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Estimation of Proximate, Fatty Acid, Mineral Content and Proline Level in Amaranth using Near Infrared Reflectance Spectroscopy

(Anggaran Proksimat, Asid Lemak, Kandungan Mineral dan Tahap Prolin dalam *Amaranth* menggunakan Spektroskopi Pemantulan Inframerah Dekat)

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ABSTRACT

For successful development of new amaranth varieties, it is important to find inexpensive and rapid analysis methods for the measurement of proximate, fatty acid, mineral content, and proline level in seeds. In this study, calibration equations in NIR spectroscopy were developed to estimate for the fatty acid, mineral content and proline level of amaranth using the modified partial least squares (MPLS) regression method. The calibrations estimated by NIR spectroscopy were consistent with the correlations between reference values at external validation. The equations developed were evaluated based on the relative estimate determination results for external validation (RPDv). The equations for total protein (RPDv = 2.967), fat (RPDv = 4.396), Zn (RPDv = 3.668), proline (RPDv = 6.692), oleic acid (RPDv = 3.366) and linoleic acid (RPDv = 2.086) showed high accuracy, while the equations for ash (RPDv = 1.675) and Fe (RPDv = 1.565) showed relatively high accuracy. When calculated with the same validation factors, the level of Ca (RPDv = 0.268), palmitic acid (RPDv = 1.434), stearic acid (RPDv = 0.949), linolenic acid (RPDv = 1.244) and arachidic acid (RPDv = 0.402) were lower than the estimated value. Protein, oil, ash, Fe, Zn, proline, oleic acid and linoleic acid can be used as reliable users, while equations developed for Ca, palmitic acid, stearic acid, linolenic acid and arachidic acid can be reliably used to screen samples for amaranth breeding programmes.

Keywords: Calibration; fatty acids; minerals; near-ınfrared reflectance spectroscopy; proline

ABSTRAK

Bagi mencapai kejayaan pembangunan varieti *amaranth* baru, adalah penting untuk mencari kaedah analisis yang murah dan pantas untuk pengukuran proksimat, asid lemak, kandungan mineral dan tahap prolin dalam benih. Dalam kajian ini, persamaan penentukuran spektroskopi NIR telah dibangunkan untuk menganggar asid lemak, kandungan mineral dan tahap prolin *amaranth* menggunakan kaedah regresi separa terkecil (MPLS) yang terubah suai. Penentukuran yang dianggarkan oleh spektroskopi NIR adalah tekal dengan korelasi antara nilai rujukan pada pengesahan luaran. Persamaan yang dibangunkan telah dinilai berdasarkan keputusan penentuan anggaran relatif untuk pengesahan luaran (RPDv). Persamaan untuk jumlah protein (RPDv = 2.967), lemak (RPDv = 4.396), Zn (RPDv = 3.668), prolin (RPDv = 6.692), asid oleik (RPDv = 3.366) dan asid linoleik (RPDv = 2.086) menunjukkan ketepatan yang tinggi manakala persamaan untuk abu (RPDv = 1.675) dan Fe (RPDv = 1.565) menunjukkan ketepatan yang agak tinggi. Apabila dihitung dengan faktor pengesahan yang sama, paras Ca (RPDv = 0.268), asid palmitik (RPDv = 1.434), asid stearik (RPDv = 0.949), asid linolenik (RPDv = 1.244) dan asid arakidik (RPDv = 0.402) adalah lebih rendah daripada nilai anggaran. Protein, minyak, abu, Fe, Zn, prolin, asid oleik dan asid linoleik boleh digunakan sebagai pengguna yang boleh dipercayai, manakala persamaan yang dibangunkan untuk Ca, asid palmitik, asid stearik, asid linolenik dan asid arakidik boleh digunakan dengan pasti untuk menyaring sampel untuk program pembiakan *amaranth*.

Kata kunci: Asid lemak; mineral; penentukuran; prolin; spektroskopi pemantulan inframerah dekat

INTRODUCTION

Amaranth can be grown in arid and low fertility soils and cultivated with maize, beans, peppers and squash (Reta Alemayehu et al. 2015). Amaranth required 53-58% less water than wheat, 40-50% less than corn, and 21% less water than cotton (Brenner et al. 2010). Amaranth has low nitrogen requirements so it can be grown in crop rotation with a legume, and can be used as a cover crop in pastures,

thereby providing sufficient available nitrogen to the soil (Ejieji & Adeniran 2010). The nutritional properties of amaranth seeds and leaves, especially the high content of lysine, arginine and histidine, and a unique protein composition make it possible to use it as a nutritional supplement to treat malnutrition in children (Garcia et al. 2011; Reta Alemayehu et al. 2015).

