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Developing an NIRS Prediction Model for Oil, Protein, Amino Acids and Fatty Acids in Amaranth and Buckwheat

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Abstract: Amaranth and buckwheat are two pseudo-cereals preferred for their high nutritional value, are gluten free and carry religious importance as fasting food. Germplasm resources are the reservoir of diversity for different traits, including nutritional characteristics. These resources must be evaluated to utilize their potential in crop improvement programs. However, conventional methods are labor-, cost- and time-intensive and prone to handling errors when applied to large samples. NIRS-based machine learning to predict different nutritional traits is applied in different food crops for multiple traits. NIRS prediction models are developed in this study using the mPLS regression technique for oil, protein, fatty acids and essential amino acid estimation in amaranth and buckwheat. Good RSQ external (power of determination) values were obtained for the above traits ranging from 0.72 to 0.929. Ratio performance deviation (RPD) value for most of the traits ranged between 2 and 3, except for valine (1.88) and methionine (3.55), indicating good prediction capabilities in the developed model. These prediction models were utilized in screening the germplasm of amaranth and buckwheat; the results obtained were in good agreement and confirmed the applicability of developed models. It will enable the identification of a trait-specific germplasm as a potential gene source and aid in crop improvement programs.

Keywords: machine learning; RSQ; RPD; mPLS; WINISI; validation

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1. Introduction

Pseudo-cereals are non-grass, dicotyledonous plants that produce seeds and have a similar physical appearance to those of true cereals and are consumed in a similar way. Among the prevalent dominance of major cereal crops, such as wheat, rice and maize, in the diet, pseudo-cereals have not gained much attention. However, the remarkable nutritional and phytochemical profile of pseudo-cereals, low gluten content, and their resistance potential toward adverse climatic conditions have sparked growing interest in pseudo-cereals among scientific communities over the last few years. As pseudo-cereals can grow under adverse environmental conditions and a small harvest cycle, they may provide an alternative option to meet nutritional and food security concerns, so they are required to bring under a staple diet.

Amaranth (*Amaranthus* L. spp.) and buckwheat (*Fagopyrum esulentum* Moench and *Fagopyrum tataricum* (L.) Gaertn) are among the most popular pseudo-cereals [1]. Just like cereals, they also have high economic value and can be used in many ways, such as for the preparation of gluten-free bakery foods, food supplements, beverages, sweets, etc. An amaranth- and buckwheat-enriched diet is recommended for celiac disease patients because of their gluten-free properties and for baby food as an alternative to rice due to its low allergenicity [2]. From 2019 to 2025, the production of gluten-free food is assumed to be expanding at an annual growth rate of 9.1% [3].

In general, gluten-free food has high fats, sugar and sodium and a lower content of protein and minerals. Despite being gluten free, pseudo-cereal seeds offer complex supplementation for nutritional deficiencies [4] as they are rich in minerals, essential amino acids, such as lysine, cysteine and methionine, as well as in proteins, such as albumin and globulin. In addition, pseudo-cereals are also low in starch and anti-nutritional factors, such as phytate, tannins, and saponins, which affect the nutrient absorption and utilization compared to cereals. The high caloric content and balanced amino acids in pseudo-cereal crops are advantageous for coping with micronutrient deficiency in developing and underdeveloped countries.

In addition, the nutritional superiority of amaranth and buckwheat species, as well as their medicinal properties, have been reported, such as antioxidant, analgesic, hypoglycemic, anti-inflammatory, antipyretic, antihypertensive, anti-hypercholesterolemic, diuretic and laxative effects, and also valuable for bronchitis, leucorrhoea, burns, wound healing, rheumatism, suppressing gallstones and tumor [5–9]

Hence, the nutrient profile of pseudo-cereals, such as amaranth and buckwheat, makes them suitable for gluten-free and nutritionally rich diets. Both crops have a substantial genetic diversity, so large variability in nutritional characteristics within the crop also exists. To bring these non-traditional crops into the popular food system, developing improved varieties with the best possible nutrient profile is of the utmost importance. The national gene bank at ICAR-National Bureau of Plant Genetic Resources has 4950 accessions of amaranth and 1057 accessions of buckwheat. For full utilization of these germplasm resources, it is essential to evaluate them for nutritional traits. However, major bottlenecks for evaluation are technically complex, time-consuming and costly methods. Near-Infrared spectroscopy (NIRS) is an established technique for rapid and non-destructive evaluation of multiple nutrients. It has been credited as the most powerful analytical technique for estimating the major nutrients in agricultural and food products. It offers the advantages of easy sample presentation, rapid data collection, low cost and accurate measurement. It provides quick and precise information from the high-resolution spectra of solids without prior sample preparation [10]. NIR spectra data are pre-processed for reducing the influence of noise and to increase the signal-to-noise ratio. Scatter correction of reflected light due to the influence of sample surface features and path lengths is corrected through commonly used methods, including Savitzky–Golay, standard normal variate and detrend (SNV-DT), multiplicative scatter correction (MSC), weighted MSC and Inverted MSC. Spectra are derivatized to 1st, 2nd, 3rd or 4th order for enhancing the peaks and valleys for feature extraction and correlation [11]. Several machine learning approaches, including linear regression, such as partial least square (PLS), modified PLS (mPLS), orthogonal PLS (OPLS), principal component regression (PCR) and non-linear regression, including artificial neural network (ANN), support vector regression (SVR), random forest (RF) and decision tree (DT), are adopted for developing prediction models [12]. Linear regressions are primarily used in NIR prediction modeling and mPLS has been reported to provide better results over PCR and other PLS techniques [13,14]. The availability of efficient chemometric evaluation tools and software, such as Winisi, Unscrambler and Matlab, has made NIRS a method of choice for the rapid and non-destructive evaluation of germplasm resources.

