samples than less abundant amino acids—the only exception being methionine. The three basic amino acids investigated show a low abundance. Looking at polar amino acids, only glutamic acid/glutamine is highly abundant. All remaining polar amino acids are in the mid-abundance range relative to the other amino acids. The non-polar amino acids vary from high to low abundance in the samples. Leucine and alanine are highly abundant in the samples, but methionine is not.

The results were in good agreement with previous studies [27–29] and other literature [30]. Furthermore, the results were compared to amino acid compositions found in the literature for wheat, rice and maize because those are the three most commonly used crops [31, 32]. Rice and maize are both gluten-free crops whereas wheat is the most commonly consumed gluten-containing crop in the world. Table 1 lists the amino acid compositions of wheat, maize, rice and proso millet.

Regarding the sum of the essential amino acids, proso millet contains approximately the same amount as wheat. Additionally, proso millet comprises in total significantly more essential amino acids than rice and maize. Furthermore, proso millet has the highest sum of N-EAA of the listed gluten-free crops. Only wheat holds more N-EAA, which is mostly due to the high amount of glutamic acid/glutamine in wheat. Proso millet also has the highest total sum of amino acids of the listed gluten-free grains.

The limiting essential amino acid for wheat and maize is methionine. For proso millet lysine and methionine are limiting essential amino acids and for rice histidine and methionine are limiting. Table 1 shows that proso millet holds more threonine, leucine, serine, alanine and tyrosine than the three most commonly used crops. However, proso millet also holds the lowest amounts of lysine and glycine. For most amino acids, wheat exhibits the highest abundances and rice the lowest.

In the next step, it was investigated whether or not certain cultivars exhibit exceptionally high or low amounts of certain amino acids. These cultivars would then be suitable for food processing, as well as good choices for breeding new proso millet cultivars. Figure 3 depicts the relative abundance of all amino acids in all samples. The results were obtained by calculating the mean of each amino acid and setting it as 1 and then expressing the abundance of each amino acid of each sample as a multiple of the mean.

The most average sample is probably 30-Sunrise-H. All amino acids except for threonine and methionine are close to the respective means. It is also apparent that methionine has the largest variance among the samples. Furthermore, Fig. 3 shows that if a sample has a high amount of amino acids this is not attributed to just one amino acid, but to all amino acid levels being elevated. The only exception is methionine, as it is not correlated with the rise or decline of the remaining amino acids. This is also confirmed in Table 2, where the Pearson correlation coefficient between amino acids and sum of amino acids is listed.

Methionine correlation to the total amount of amino acids is close to zero. Threonine and lysine also have a very low correlation to the overall amount of amino acids

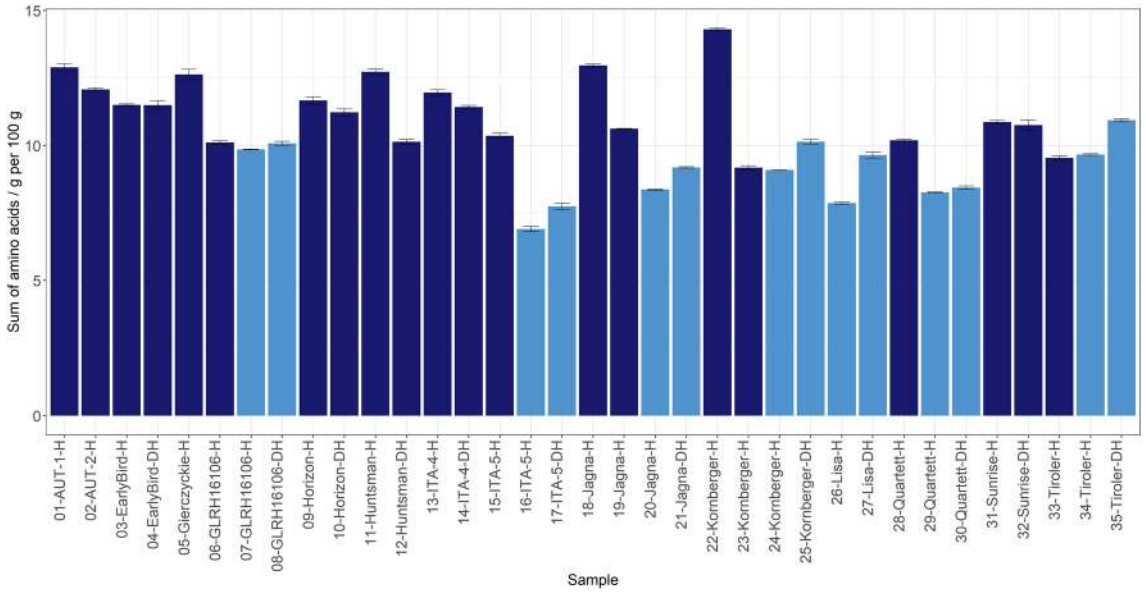

Fig. 1 Sum of amino acids for each sample. Dark blue—2016, year of harvest; light blue—2017, year of harvest. *H* whole samples, *DH* dehulled samples

in the sample. In Fig. 2 it is evident that lysine concentration is approximately the same for all the samples, which explains the low correlation to the total amino acid concentration. Additionally, glycine and arginine have a correlation value of about 0.9. Both amino acids have a rather low abundance in proso millet samples and also have a low variance across samples.