The amaranth seed is considered a good source of mineral with amount of iron at 72-174 ppm, calcium at 1300-2850 ppm, sodium at 160-480 ppm, magnesium at 2300-3360 ppm and zinc at 36.2-40.0 ppm (Becker et al. 1981). The oil fraction of amaranth is similar to cereals with 77%; unsaturated fatty acids with linoleic acid as the predominant fatty acid (Barba de la Rosa et al. 2009; Repo-Carrasco-Valencia et al. 2009). Amaranth has high consumption potential among consumers such as children, high-performance athletes, diabetics, celiac patients, and people with gluten or lactose intolerance (Valcàrcel-Yamani & Lannes 2012).

In many studies, proline accumulation has been observed in response to abiotic stress conditions such as drought and salt stress (Gregorova et al. 2015; Hasegawa et al. 2000; Maritim et al. 2015; Muscolo et al. 2015). Proline may have different roles in salt and drought stress. It can balance low turgor pressure inside the cell (Delauney et al. 1993) or reduce reactive oxygen species (Wutipraditkul et al. 2015). In this case, it can be concluded that there may not be a positive relationship between proline level and drought stress (Montesinos Pereira et al. 2014).

In IR region, near infrared (NIR) region covers wavelengths from 780 nm up to 2.5 μm, mid infrared (MIR) covers the region from 2.5 to 25 μm (Herold et al. 2009). The overtones and combinations of fundamental vibrations of C-H, N-H, O-H and S-H bonds in organic molecules observed in the mid-infrared (MIR) range from the most dominant absorption bands in the NIR spectra of biological samples (Porep et al. 2015).

The spectra of the sample are collected and the quality parameters are determined by conventional techniques before starting the calibration. The calibration model obtained may be used to accurately predict the quality characteristics in samples using the rapid method, therefore replacing the conventional method through multivariate calibrations by applying appropriate mathematical modelling (Herold et al. 2009). NIR spectra can provide information on the physical properties of the sample under investigation, as well as showing fingerprint properties for its chemical composition (Siesler 2008). The sample parameters are introduced to an instrument and computer system by choosing the correct calibration methods and converted to the NIR analysis method (Porep et al. 2015). The NIR spectrum contains noise and background interference, including overlapping bands. Different light scattering effects occur when recording its spectrum; therefore, spectral information becomes complex and lacks the detailed structure required for analysis (Guo et al. 2016). The chemo metrics was required to correlate the spectral features and quality parameters of samples from NIR spectra and to construct calibration models (Qu et al. 2015). The collection of NIR spectra of known samples; pre-processing of original spectra; elimination of abnormal samples; creation and validation of the model using chemometric methods; estimating target parameters of unknown samples are the process of near-infrared analysis (Li et al. 2020). Major absorptions in the NIR spectra of the forages include with two bands water at 1940 nm and 1450 nm; aliphatic carbon bands (lipids) at 2310, 1725, 1400, and 1210 nm; and oxygen bands (carbohydrates) around 2100 and 1600 nm (Conzen 2006).

Unlike most conventional analytical methods, NIR spectroscopy is fast and non-destructive; it does not use chemicals, does not generate chemical wastes that require disposal and is also multiparametric analysis because of several parameters can be determined simultaneously in the same measurement (Acosta et al. 2020). Some studies have been conducted using NIR spectroscopy for betacyanin and moisture content in fresh or dried samples from different parts of *Amaranthus* plants (Cai & Cork 2001), determining the total arsenic content in prostrate amaranth (Font et al. 2004), and carotenoid content in amaranth leaf (Aditya et al. 2018). However, no study has determined amaranth grain quality using NIR spectroscopy for seed cultivar improvement and breeding. Today, wheat, maize, rice and potatoes are the main food sources in more than half of the World (Savary et al. 2019). It is important to grow alternative plants to these products in terms of changing climatic conditions. Amaranth is an alternative plant especially for cereals. In addition to its excellent nutritional value, its ability to grow in temperate climates, that is, in all conditions where wheat and corn can grow, adds a special importance to the plant and shows the possibility of using it as an alternative plant in rural areas. It is important to get accurate and quick results from the large amount of initial breeding materials in field crop breeding studies. The objectives of this study were to develop a non-extractive, rapid and accurate measurement analysis method of proximate composition (protein, oil, ash), mineral (Ca, Fe, Zn), oil composition (palmitic, stearic, oleic, linoleic, linolenic and arachidic) and proline level of amaranth.

Materials and Methods

This study was carried out with a total of 76 amaranth cultivars in the Cukurova region of Turkey between November-February (2019-2020). Three different amaranth cultivars (*Amaranthus hypochondriacus* (30) and *Amaranthus caudatus* (26), *Amaranthus paniculatus* (20)) with different seed and leaf colours and grown as cereals were used. The harvest was carried out in the period end of June-July 2020. Surface and subsoil drip irrigation systems were used in the experiment. In the surface drip system, the main line, manifold and laterals used in the transmission system were made of polyethylene pipes. In-line drippers were spaced at 30 cm intervals on the laterals with a diameter of 16 mm. The dripper flow rate was 2 L/hour at 100 kPa operating pressure. Laterals are placed in each plant row. In the subsoil drip irrigation system, the laterals were placed 25 cm below the soil surface at the level of the plant row. Drippers are spaced at 30 cm intervals on the 16 mm diameter laterals. The dripper flow rate was 2.1 L/hour at 100 kPa operating pressure. The seed samples were stored in plastic cups with screw caps or sealed plastic bags at 4 °C in a refrigerator. Approximately 100 g of fresh leaf samples were dried in the oven at 80 °C for 48 h for proline analyses. All samples were ground in a mill (with a 1 mm pore spacing) and wet chemistry analyses were performed at the specified parameters. To prevent insect infestation and chemical change, the samples were stored in a deep freezer in plastic containers with lids until analysis.