In this study, we attempted to develop an NIR-based prediction model (simple, rapid and green method) for estimating oil, proteins, essential amino acids and fatty acids in the amaranth and buckwheat germplasm. Otherwise, biochemical analysis for these traits is tedious, time-consuming, costly and requires a large number of chemical reagents and a high degree of technical expertise. The developed model will also be used in crop improvement programs for studying trait inheritance in breeding populations and may also be used by the food and seed industry for quality assessment.

2. Materials and Methods

2.1. Sample Collection

In total, 150 accessions each of amaranth and buckwheat were obtained from NBPGR, Regional Station Shimla, India, of which 100 accessions were used as a training set for developing the NIRS prediction models to estimate the oil, protein, fatty acids and essential amino acids content in amaranth and buckwheat. The remaining fifty accessions were used as a testing set for validation of the developed NIRS prediction model. The wet chemistry data were generated for all 150 accessions following standard methods (Section 2.2), and used as reference values in the training and testing of models. The robustness and applicability of developed models were further tested on an additional set of 600 accessions of amaranth and 190 accessions of buckwheat.

2.2. Reference Analysis for the NIRS Prediction Models

2.2.1. Total Oil Content of Seed

All the amaranth and buckwheat seeds were oven dried to 4–5% moisture level at 108 °C for 16 to 18 h. The oil content of the seed samples was determined by a non-destructive method using a Newport NMR analyzer (Model-4000) from Oxford Analytical Instruments Ltd. U.K, equipped with 40 mL coil assembly. The instrument was kept in a room of constant temperature (23–25 °C). Pure seed oil required to calibrate the instrument was extracted by solvent extraction method. The instrument was run by adjusting audio frequency (AF) gain of 400 and radio frequency (RF) current of 225 µA with a gate width of 1.5 gauss. The NMR responses (signal/mass) of the seed samples were compared to NMR response of pure oil (100%) for obtaining oil percentage of the amaranth and buckwheat accessions.

2.2.2. Fatty Acid Profiling

Amaranth and Buckwheat seed samples were freshly ground using a cyclotech mill and weighed so that 40 mg oil is obtained when extracted with a 10 mL solvent mixture of chloroform: hexane: methanol (8:5:2 $v/v/v$). The extracts obtained were dried at 60 °C in nitrogen gas for 30 min. Methyl esters of oil samples were prepared according to the method described by [15] with slight modifications applied for Cruciferous species [16]. Thus, 1 µL of the hexane extract was injected into a highly polar HP Innowax capillary column of 30 m length (inner diameter: 0.32 m, film thickness: 0.5 µm, split: 1:80). A Hewlett Packard gas chromatograph, model 6890, equipped with flame ionization detector (FID) was used. The injector and detector temperatures were 260 °C and 275 °C, respectively. The oven temperature was programmed from 150 °C holding at 1 min to 210 °C at a rate of 15 °C/min, followed by 210 °C to 250 °C at a rate of 5 °C/min for 12 min. Peaks of fatty acid methyl esters were identified by comparing their retention time with the known standards under similar separation conditions, and peak integration was performed by applying HP3398A software.

2.2.3. Total Protein Content

Nitrogen contents of the Amaranth and Buckwheat seed samples were determined by following the conventional Kjeldahl method (AOAC, 984.13) [17] using Kjeltec analyzer (Model-2300) from Foss Tecator, Sweden. Jones' conversion factor 6.25 was used to convert percent nitrogen to percent protein.

2.2.4. Amino Acid Profiling

The amino acids, including Arginine (Arg), threonine (Thr), leucine (leu), methionine (Met), phenylalanine (Phe), valine (Val), cysteine (Cys), histidine (His), isoleucine (Ile) and lysine (Lys), were investigated. Amino acid analysis was carried out using an HPLC-based pre-derivatization technique because most amino acids do not yield fluorescence. Therefore, the derivatization of amino acids with appropriate reagents transforms primary and secondary amines into highly stable and sensitive fluorescence derivatives, necessary

for the detection of amino acids. Fluorescent active reagent 6-aminoquinolyl-N-hydroxy succinimidyl carbamate was used for derivatization with protein hydrolysate amino acids.

Hydrolyzing Samples

For the sample hydrolysis, 10 mg homogenized sample flour was taken in a 6 × 50 mm sample tube and placed in a reaction vial containing 200 µL of constant boiling HCl (6 N) and a crystal of phenol. Samples were kept in an oven at 112 °C to 116 °C for 20 to 24 h. After complete hydrolysis, excess HCl was removed, and tubes were dried under a vacuum.

Derivatizing the Samples

After hydrolysis, vacuum-dried samples were dissolved in 750 µL, 20 mM HCl solution. The derivatization process was performed by adding 20 µL of protein hydrolysate amino acid solution and 20 µL of AccQ. Fluor ^{TM} reagent (waters Part No. WAT052880) and 60 µL of AccQ. Fluor^{TM} Borate buffer (waters Part No.WAT052880) Milford, MA, United States. The reaction vials were vortexed and heated for 10 min at 55 °C.