Nutritional Implications

In Europe wheat and corn dominate the crop market, but recently proso millet and other gluten-free grains gained popularity. Table 1 clearly shows that proso millet holds more amino acids than other gluten-free crops. Table 3 below shows the estimated essential amino acid requirements of human adults [34] in comparison to the found amino acid concentrations in the samples. Phenylalanine and tyrosine as well as methionine and cysteine are listed as one parameter because cysteine and tyrosine can partly replace methionine and phenylalanine in the human body [34, 35].

The two most abundant essential amino acids in the investigated proso millet samples and the two most highly required EAA are leucine and phenylalanine/tyrosine. Lysine, histidine and methionine/cysteine are also required in the human diet, however, the former three EAA are not highly abundant in the investigated proso millet samples. The content of cysteine was not measured in this study. Consequently, a diet which integrates proso millet should be complemented with other lysine-, histidine- and methionine-rich foods. By choosing specific cultivars of proso millet, the methionine intake can also be influenced.

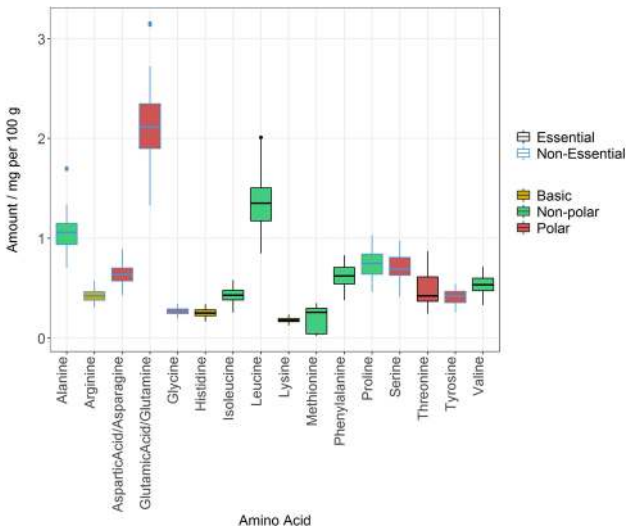

Fig. 2 Box plot with error bars of the distribution of each amino acid in all samples. The bold line in the box indicates the median and the box refers to the 2nd and 3rd quartile. The 1st and 4th quartiles are indicated by the lines below and above each box

Additionally, asparagine can be transformed into acrylamide when sugar is present during food processing [36]. Hence, a low asparagine content is beneficial for health and lowers the risk of acrylamide formation. In general, proso millet has a low aspartic acid/asparagine concentration.

Table 1 Comparison of amino acid compositions of durum wheat, maize, medium-grain unenriched white rice and proso millet

Essential/non-essential	Amino acid	Wheat[a]/g per 100 g	Maize[a]/g per 100 g	Rice[a]/g per 100 g	Millet[b]/g per 100 g
EAA	Threonine	0.37	0.35	0.24	0.49
	Valine	0.59	0.48	0.40	0.54
	Methionine	0.22	0.20	0.16	0.18
	Isoleucine	0.53	0.34	0.29	0.43
	Leucine	0.93	1.16	0.55	1.35
	Phenylalanine	0.68	0.46	0.35	0.63
	Histidine	0.32	0.29	0.16	0.25
	Lysine	0.30	0.27	0.24	0.18
N-EAA	Aspartic acid/asparagine	0.62	0.66	0.62	0.64
	Serine	0.67	0.45	0.35	0.71
	Glutamic acid/glutamine	4.74	1.77	1.29	2.12
	Proline	1.46	0.82	0.31	0.75
	Glycine	0.50	0.39	0.30	0.27
	Alanine	0.43	0.71	0.38	1.06
	Tyrosine	0.36	0.38	0.22	0.42
	Arginine	0.48	0.47	0.55	0.42
Sum EAA		3.94	3.55	2.39	4.05
Sum N-EAA		9.26	5.65	4.02	6.39
Total sum		13.20	9.20	6.41	10.44