Proximate composition, mineral and proline analyses

The crude protein content (calculated as N × 5.83) was determined on amaranth samples by the standard Kjeldahl procedure (AOAC 2005). Organic materials were burned at 550 °C for ash determination, the remaining amount was expressed as a percentage (AOAC 2005). The samples prepared as a result of ash determination were read in an Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES) device and the concentrations of mineral (Ca, Fe, Zn) were determined (AOAC 2005). Crude oil was extracted with petroleum ether using a Soxhlet apparatus for 4 h. Fatty acid methylesters obtained by methylation of total lipids were analysed by the American Oil Chemists' Society according to the method described in the analysis methods of Ce1-62 (AOCS 2005).

To determine the proline concentration, 20 mg of sample was dissolved in 10 mL of distilled water, kept at 100 °C for 20 min and filtered. The filtrate was kept in a dark and cool environment for 24 h, 2 mL of this filtrate was taken and 2 mL of acid ninhydrin and 2 mL of glacial acetic acid were added to it and kept in a water bath at 100 °C for 1 h. An ice bath was used to stop the reaction. Then, 4 mL of cold toluene was added to the solution in the tubes, mixed with a stirrer, and the absorbance of the toluene-containing fraction aspirated from the liquid phase was taken at 520 nm in a UV-VIS spectrophotometer. The resultant proline concentration was expressed as μg g^{-1}dry weight (DW) (Barickman et al. 2019).

Spectra collection and pretreatment

XDS- NIR spectroscopy rapid content analyzer (FOSS Analytical, Slangerupgade, Denmark) and ISI scanning program was used to collect the spectra of the amaranth. The spectrum of milled amaranth between 400 and 2500 nm wavelengths was scanned by measuring the absorptions every 2 nm. Each sample was subsequently scanned 32 times and an average spectrum was collected to process calibration and external validation. The spectra were collected and managed using ISI scan software (Infrasoft International Port Matilda, PA, USA).

Calibration

The calibration models were developed using WinISI III software (version 1.61). The calibration was performed using the recommended modified partial least squares (MPLS) in developing compatible calibrations for amaranth components. In order to eliminate reflection irregularities in absorbance spectra, different smoothing amounts were used, from which the difference was calculated. For an acceptable equation, nine different mathematical treatments were tried. The first and second derivatives of log *I/R* were also included in the selection of mathematical treatments. The spectra were collected and managed using ISI scan software (Infrasoft International Port Matilda, PA, USA). For each component, the samples from the calibration population were randomly selected by the software and used to test the equations. The SD and mean of the validation set data obtained by wet chemistry analysis were compared with the SD and mean of the calibration set. To select the most appropriate equation, multiple distribution correction (MSC), standard normal variable and trend shift (SNV+D), standard normal variable (SNV), trend reversal (DET), standard multiple distribution correction (S-MSC), weighted multiple scatter correction (W-MSC) and inverse multidistribution correction (I-MSC) scatter correction algorithms were used (Barnes et al. 1989).

External validation

The optimum combination of SE (standard error) and r^2 (the coefficient of determination in calibration) of the

calibration and validation sets and the bias and slope of the validation sets were selected for the best equations. In addition, the points corresponding to the known absorbance peaks of the wavelengths related to the studied components were also evaluated. The standard error of calibration (SEC), the coefficient of determination in calibration (R^2), the standard error of cross validation (SECV), and one minus the ratio of unexplained variance to total variance (1 – VR) were calculated to evaluate the predictive ability of the models (Williams & Norris 2001). Relative predictive determination for cross-validation (RPDc) was calculated (SD of reference data/SECV) to test the accuracy of the calibration models developed (Font et al. 2003; Patil et al. 2010). The equations for each amaranth parameter were screened based on minimizing the SEC and SECV, and maximizing the 1 – VR. The R^2 and RPDc values were used as criteria for evaluation of the optimal performance of the calibration equations (Patil et al. 2010).

To independently check the NIR calibration equations, samples were randomly selected from 76 calibration sample sets using the Monitor program in WinISI. Hence, an independent test set representing a complete range of proximate (protein, oil, ash), mineral (Ca, Fe, Zn), fatty acid composition (palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid and arachidic acid) and proline levels were used for validation of each model.