Chromatographic Conditions

The HPLC system consisted of two pumps (Waters 515), autosampler (Waters), a column (Waters AccQ. Tag ^{TM} Milford, MA, United States of length 3.9 × 150 mm) and fluorescence detector (Waters 2475). A mobile gradient phase consisted of two eluents: mobile phase A contains a 10% solution of AccQ. Tag ^{TM} concentrate (Part No. WAT052890) and mobile phase B contains 60% HPLC-grade Acetonitrile. The gradient was initially A = 100%, 2 min = 98%, 15 min = 93%, 19 min = 90%, 32 to 37 min = 67% followed by a wash with 100% eluent B for 13 min and re-equilibration for 10 min at 100% by eluent A. Individual amino acid detection was carried out by fluorescence detector (Waters 2475) with excitation at 250 nm and emission at 395 nm with a bandwidth of 18 nm. The polarity of the detector was kept positive with a gain of 10 and a sampling rate of 1.

2.3. NIR Reflectance Spectroscopy Spectra Acquisition

2.3.1. Spectroscopic Analysis

About 5 g intact seeds was uniformly placed in a small circular ring cup (φ 3.8 cm and 1 mm thickness) for scanning on a Monochromator-based FOSS NIRS 6500, and reflectance spectra (Log 1/R) from 400 to 2500 nm were recorded at 2 nm interval. The NIRS spectra normalization, scatter correction, mathematical processing and statistical analysis were performed using the Global program in WINISI software version 3.10 (Infrasoft International, Port Matilda, PA, USA)

2.3.2. Outlier Identification

Outlier detection technique was used to predict the 'uniqueness' of a sample using neighborhood Mahalabonis distance (NH) and spectral distance from the mean spectrum of the population (GH). The NH estimates the proximity of each sample to every other sample in the population and GH ascertains the ability of the calibration model to predict the accuracy of an unknown sample and to allow for the removal of superfluous spectra from the calibration population [18]. The scoring algorithm ranks spectra according to H distance from the average spectrum. It provides spectral boundaries to eliminate outliers with GH > 2.5 and NH < 0.6 (Figure 1). Therefore, the final number of samples was variable for each parameter, depending on the spectral and chemical variability in the samples used in the population for NIRS estimation.

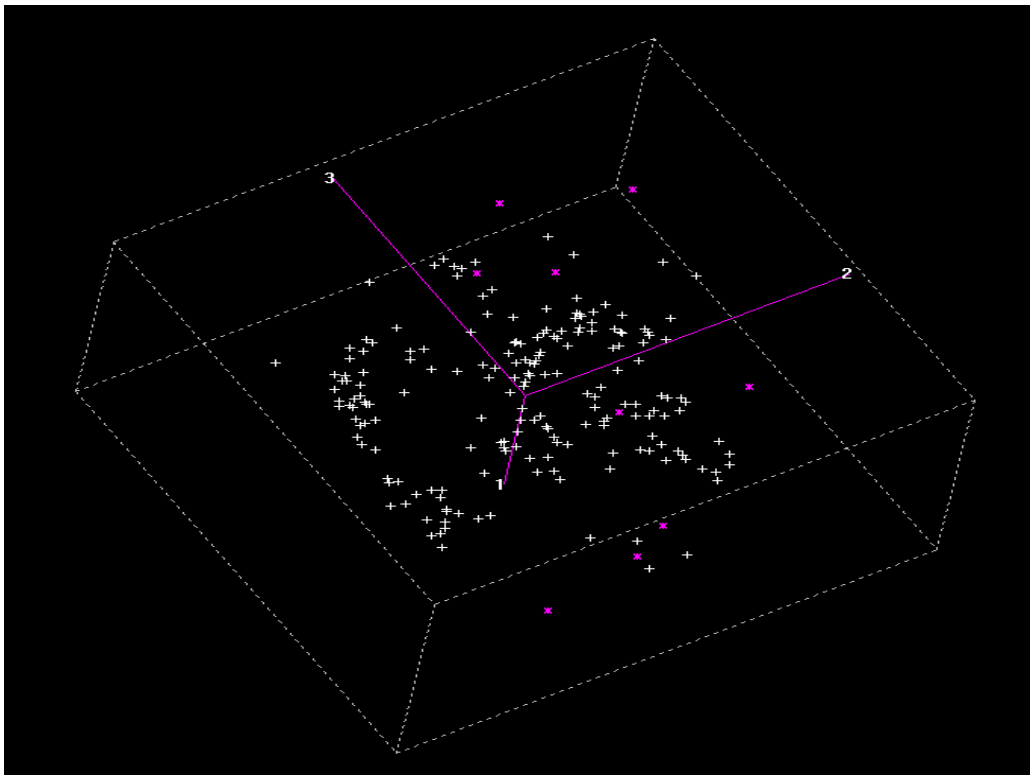

Figure 1. 3D view showing outlier samples.