EAA essential amino acids, *N-EAA* non-essential amino acids

[a]Data was taken from the National Nutrient Database for Standard Reference from USDA [33]

[b]Averages of measured data

Reliability of Analysis

Proso millets, just like other cereals, are dependent on the nutrients from the soil it grows on. Hence, the soil greatly influences nutritive properties of the harvested grains. To combat this, some samples were harvested in two consecutive years, to investigate if the amino acid profiles change from one year to another. Samples were grown in the same fields with the same soil in both years. The fields were located in Italy and Austria and had varying growing conditions. Some fields were located close to the sea, others in the Alps. Some fields were in a valley, others on a hillside. This way, a realistic experimental set-up was ensured, as on the European market proso millet is always only available as a mixture from a variety of fields. As already mentioned in Sect. 3.1, the samples harvested in the first year have a slightly higher amount of amino acids than the samples from the second year. However, this is not due to an increase of a specific amino acid but related to an overall increase of amino acid concentrations. Consequently, the soil influenced the amount of total amino acids (see Fig. 1), but did not change the profile (see Fig. 3). This indicates that the overall growing conditions were better in the second year, which promoted higher amino acid abundances.

Hydrolysing proteins and measuring the amino acid profile afterwards is very precise, but has a few drawbacks. First of all, some amino acids are unstable during hydrolysis. Asparagine and glutamine are de-aminated and transformed to aspartic and glutamic acid. This was also the case in this study, but since the deamination affects these amino acids fully, the glutamic acid and aspartic acid peaks are used to determine the respective concentrations [37]. Serine and threonine are partially destroyed during hydrolysis, which is accounted for by internal standards and multiplication with a factor. Methionine is sometimes oxidized during hydrolysis. This is why derivatives of those amino acids are usually also monitored during analysis [37]. Furthermore, ion exchange chromatography is not able to distinguish between L- and D-amino acids. Consequently, the chirality is not specified. Additionally, the reaction with ninhydrin is often not complete. This does not pose a problem, as long as the ratio of derivatized amino acids is always the same [37].

Lastly, the measurements can vary from run to run and from day to day. This is why an interday and an intraday assay was used to determine the accuracy of the analysis (see supplementary Table 2). Five standards, comprising all amino acids investigated at a concentration of 2.5 µmol mL^{-1}