There are two control limits to determine whether there is a significant bias and a significant increase in unexplained error. The results contain deviation limits and values for global and neighbourhood spectral distances. The coefficient of determination in validation (r^2), SEP (standard error of performance), SEP(C) (standard error of prediction corrected for bias), the bias (mean difference between NIR predicted and reference concentration), RPDv (SD of the external validation set data/SEP(C)) to determine the accuracy of prediction (Williams & Norris 2001), and the range to error ratio were used to evaluate the predictive ability of the models.

Results and Discussion

PRETREATMETS

The means, standard deviations and range values for proximate composition, mineral and fatty acids, and proline level in the calibration and validation sets are shown in Table 1. The value of protein, ash, oil fatty acid

TABLE 1. Relative compositions of proximate, mineral and fatty acids and proline level in amaranth used in calibration and validation

Constituent	Calibration set					External validation set				
	Number	Min	Max	Mean%	SD*	Number	Min	Max	Mean%	SD*
Protein %	76	11.868	13.909	12.889	0.340	31	11.669	13.773	12.808	0.328
Oil %	76	1.771	3.292	2.531	0.294	32	1.739	3.323	2.513	0.321
Ash %	76	2.003	4.456	3.229	0.409	32	1.991	4.115	3.229	0.449
Ca%	76	0.065	0.544	0.304	0.079	32	0.064	0.547	0.306	0.089
Fe(ppm)	76	21.703	113.829	67.767	15.354	32	21.301	106.973	67.557	17.139
Zn(ppm)	76	3.585	60.182	31.883	9.433	29	3.306	55.005	31.662	10.888
Palmitic acid%	76	16.799	20.582	18.690	0.631	32	14.399	1.607	16.338	0.663
Stearic acid%	76	2.234	4.869	3.552	0.439	32	2.289	4.849	3.502	0.467
Oleic acid%	76	25.704	32.453	29.078	1.625	32	25.348	32.621	29.216	1.821
Linoleic acid%	76	16.841	44.019	30.430	4.529	32	14.435	3.437	26.599	0.652
Linolenic acid%	76	0.519	1.710	1.115	0.198	31	0.494	1.543	1.122	0.190
Arachidic acid%	76	0.527	0.807	0.667	0.017	32	0.500	0.728	0.671	0.016
Proline ($\mu g\ .g^{-1}$)	76	5.035	20.538	12.786	5.084	32	4.950	20.337	12.706	4.909

*Standart deviation

composition, Ca and Fe of the samples used in this study are similar to those reported by other authors (Nasirpour-Tabrizi et al. 2020; Olaniyi et al. 2004; Reta Alemayehu et al. 2015).

The values between minimum and maximum in calibration and validation sets are high in all parameters except % Ca and arachidic acid (C20-0). The difference between the minimum and maximum values was determined at the highest Fe (ppm) content, followed by Zn (ppm), proline (μg g^{-1}), linoleic acid (C18-2), oleic acid (C18-1), palmitic acid (C16-0), stearic acid (C18-0), ash, protein, oil, and linolenic acid (C18-3), respectively. Standard deviation values ranged between 0.017% and 15.354% in the calibration set and between 0.016% and 17.139% in the validation set. The content with the highest variability was Fe, followed by Zn, proline, linoleic acid, oleic acid, palmitic acid, stearic acid and linolenic acid, and ash, protein, and fat contents. The values of Ca and arachidic acid had the lowest in terms of variability. It was observed that the differences between the means and standard deviations between the calibration set and the validation set were minimal. It has been observed that the linolenic acid values have a relatively wider range in the calibration set compared to the validation set. The ranges of the components in the calibration set were found to be similar to those in the validation set except for linolenic acid. In this case, it can be seen that both the calibration and validation set parameters for NIR spectroscopy analysis represent the total variation for the analysed constituents.

DETERMINING THE BEST SCATTER CORRECTION PROCEDURE AND MATHEMATICAL TREATMENT

The original NIRS spectra of all amaranth samples used in this study are shown in Figure 1(a). The original spectra have large peaks and significant baseline shifts (vertical shifts). A series of mathematical operations combined with scatter correction algorithms are attempted to resolve the broad peaks of the spectra and eliminate baseline and parallel shifts (Uddin et al. 2006).

To develop the calibration equation for the best scatter correction procedure and mathematical treatment, one proximate component (protein), one mineral compound (Fe), one fatty acid (linoleic acid) and proline content were randomly selected. These components were used for different mathematical treatments with different scatter correction algorithms in the MPLS regression method. This method was useful for agricultural crops such as soybean and chickpea (Font et al. 2021; Han et al. 2014).