2.3.3. Spectral Data Processing and Normalization

The standard normal variate and detrend (SNV-DT) scatter correction procedure was applied to the spectral data to reduce the difference in spectra related to physical characteristics, such as particle size and path length of samples. Mathematical treatments using the raw optical spectrum (log 1/R), first to fourth derivatives of the 1/R data combined with smoothing and gap size were used to maximize the signal to noise ratio. The multiple combinations of different mathematical treatments were as follows: 2,4,4,1; 2,4,2,2; 2,5,4,1; 2,5,2,2; 2,6,4,1; 2,6,2,2; 2,7,4,1; 2,7,2,2; 2,8,4,1; 2,8,2,2; 3,5,6,1; 3,5,3,2; 3,5,6,2; 3,6,6,1; 3,6,3,2; 3,6,6,2; 3,7,6,1; 3,7,3,2; 3,7,6,2; 3,8,6,1; 3,8,3,2; 3,8,6,2; 4,5,6,1; 4,5,3,2; 4,5,6,2; 4,6,6,1; 4,6,3,2; 4,6,6,2; 4,7,6,1; 4,7,3,2; 4,7,6,2; 4,8,6,1; 4,8,3,2; 4,8,6,2. Further, SNV-DT and mPLS (modified partial least square) regression were tested to obtain the best-fit model. In mathematical treatment, the first number indicates the order of derivative function (two is the second derivative of log 1/R), the second no. is the gap (the length in nm) in data points over which the derivatives is calculated, the third number represents the number of data points (segment length) used in first smoothing and the fourth number is the number of data points in the second smoothing [13,14]. The main absorption bands were observed at 1204 nm related to C-H stretching 2nd overtone (-CH_3), 1460 nm related to N-H stretching 1st overtone (CO-NH_2), 1760 nm related to CH (oil), 1938 nm related to OH bending 2nd overtone (water) and 2302 nm related to C-H bending 2nd overtone (protein) (Figure 2). Lipids have an absorption band around 1360 nm, 1762 nm. The information on functional groups in the spectrum was generated from WinISI II software.

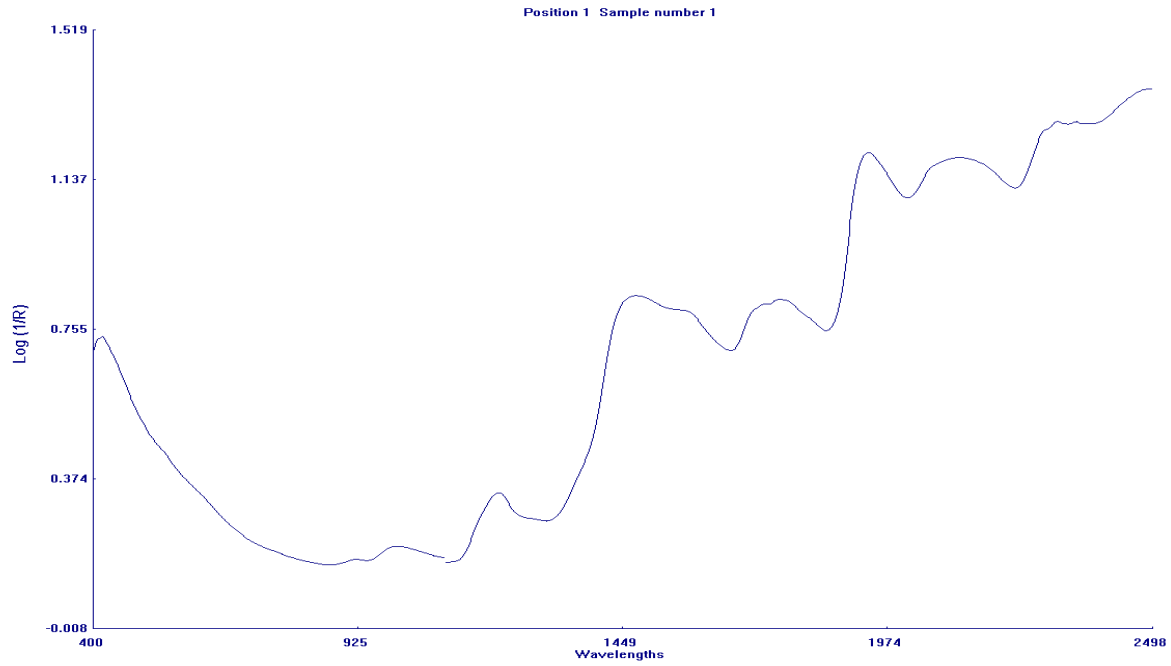

Figure 2. Average reflectance spectrum of amaranth and buckwheat homogenized flour with five absorption bands.

2.3.4. Development of Calibration Equation

The modified partial least square (mPLS) regression technique was used to develop the NIRS calibration using a spectral range from 400 to 2500 nm. The sample set with reference analytical values was split into two subsets in a ratio of 2:1, where the bigger subset of about 100 samples was used as the training set and the small subset of 50 samples for testing the calibration equations. The normalized spectral information was correlated to laboratory reference values for the constituents, and the best equations were selected based on having a low value of the standard error of cross-validation and a high value of the coefficient of determination (r^2) [19] (internal validation). The performance of calibration and accuracy of the equation were further confirmed by RPD (ratio of the standard deviation of reference data to the corrected standard error of prediction (SEP(C)) [20,21] obtained in testing (external validation)). The results for only the best-fit model confirmed in external validation, out of multiple combinations of math treatment tested for each trait, are presented in this manuscript.

For amino acids, a limited number of samples was analyzed on HPLC. Hence, amaranth and buckwheat were combined to develop the calibration equations for essential amino acids.

2.3.5. Statistical Analysis of NIRS Prediction Models

All the calibration and predictions were performed using Win ISI III Project Manager Software Version 1.50, which applied various mathematical treatments based on spectral and analytical data. Reference and predicted values were monitored using Win ISI Project Manager Software V 1.50 with the developed equation. Using global statistical values, such as RSQ, slope, bias, RPD and SEP(C), the accuracy and predictive capacity of the model were evaluated [11,13,14].