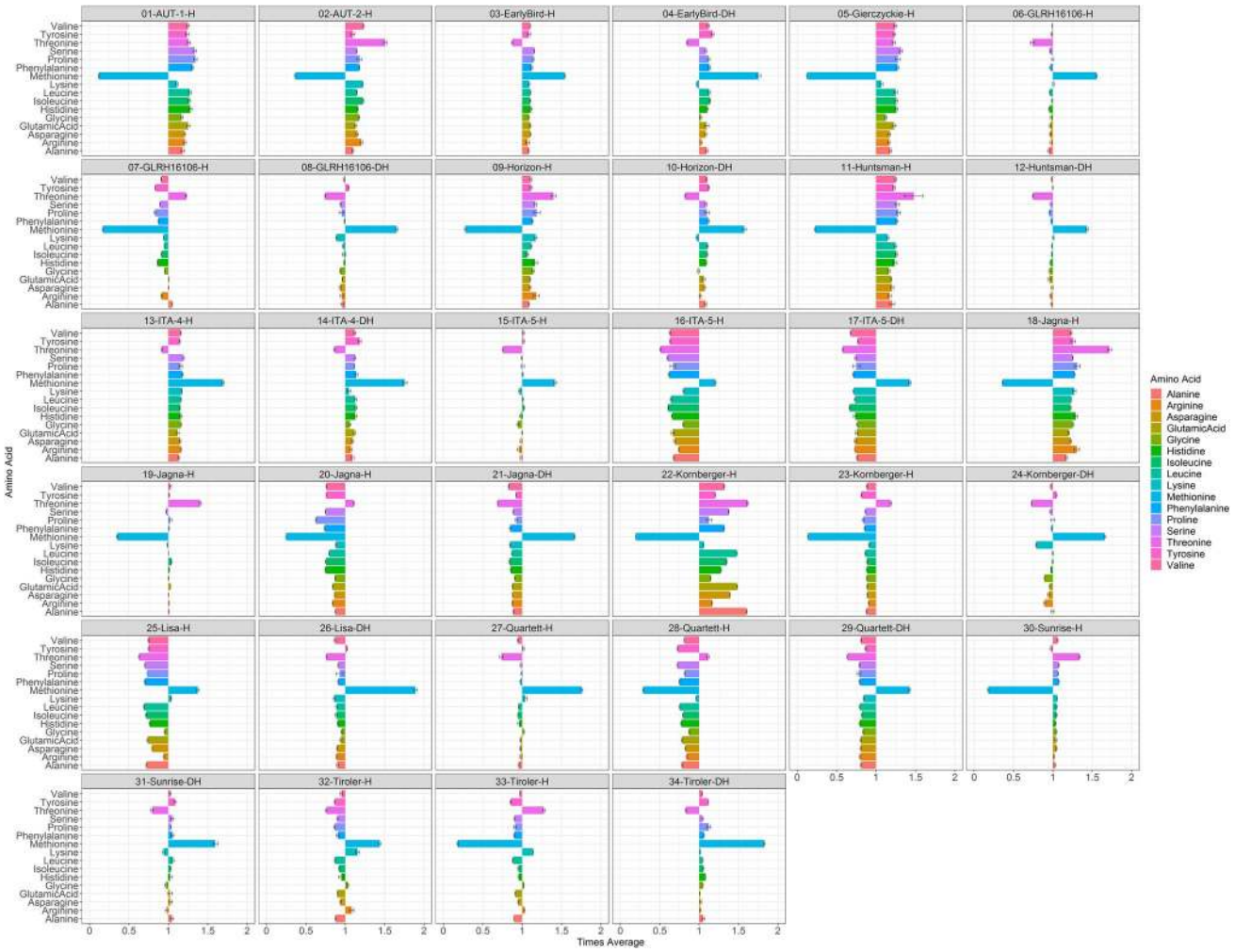

Fig. 3 Relative abundances of each amino acid in each sample, expressed as multiples of the mean, which is set as 1

in acidified water, were analysed for the inter- and for the intraday assay.

Conclusion

This study investigates 35 proso millet (*Panicum miliaceum* L.) samples regarding their amino acid profiles and compositions using ion-exchange chromatography. The results showed that although the amino acid profile is quite similar for all cultivars examined in this study, the concentrations vary greatly. Consequently, to assess the suitability of different types of millet (e.g. proso, pearl, finger millet) for consumption a variety of cultivars of each type need to be tested, as they already vary greatly in their nutritional parameters.

The obtained results were also compared to values for gluten containing and gluten-free grains. This revealed that proso millet holds the highest concentration of essential amino acids and thus is a valid alternative to durum wheat, maize or white rice. The amino acid profiles of proso millet were put in contrast to the estimated requirements of essential amino acids for adult humans. The results show that proso millet has high concentrations of phenylalanine/tyrosine and leucine, which matches the high requirement of humans for these amino acid. Additionally, the methionine intake from proso millet can be regulated by choosing cultivars with high or low concentrations of this amino acid.

Lastly, this study highlights the statistical evaluation of the influence of different harvest years and pre-processing steps such as dehulling on the amino acid profiles and concentrations. Dehulled samples did not differ significantly from the whole samples. The year of harvest on the other hand, had a significant impact on the sum of amino acids in the samples.

Table 2 Pearson correlation coefficient between the total amino acid and a specific amino acid

	Pearson correlation coefficient
TAA—alanine	0.955
Taa—arginine	0.900
Taa—aspartic acid/asparagine	0.990
TAA—glutamic acid/glutamine	0.985
TAA—glycine	0.899
TAA—histidine	0.983
TAA—isoleucine	0.986
TAA—leucine	0.993
TAA—lysine	0.681
TAA—methionine	− 0.224
TAA—phenylalanine	0.989
TAA—proline	0.932
TAA—serine	0.989
TAA—threonine	0.622
TAA—tyrosine	0.935
TAA—valine	0.985

TAA total amino acids

Table 3 Comparison between the estimated essential amino acid requirements of a human adult [34] and the found amino acid concentration in the proso millet samples

Amino acid	Millet/g per 100 g	Estimated requirement/mg per kg body weight
EAA		
Threonine	0.49	7
Valine	0.54	10
Methionine and cysteine	0.18	13
Isoleucine	0.43	10
Leucine	1.35	14
Phenylalanine and tyrosine	1.04	14
Histidine	0.25	8–12
Lysine	0.18	12

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Author Contributions Conceptualization: VW, SS-B, DK, CH; methodology: VW, SS-B; formal analysis an investigation: VW; writing—original draft preparation: VW; writing—review and editing: VW, SS-B, DK, CH; funding acquisition: VW, SS-B, DK, CH; resources: SS-B, D, CH; supervision: SS-B, CH.

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Availability of data and material The data is available upon request. The sample material will be available until 06/2021.

Compliance with Ethical Standards

Conflicts of interest The authors declare no conflicts of interest.

Ethical approval The research involved no ethical issues concerning living beings. The authors complied with the ethical standards in publishing.

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