Calibrations were first performed with nine different mathematical treatments (0,0,1,1; 1,4,4,1; 1,6,8,1; 1,10,10,1; 1,12,12,1; 2,4,4,1; 2,6,8,1; 2,10,10,1; 2,12,12,1) (Table 2). RPDc valuesto indicate the standardization of the SECV and the performance of the equation were used to determine the robustness of the equation. RPDc values more than 3 represent perfectly calibrated equations, while values between 2 and 3 can be considered equations with very reliable estimates. The values between 2 and 1.5 indicate limited predictive values, while values below 1.5 indicate equations that can be used for scanning (Williams & Norris 2001). It was observed that the components had significant effects on the calibration equations when compared with the mathematical treatment 0.0,1,1 used as a control. Good calibration models for protein and proline content were developed. They had higher RPD values with maths treatment 1,4,4,1 (RPDc = 2.589) and 2,6,8,1 (RPDc = 2.727) in protein and 1,4,4,1 (RPDc = 2.647) and 2,6,8,1 (RPDc = 2.788) in proline content, respectively. However, relatively lower calibration results were obtained for Fe and linoleic acid (1,6,8,1 (RPDc = 1.168) in Fe, 2,6,8,1 (RPDc =1.275) in linoleic acid). Therefore, treatment 2,6,8,1 was chosen as the mathematical treatment that produces optimum equations for other values. MSC, SNV + D, SNV, DET, S-MSC, W-MSC and I-MSC scatterings for all components were used to reduce the parallel shifts caused by the scattering of the samples along with the 2.6, 8.1 treatment as shown in Table 3. The treatment of 2,6,8,1 with SNV+D was determined as the most suitable method giving the best results for protein (RPDc = 3.118), linoleic acid (RPDc = 1.459) and proline (RPDc = 3.189), since this combination was used to match the reference values of the spectral data of the other components.

Using standard normal variable and de-trending (SNVD) transformations after derivation to correct for baseline deviations due to particle size and path length differences between samples helped refine the calibration model (Font et al. 2021). Figure 1(b) shows spectra in which spectral differences are corrected for baseline shift by mathematical treatment 2,6,8,1. In Figure 1(c), the spectra are closer because the scattering of the spectra processed with the mathematical operation 2,6,8,1 combined with SNV+D is corrected and their variations are corrected.

The absorbances of spectra peaked between 1460-1934 nm wavelengths (Figure 1(a)). These wavelengths gave information about O-H and C-H molecule bonds. Maximum were found at 950, 1130, 1224, 1326, 1390,

1672, 1882, 2020, 2234, and 2330 nm and minimum were found at 1204, 1360, 1428, 1586, 1700, 1824, 1922, 2054, 2278, and 2346 nm in second derivative (Figure 1(c)). In the NIR segment of the spectrum, the absorption bands were displayed at 1224 nm, which has been attributed to C-H second overtone; 1390 nm related to C-H combination of methylene groups, 2054 nm which has been assigned N-H bend second overtone or N-H bend/N-H stretch combination band of protein; 2278 nm was due to C-H stretch/CH^2 deformation band of starch (Shenk et al. 2008). Kamboj et al. (2017) specified the absorbance between 1620-2345 nm for carbohydrates, 1180-1590 nm and 1860-2094 nm for fat and 1700-2345 nm for proteins in chickpea flours. Minerals of plant were found as organic complexes, chelates with other mineral salts, and in ionic forms. The minerals that do not have absorption bands for the NIR region can be detected by changes in the hydrogen bonding of salts in high humidity samples, theoretically (Shenk et al. 2008).