3. Results and Discussion

3.1. Quantification of Biochemical Parameters

Biochemical parameters, including oil, total protein, essential fatty acids (palmitic acid, oleic acid, linoleic acid, linolenic acid) and essential amino acid content (arginine + threonine, histidine, valine, cysteine, methionine, lysine, isoleucine, leucine, phenylalanine)

were established for 150 amaranth and 150 buckwheat accessions. Descriptive statistics are given in Tables 1 and 2. All 150 accessions were divided into two subsets. One of them, called the calibration set (100 accessions), is used to build the model, and the second set (50 accessions), the validation set, is used to test the robustness of the model.

Amaranth oil content was found to be significantly higher (7.8% to 11.6%) compared to buckwheat (1.5% to 2.9%). Amaranth oil is a good source of tocopherols and phytosterols, which plays a significant role in blocking cholesterol absorption and decreasing LDL-cholesterol. Protein was found to range from 8.2% to 14.1% in amaranth [2]. The protein content in amaranth and buckwheat ranged between 13.4% and 16.5% as well as 12.0 and 18.9%, respectively. Reference [22] reported protein content in buckwheat seeds as 8.81–18.7%. Protein in amaranth is located in the embryo, unlike corn and rice, in which protein is mainly found in endosperm. Protein content in amaranth was reported to be higher than in barley, corn, wheat, sorghum and rice. References [23–25] also reported 13.2–18.2% protein in amaranth. The fatty acid content in amaranth, including palmitic acid, oleic acid, linoleic acid and linolenic acid, varied from 15.4 to 21.8%, 22.5 to 37.6, 39.1 to 51.8% and 1.74 to 4.48%, respectively. Our results agree with [23,24,26], stating that linolenic acid is the most prominent fatty acid, followed by oleic and palmitic acids. In contrast, linolenic is found in traces only in amaranth seeds. The biochemical values as range and mean values of calibration and validation set obtained from the laboratory method with the NIRS method were similar.

Pseudo-cereals are considered functional food due to their high-quality protein, contributed by their rich essential amino acid composition. In the present study, the amino acid profile of amaranth and buckwheat was analyzed and is depicted in Table 3. The results show a good amount of essential amino acid content in all the amaranth and buckwheat accessions. The amino acid composition in amaranth and buckwheat shows the presence of essential amino acids, namely, arginine, threonine, histidine, valine, cysteine, methionine, lysine, isoleucine, leucine and phenylalanine. The arg+thr (8.78–15.5 g/16 g/N) content in amaranth and buckwheat was found to be highest, followed by lysine (5.32–8.13 g/16 g/N), leucine (4.27–5.54 g/16 g/N) and valine (3.38–4.66 g/16 g/N). Phenylalanine content was significantly varied from 1.39 to 5.31 g/16 g/N. Histidine is also found in reasonable amounts (3.05 to 6.08 g/16 g/N) required for child development. Methionine and cysteine are found in the lowest concentration range from 0.75 to 2.27 g/16 g/N and 1.06 to 2.68 g/16 g/N, respectively.

Pseudo-cereals are rich sources of essential amino acids that can be used as an alternative to cereal [27]. Lysine is considered as the most limiting amino acid in cereal proteins and cereal-based diets [28]; however, in buckwheat, it is reported as lysine-rich pseudo-cereal. References [29–31] also reported the presence of essential amino acid in buckwheat, mainly lysine (3.74–4.9 g/100 g) followed by isoleucine (0.19–1.3 g/100 g) and leucine (0.78–1.6 g/100 g). Amaranth is rich in amino acids, namely, isoleucine, alanine, tryptophan, valine, leucine, arginine, phenylalanine, methionine, α-aminobutyric acid and serine [32].

Table 1. Statistics for different traits of amaranth used in calibration and validation.

Traits	Amaranth												
	Laboratory Value (n = 100)	Calibration of NIRS Model					Validation Set (n = 50)	External Validation					
	Range (%)	Range (%)	$RSQ_{Internal}$	SECV	RSC	Math Treatment	Range (%)	Mean	$RSQ_{External}$	SEP	Bias	Slope	RPD
Oil	7.80–11.6	6.77–12.7	0.94	0.32	4.28	2,4,4,1	7.17–10.6	9.14	0.87	0.18	−0.00	0.95	2.72
Protein	8.20–14.1	6.94–14.1	0.75	0.75	2.04	2,4,4,1	8.56–13.1	10.5	0.84	0.51	−0.04	1.01	2.54
Palmitic acid	15.4–21.8	17.8–22.1	0.84	0.34	2.50	3,6,6,1	19.1–20.7	20.0	0.77	0.17	−0.05	0.91	2.01
Oleic acid	22.5–37.6	19.3–33.3	0.86	1.33	2.65	3,6,6,1	22.5–37.1	25.75	0.85	0.99	0.45	0.93	2.34
Linoleic acid	39.1–51.8	40.5–56	0.86	1.32	2.71	2,4,4,1	39.9–52.0	49.0	0.87	1.04	−0.53	0.90	2.39
Linolenic acid	0.58–1.63	0.58–1.52	0.85	0.073	2.56	3,6,6,1	0.71–1.19	1.03	0.81	0.04	−0.00	0.90	2.27

Table 2. Statistics for different traits of buckwheat used in calibration and validation.

Traits	Buckwheat											
	Laboratory Values (n = 100)	Calibration of NIRS Model					Validation Set (n = 50)	External Validation				
	Range (%)	Range	$RSQ_{Internal}$	SEC (V)	RSC	Math Treatment	Range (%)	$RSQ_{External}$	SEP	Bias	Slope	RPD
Oil	1.56–2.92	1.24–3.38	0.77	0.18	2.07	2,4,4,1	1.40–3.05	0.85	0.15	−0.05	1.09	2.46
Protein	9.58–19.2	9.76–19.2	0.977	0.520	6.31	2,4,4,1	10.1–19.1	0.804	0.97	−0.003	0.944	2.26
Palmitic acid	13.6–17.4	12.4–18.6	0.86	0.48	2.71	3,6,6,1	13.2–18.5	0.78	0.43	0.02	1.05	2.19
Stearic acid	2.37–3.57	2.03–3.84	0.73	0.16	1.93	2,4,4,1	2.34–3.55	0.72	0.14	0.02	1.02	2.05
Oleic acid	36.3–45.2	34.5–46.0	0.77	1.27	2.11	3,6,6,1	38.4–45.7	0.82	0.72	−0.26	0.88	2.17
Linoleic acid	32.9–41.8	31.3–43.6	0.71	1.25	1.93	2,4,4,1	32.8–39.0	0.83	0.71	0.41	0.98	2.04
Linolenic acid	1.74–4.84	0.99–5.62	0.75	0.43	2.01	3,6,6,1	1.98–4.48	0.74	0.32	−0.06	0.96	2.02

Table 3. Statistics for essential amino acids of combined samples of amaranth and buckwheat used in calibration and validation.

Traits	Amino Acid Profiling Amaranth and Buckwheat											
	Laboratory Values (n = 100)	Calibration of NIRS Model					Validation Set (n = 50)	External Validation				
	Range (%)	Range	$RSQ_{Internal}$	SEC (V)	RSC	Math Treatment	Range (%)	$RSQ_{External}$	SEP	Bias	Slope	RPD
Arginine+Threonine	8.78–15.5	8.61–14.96	0.971	0.28	5.87	2,4,4,1	9.97–15.0	0.793	0.42	0.146	1.02	2.06
Histidine	3.05–6.08	2.33–6.62	0.89	0.26	3.24	2,4,4,1	2.87–5.20	0.792	0.18	0.00	1.10	2.16
Valine	3.55–4.77	3.38–4.66	0.78	0.10	2.73	2,4,4,1	3.57–4.97	0.842	0.06	−0.05	0.95	1.88
Cystine	1.06–2.68	1.50–2.17	0.90	0.04	2.23	2,4,4,1	1.20–2.30	0.784	0.18	−0.02	0.98	2.04
Methionine	0.75–2.27	1.14–2.36	0.98	0.06	7.41	2,4,4,1	0.82–2.32	0.929	0.06	0.01	1.09	3.55
Lysine	5.32–8.13	4.88–9.05	0.85	0.29	2.61	2,4,4,1	4.86–8.33	0.782	0.42	−0.09	1.10	2.01
Isoleucine	2.05–3.50	1.64–3.51	0.70	0.18	3.00	2,4,4,1	2.25–2.84	0.804	0.05	0.01	0.89	2.18
Leucine	4.27–5.54	4.13–5.34	0.85	0.10	2.58	2,4,4,1	4.11–5.96	0.839	0.07	0.02	0.82	2.14
Phenylalanine	1.39–5.31	0.65–4.25	0.86	0.23	7.41	2,4,4,1	2.14–4.12	0.837	0.13	0.02	0.91	2.38

3.2. NIR Reflectance Spectra

The average NIR reflectance spectrum of all the accessions of amaranth and buckwheat in an NIR wavelength range of 400–2490 nm is shown in Figure 2, which gave five major bands at wavelengths 1204, 1460, 1760, 1938 and 2302 nm.

The information on functional groups in the spectrum was generated from Win ISI II software. The 1204 nm band arises due to the C-H second overtone corresponding to aliphatic hydrocarbons. The 1460 nm band arises due to the N-H functional group from starch at the first overtone ($CO\text{-}NH_2$), the 1760 nm band is related to oil (CH), the 1938 nm band arises due to the O-H bending at the second overtone related to water and the 2302 nm band is due to the C-H bending at the second overtone related to protein. Absorption bands around 1360 nm and 1762 nm are related to lipids.

3.3. Calibration Equation Data for Amaranth and Buckwheat Germplasm

The results of the calibration model by MPLS for oil, protein, palmitic acid, oleic acid, linoleic acid and linolenic acid, with a calibration set of 100 accessions of amaranth, showed close relationships between NIRS and reference values, as depicted in Table 2. $RSQ_{internal}$ for oil (0.9455), protein (0.7502), palmitic (0.8404), oleic (0.8572), linoleic acid (0.8645) and linolenic acid (0.8472) was obtained after the given mathematical treatments "2,4,4,1", "2,4,4,1", "3,6,6,1", "3,6,6,1", "2,4,4,1" and "3,6,6,1", respectively. The established mean value for oil content was 9.75%, ranging from 6.77% to 12.73%, having an RSC value of 4.28. Similarly, the established mean value for protein, palmitic, oleic, linoleic and linolenic was 10.51%, 19.92%, 26.25%, 48.54% and 1.057%, having an RSC value of 2.04, 2.50, 2.65, 2.71 and 2.56, respectively. The calibration equation was based on the highest 1-VR and $RSQ_{internal,}$ lowest SEC(V) values. The spectra resolution was improved by using derivatives 2 and 3, for the elimination of baseline shifts and superimposed peaks. Calibration equations were generated by removing a few outliers (<10), which may occur during scanning or analytical error.