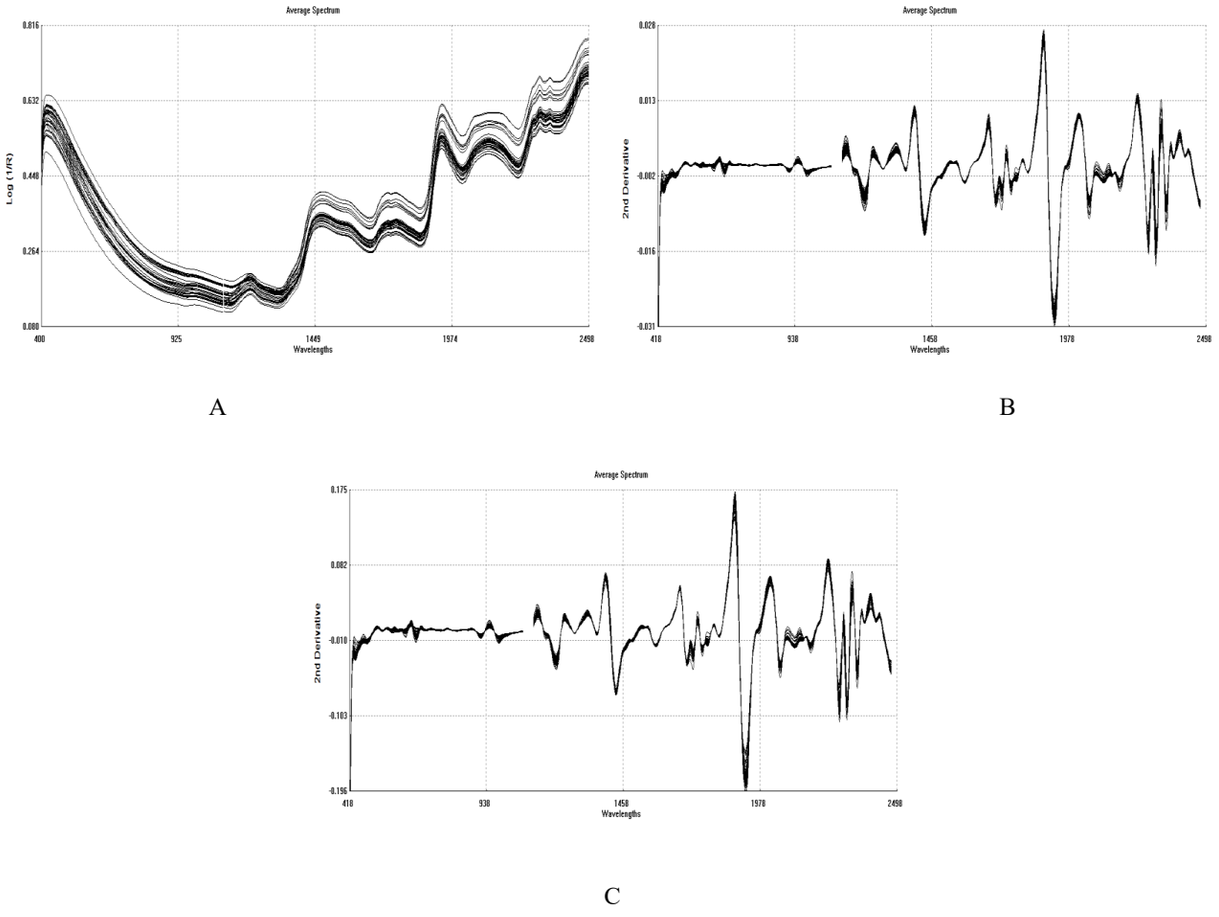

FIGURE 1. Pre-processing stages of amaranth spectra. A. Original spectra of amaranth samples in the 400 to 2,490 nm wavelength range, B. 2,6,8,1 treated spectra of samples in the 400 to 2,490 nm wavelength range, C. 2,6,8,1 and SNV+D treated spectra of samples in the 400 to 2,490 nm wavelength range

TABLE 2. Comparative results from calibration equations for protein%, Fe(ppm), linoleic acid and proline content with different mathematical treatments

Constituent	M[1]	Calibration		Cross validation		RPD[5]*	Constituent	M	Calibration		Cross validation		RPD
		SEC[2]	R^{2*}	SECV[3]	1-VR[4]				SEC	R^2	SECV	1-VR	
Protein%	0,0,1,1	0.156	0.788	0.151	0.184	2.259	Linoleic acid%	0,0,1,1	2.999	0.653	4.199	0.493	1.057
	1,4,4,1	0.123	0.894	0.131	0.202	2.589		1,4,4,1	2.364	0.740	3.666	0.543	1.211
	1,6,8,1	0.142	0.832	0.146	0.186	2.325		1,6,8,1	2.723	0.689	4.080	0.499	1.088
	1,10,10,1	0.142	0.832	0.146	0.186	2.325		1,10,10,1	2.723	0.689	4.080	0.499	1.088
	1,12,12,1	0.152	0.799	0.155	0.178	2.197		1,12,12,1	2.916	0.662	4.317	0.477	1.028
	2,4,4,1	0.111	0.922	0.144	0.192	2.369		2,4,4,1	2.158	0.764	4.004	0.515	1.108
	2,6,8,1	0.112	0.918	0.125	0.205	2.727		2,6,8,1	2.145	0.760	3.479	0.551	1.275
	2,10,10,1	0.138	0.842	0.142	0.190	2.399		2,10,10,1	2.646	0.697	3.953	0.510	1.122
	2,12,12,1	0.150	0.809	0.150	0.184	2.269		2,12,12,1	2.878	0.670	4.182	0.494	1.061
Fe(ppm)	0,0,1,1	8.239	0.767	12.804	0.559	1.032	Proline ($\mu g \cdot g^{-1}$)	0,0,1,1	0.931	0.836	2.292	0.532	2.310
	1,4,4,1	7.661	0.771	11.942	0.563	1.106		1,4,4,1	0.734	0.948	2.001	0.586	2.647
	1,6,8,1	7.525	0.772	11.308	0.566	1.168		1,6,8,1	0.846	0.883	2.227	0.539	2.378
	1,10,10,1	7.931	0.769	11.790	0.564	1.120		1,10,10,1	0.846	0.883	2.227	0.539	2.378
	1,12,12,1	8.066	0.769	12.069	0.563	1.095		1,12,12,1	0.906	0.848	2.357	0.514	2.247
	2,4,4,1	7.448	0.772	12.779	0.558	1.034		2,4,4,1	0.670	0.979	2.186	0.556	2.423
	2,6,8,1	7.101	0.775	11.562	0.564	1.143		2,6,8,1	0.666	0.974	1.899	0.595	2.788
	2,10,10,1	7.352	0.774	11.511	0.565	1.148		2,10,10,1	0.822	0.893	2.158	0.550	2.454
	2,12,12,1	8.741	0.763	12.424	0.561	1.063		2,12,12,1	0.894	0.859	2.283	0.533	2.319

[1]Mathematical treatment, [2]Standard error of calibration, [3]Standart error of cross validation, [4]Coefficient of determination in cross validation
*SD/SECV

TABLE 3. Statistics on scatter correction algorithms for calibration equations for protein%, Fe(ppm), linoleic acid and proline content combined with 2,6, 8, 1 mathematical treatment

Constituent	S[1]	Calibration		Cross validation		
		SEC	R^2	SECV	1-VR	RPDc
Protein%	SNV+D[2]	0.126	0.863	0.109	0.204	3.118
	SNV[3]	0.121	0.877	0.109	0.208	3.105
	DET[4]	0.109	0.898	0.098	0.223	3.476
	S-MSC[5]	0.124	0.877	0.112	0.208	3.039
	W-MSC[6]	0.123	0.878	0.112	0.208	3.039
	I-MSC[7]	0.121	0.877	0.110	0.207	3.092
Fe(ppm)	SNV+D	8.013	0.728	10.111	0.559	1.307
	SNV	8.049	0.728	10.111	0.559	1.307
	DET	4.543	0.741	13.011	0.570	1.015
	S-MSC	8.049	0.728	10.111	0.559	1.307
	W-MSC	8.049	0.728	10.007	0.560	1.320
	I-MSC	8.013	0.728	10.111	0.559	1.307
Linoleic acid%	SNV+D	2.421	0.714	3.043	0.547	1.459
	SNV	2.319	0.726	3.056	0.557	1.452
	DET	2.095	0.743	3.392	0.599	1.308
	S-MSC	2.381	0.726	3.122	0.559	1.421
	W-MSC	2.365	0.727	3.122	0.559	1.421
	I-MSC	2.319	0.726	3.069	0.555	1.446
Proline (μg .g^{-1})	SNV+D	0.752	0.915	1.661	0.589	3.189
	SNV	0.720	0.930	1.668	0.601	3.175
	DET	0.651	0.952	1.852	0.646	2.860
	S-MSC	0.739	0.930	1.704	0.603	3.107
	W-MSC	0.734	0.931	1.704	0.603	3.107
	I-MSC	0.720	0.930	1.675	0.599	3.161

[1]Scatter correction algorithm, [2]Standard normal variate + detrending, [3]Standard normal variate, [4]Detrending, [5]Standard multiple scatter correction, [6]Weighted multiple scatter correction, [7]Inverse multiple scatter correction

CALIBRATION

Calibration equations developed using 2,6,8,1 mathematical operations and SNV+D scatter correction method for values other than protein, Fe, linoleic acid and proline values are given in Table 4. The coefficients of determination (R^2), SEC, SECV and 1-VR were obtained for all equations. The equation for oleic acid with the highest RPDc value (6.176) and the best equation had low SEC (0.331) and SECV (0.279) and high R^2 (0.913) and 1-VR (0.822). While RPD_C values of oil (3.301) and Zn (3.066) were high, R^2, 1-VR, SEC and SECV values were 0.930, 0.965; 0.893, 0.909; 0.067, 1.762; 0.088, 2.929, respectively. The ash had an acceptable RPDc (1.612) value, with an R^2 value of 0.707, 1-VR value of 0.541, SEC value of 0.221, and SECV value of 0.271. Ca, palmitic acid, stearic acid, linolenic acid and arachidic acid had RPDc (0.878, 1.071, 1.279, 1.144 and 0.425, respectively) and very low R^2 (0.453, 0.439, 0.698, 0.677 and 0.353, respectively) and 1-VR (-0.182, 0.177, 0.534, 0.619 and 0.318), respectively. These calibration equations can be used for screening because they are within the limits of reliable predictability with low SEC (0.059, 0.472, 0.241, 0.113 and 0.037, respectively) and SECV (0.089, 0.569, 0.331, 0.125 and 0.389, respectively). The values between 2 and 1.5 indicate usable with caution for many applications, while values below 1.5 indicate equations that can be used for scanning and some other 'approximate' applications (Williams & Norris 2000). The oil and fatty acid results were similar to study on olive oil and soybean (Han et al. 2014; Mailer 2004). The authors analyzed the reflectance spectral data and obtained R^2 values of oleic acid and linoleic acid greater than 0.97. However, the correlation between stearic acid and linolenic acid were poor and could not be used for the prediction of its content.