Table 2 depicts the results of calibration equation data for quality traits of buckwheat germplasm for oil, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid. $RSQ_{internal}$ for oil (0.7675), palmitic (0.8632), stearic (0.7300), oleic (0.7763), linoleic acid (0.7155) and linolenic acid (0.7521) for the mathematical treatments "2,4,4,1", "3,6,6,1", "3,6,6,1", "3,6,6,1" and "2,4,4,1,", respectively. The calibration equation was generated by removing a few outliners (<10), which may have occurred during scanning or analytical error. The established mean value for oil content was 2.31%, having an RSC value of 2.07. Similarly, the established mean value for palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid was 15.5%, 2.93%, 40.3%, 37.4% and 3.04%, having RSC values of 2.71, 1.93, 2.11 and 2.01, respectively.

Table 3 summarizes the calibration equation of essential amino acids, showing close relationships between NIRS and HPLC data on the calibration sample of amaranth and buckwheat homogenized flour. The $RSQ_{internal}$ values are as follows: arginine + threonine (0.9710), leucine (0.7232), methionine (0.9817), phenylalanine (0.8660), valine (0.7983), cystine (0.7988), histidine (0.8880), isoleucine (0.7416) and lysine (0.8248). The established range for arginine and threonine varied from 8.608 to 14.95 (g/16 gN), having an RSC value of 5.876, for leucine (4.13–5.34, RSC 2.58), methionine (1.14–2.36, RSC 7.1), phenylalanine (0.65–4.25, RSC 2.73), valine (3.38–4.66, RSC 2.23), cystine (1.50–2.17, RSC 3.24), histidine (2.33–6.62, RSC 3.00), isoleucine (1.64–3.51, RSC 1.84) and lysine (4.88–9.05, RSC 2.61) values.

3.4. Validation of the NIRS Model for Oil, Protein, Fatty Acid and Amino Acid Composition

The best-fit models were selected based on higher $RSQ_{external}$, RPD and low SEP, SD, slope and bias values. RSQ value determines the validity and accuracy of prediction based on the correlation between the predicted and reference value about the straight line [30]. Apart from high RSQ values, the RPD value also governs the prediction accuracy of the models. For the authentication of the model's validity, the RPD value was used, which

considers both SEP and variation in values and is more precise than SEP(C). RPD values are defined as the ratio of prediction to standard deviation of reference values, where for RPD < 1.5, the model is said to be unreliable, in between 1.5 and 2.0 indicates the capacity of a model to distinguish high and low values, in between 2 and 2.5 indicates approximate quantitative prediction, in between 2.5 and 3.0 indicates good-quality prediction. If it is greater than 3, then the prediction is excellent [33]. The RSQ values for oil, palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid in the amaranth sample are 0.85, 0.79, 0.71, 0.81, 0.83 and 0.75, respectively. However, in buckwheat, RSQ values for oil, palmitic acid, oleic acid, linoleic acid and linolenic acid are 2.72, 2.54, 2.01, 2.34, 2.39 and 2.27. For amaranth models for oil, proteins, palmitic, oleic acid, linoleic acid and linolenic acid displayed RPD values of 4.308, 2.09, 2.56, 2.81 and 2.54, respectively, denoting the model's excellent prediction power. Similarly, the buckwheat model for oil, palmitic acid, stearic acid, oleic acid and linoleic acid displayed RPD values of 2.12, 2.64, 2.01, 2.25, 2.49 and 2.05, respectively, denoting the model's prediction power. Slope denotes the change in predicted values with a unit change in reference values. The ideal value of the slope is 1, and any value close to 1 indicates an accurate model [33]. In amaranth accessions, slope values are 1.09 (protein), 1.05 (palmitic acid), 1.02 (stearic acid), 0.88 (oleic acid), 0.98 (linoleic acid) and 0.96 (linolenic acid). Similarly, in buckwheat accessions, slope values are 0.96 (oil), 1.01(protein), 0.91 (palmitic acid), 0.93 (oleic acid), 0.90 (linoleic acid) and 0.90 (linolenic acid).

To determine the accuracy of the model, the bias value is also an important indicator of similarity between the reference and predicted values of the model [34]. When the reference and predicted values are the same, the bias is equal to zero, which is the ideal value for bias. An underestimating model will be signified by negative bias, and an overestimating model will be signified by positive bias [35]. The values of bias for different traits in amaranth were −0.00 (oil), −0.04 (protein), −0.05 (palmitic acid), 0.45 (oleic acid), −0.53 (linoleic acid) and −0.00 (linolenic acid). All the developed models were found to be slightly underestimated, except oleic acid, which is found to be slightly overestimated. In buckwheat samples, values of bias are −0.05 (oil), −0.26 (oleic acid) and −0.06 (linolenic acid), slightly underestimated.