TABLE 4. Statistics on calibration equations representing proximate, mineral and fatty acids and proline level in amaranth samples with 2,6,8,1 mathematical treatments and SNV+D scatter correction method

				Calibration		Cross Validation		
Constituent	N	SD	Range	SEC	R^2	SECV	1-VR	RPDc
Oil %	74	0.291	1.632-3.487	0.067	0.930	0.088	0.893	3.301
Ash %	74	0.449	1.962-4.410	0.221	0.707	0.278	0.541	1.612
Ca%	75	0.780	0.063-0.538	0.059	0.453	0.888	-0.182	0.878
Zn(ppm)	75	0.898	3.628-55.215	1.762	0.965	0.293	0.909	3.066
Palmitic acid%	74	0.609	16.611-18.771	0.472	0.439	0.569	0.177	1.071
Stearic acid%	72	0.423	2.089-4.825	0.241	0.698	0.331	0.534	1.279
Oleic acid%	74	1.727	24.467-34.504	0.331	0.913	0.279	0.822	6.176
Linolenic acid%	71	0.143	0.636-1.468	0.113	0.677	0.125	0.619	1.144
Arachidic acid%	74	0.165	0.532-0.740	0.037	0.353	0.389	0.318	0.425

TABLE 5. External validation statistics of NIRS predictive equations proximate, mineral and fatty acids and proline level in amaranth

Constituent	N	SD	Bias	r2 [a]	SEP[b]	Slope	RPD^{c}_{v}
Protein %	31	0.063	-0.032	0.792	0.021	0.768	2.967
Oil %	32	0.277	0.000	0.935	0.063	1.000	4.396
Ash %	32	0.405	0.000	0.744	0.242	1.000	1.675
Ca%	32	0.145	0.000	0.524	0.540	1.000	0.268
Fe(ppm)	32	0.995	0.000	0.763	0.636	1.000	1.565
Zn(ppm)	29	0.987	-0.864	0.905	0.269	1.038	3.668
Palmitic acid%	32	0.645	0.000	0.475	0.450	1.000	1.434
Stearic acid%	32	0.218	0.000	0.718	0.230	1.000	0.949
Oleic acid%	32	0.986	0.000	0.930	0.293	1.000	3.366
Linoleic acid%	32	0.472	0.000	0.742	0.226	1.000	2.086
Linolenic acid%	31	0.164	-0.014	0.538	0.132	0.925	1.244
Arachidic acid%	32	0.144	0.000	0.373	0.360	1.000	0.402
Proline ($\mu g .g^{-1}$)	32	0.445	0.000	0.932	0.066	1.000	6.692

[a]Coefficient of determination in external validation, [b]Standard error of prediction, [c]Relative predictive determinant of external validation

EXTERNAL VALIDATION

The estimation capabilities of all calibration equations with external validation were evaluated in Table 5. At this stage, as in calibration and cross-validation, optimum equations are selected based on low SEP and high coefficients of determination (r2) for external validation (Shenk et al. 2008). Except for the equations for palmitic acid, Fe and Ca minerals which had relatively high error values, all other equations had low SEPs. High coefficients of determination was obtained for the equations for oil (r2 = 0.935), proline (r2 = 0.932), oleic acid (r2 = 0.930) and Zn (r2 = 0.905). The determination coefficients of protein, ash, Fe, stearic acid, and linoleic acid were relatively high (r2 = 0.792, 0.744, 0.763, 0.718, and 0.742, respectively). The lowest values were obtained for Ca, palmitic acid, linolenic acid, and arachidic acid (r2 = 0.524, 0.475, 0.538, and 0.373, respectively). Biases varied around '0' and '1' in all equations. The highest RPDv were obtained from the equations for proline (6.692) and oil (4.396). Protein and Zn, oleic acid and linoleic acid had high RPDv values (2.967, 3.668, 3.366, 2.086, respectively). RPDv values corresponding to ash (1.675) and Fe (1.565) were relatively high while those for Ca (0.268), palmitic acid (1.434), stearic acid (0.949), linolenic acid (1.244) and arachidic acid (0.402) were low. Calibration and internal cross-validation results paralleled with minimal differences, while external validation results agreed with these results. Quampah et al. (2012) reported a high R^2cv values in predicting oil (0.993) and linoleic acid (0.963) in cottonseed kernels. RPDv of these components ranged from 11.495-5.026.

Conclusion

In this study, different constituents of amaranth plant were determined. The comparative analysis of mathematical treatments combined with different scatter correction methods should be done as a pre-treatment to develop more accurate estimation methods. The results from the external validation showed that the prediction capabilities of the calibration equations can be used for analysis. In the current experiment, protein, oil, Zn and proline and oleic acid and linoleic acid showed high RPDv (2.967, 4.396, 3.668, 6.692, 3.366, and 2.086, respectively), while ash and Fe showed acceptable RPDv (1.675 and 1.565, respectively). Protein, oil, ash, Fe, Zn, proline, oleic acid, and linoleic acid can be predicted accurately using NIR spectroscopy method in amaranth breeding programmes. The results of Ca, palmitic acid, stearic acid, linolenic acid, and arachidic acid may be used for scanning purposes. These results suggest that NIR spectroscopy could be suitably used as a standard screening method for the contents of these eight traits in large scale amaranth breeding programs where the use of conventional screening methods may be a limiting factor. The inclusion of amaranth cultivars of different geographical origins in future study may increase the robustness of the equations and lead to the sequence of a global calibration for these properties in amaranth.

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