Combined RSQ values for the amino acid composition in amaranth and buckwheat are Arg+Thr (0.793), leu (0.839), meth (0.929), Phe (0.91), Val (0.837), Cys (0.784), His (0.792), Ile (0.804) and Lys (0.782). RPD value for amino acids was greater than 2, indicating their potential use in screening amino acids in pseudo-cereals. The valine RPD value is 1.88, indicating that the model can distinguish between low and high values. For amino acids, slope values are as follows: 1.02 (Arg+Thr), 0.82 (Leu), 1.09 (Meth), 0.91 (Phe), 0.95 (Val), 0.98 (Cys), 1.10 (His), 0.89 (Ile) and 1.10 (Lys). All the above values were near 1, representing the accuracy of the model. Bias values for Arg+Thr (0.14), His (0.00), Meth (0.01), isoleucine (0.01), Leucine (0.02), and Phenylalanine (0.02) were slightly overestimated, though somewhat underestimated for Lysine (−0.09), Val (−0.05) and cystine (−0.02).

In the present study results, the RPD value for most of the traits was greater than 2, indicating potential use in screening large collections for the quantitative prediction of oil, proteins, fatty acids and essential amino acids in amaranth and buckwheat. However, the RPD value for methionine was 3.55, indicating that the model is providing results similar to wet chemical analysis.

3.5. Application of Developed Prediction Model for Screening Amaranth and Buckwheat Germplasm

Based on the developed prediction models in our study, around 600 samples of amaranth and 190 samples of buckwheat were predicted for different traits to check the applicability of the developed model. The predicted values' results are mentioned in Tables 4 and 5.

Table 4. Prediction values of amaranth and buckwheat for quality traits to check the applicability of the developed models.

Quality Traits	Amaranth (n = 600)		Buckwheat (n = 190)	
	Range (%)	Mean (%)	Range (%)	Mean (%)
Oil	6.90–11.7	9.64	1.74–2.86	2.35
Protein	8.96–13.9	10.5	8.77–19.2	13.7
Palmitic acid	17.7–21.4	19.9	14.7–17.4	16.0
Stearic acid	-	-	2.50–3.28	2.94
Oleic acid	20.0–44.5	26.9	39.5–47.7	43.4
Linoleic acid	19.9–58.0	48.0	30.4–38.64	34.5
Linolenic acid	0.31–1.55	1.08	1.50–4.00	2.85

Table 5. Prediction values of amaranth and buckwheat for essential amino acids to check the applicability of the developed models.

Essential Amino Acid	Amaranth (600)		Buckwheat (190)	
	Range (g/16 g/N)	Mean (g/16 g/N)	Range (g/16 g/N)	Mean (g/16 g/N)
Arg+Thre	8.54–15.3	11.9	11.0–13.8	12.2
Phenylalanine	1.74–3.90	2.42	3.28–4.71	4.09
Valine	3.61–4.87	4.03	3.63–4.72	4.28
Histidine	3.05–6.08	4.58	2.63–4.21	3.22
Methionine	0.93–2.75	1.89	0.69–1.59	1.19
Cystine	1.17–2.04	1.75	1.83–2.29	2.05
Leucine	4.34–5.82	4.81	4.89–5.42	5.17
Isoleucine	2.24–3.43	2.58	2.21–3.00	2.62
Lysine	5.50–7.83	7.16	5.68–6.43	6.06

The results of NIRS-predicted data of amaranth and buckwheat for different traits were similar to the studied 150 samples and agreed with previously reported studies. The predicted range of oil content in 600 accessions of amaranth seed was found to be 6.90–11.76%, while in buckwheat for 190 accessions, oil content ranged from 1.74 to 2.86%. Similar findings in amaranth seeds were reported by [36–38].

The predicted protein content among amaranth seed samples ranged from 8.96 to 13.9% and, for buckwheat, 8.77 to 19.2. Reference [39] reported that amaranth has higher protein content than common cereals. The predicted value of protein content falls within the same range, as reported by other authors [40–43].

The ratio of saturated and unsaturated fatty acid content determines edible oil's nutritional quality and stability properties. The range of predicted values of palmitic acid (17.72–21.46%), oleic acid (19.97–44.48), linoleic acid (19.91–58.03%) and linolenic acid (0.31–1.55%) in amaranth oil was comparable to the already-reported values by [43–45]. Similarly, in buckwheat, the predicted value of fatty acids, palmitic acid (14.7–17.4%), stearic acid (2.5–3.28%), oleic acid (39.5–47.7), linoleic acid (10.4–38.64%) and linolenic acid (0.31–1.55%) shows similarities to the reported ranges from [46–49].

Using NIR spectroscopy, [50] reported the content of moisture, fat and protein in buckwheat flour. The essential amino acid content of amaranth accessions in descending order reported by [51] is as follows: Lys > Val > Leu > Met > Ile > Phe > Trp > Thr.

Very few studies have been carried out for the prediction of amino acids in pseudo-cereals using the NIRS method. The predicted range of amino acids in the present work is comparable with previously reported studies [52,53].

4. Conclusions

There is emerging interest in pseudo-cereal as a potential nutritional and climate-resilient crop, so this study focused on developing a rapid prediction model for oil, protein, fatty acid and amino acids in amaranth and buckwheat accessions through the MPLS regression method based on NIR spectroscopy. The present work is the first report on

the development of an NIRS prediction model for large-scale screening of amaranth and buckwheat germplasms. Good RSQ external values and RPD values were obtained for nutritional traits, indicating the good prediction quality of the models and their potential use in screening the extensive collection of germplasm resources for quantitatively estimating nutritional traits in amaranth and buckwheat. These developed prediction models for buckwheat and amaranth could be extensively used in rapid high-throughput screening and help in identifying trait-specific germplasms for use as gene sources and in crop improvement programs.

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