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Discrimination of flax cultivars based on visible diffusion reflectance spectra and colour parameters of whole seeds

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Abstract: Discrimination of yellow and brown seeded flax cultivars was made based on visible (Vis) diffusion reflectance spectra of whole seeds. Hierarchy cluster analysis (HCA) and principal component analysis (PCA) were used for the discrimination. Multivariate analyses of Vis spectra led to satisfactory discrimination of all flax cultivars of this study. The CIE $L^*a^*b^*$ colour parameters were calculated from the diffusion reflectance Vis spectra. The values of L^* were in the range of 48.8–53.6 and 62.6–66.0% for brown and yellow seeded cultivars, respectively. Chromatic parameters a^* and b^* were in the range of 2.8–4.9 and 7.9–16.4%, respectively. A strong linear correlation (R_2 = 0.9712) was found between a^* and b^* parameters for all the flaxseed samples. The L^* and a^* parameters were sufficient for HCA clustering of the individual flax cultivars.

Keywords: colour space; electronic spectroscopy; flaxseed; multivariate statistics

Flax (*Linum usitatissimum* L.) is an important crop that has been cultivated since antiquity. The whole flaxseed and flaxseed oil have been used for consumption in European and Asian countries (Tejklová *et al*. 2011). Flaxseed contains many biologically active compounds including unsaturated fatty acids, lignans, cyclic peptides, proteins, polysaccharides, alkaloids and cyanogenic glycosides. Flax is cultivated mainly for fibre and oil production; in the latter case it is important to modify fatty acid composition in seeds by breeding. Therefore, oiliness flax varieties are cultivated for seed production and nutritional purposes (Smolová *et al*. 2017).

Amon (2007) and Raciol (2011) are oiliness yellow seeded cultivars developed by Agritec (Czech Republic). Yellow flaxseeds have thin and soft hulls, which are an advantage for use in bakery. Amon was characterised by high yield of seeds, but medium to low resistance to diseases. The content of α-linolenic acid is very low (2–4%), but the content of linoleic acid is very high (68–71%) (Smykalova *et al*. 2013). Raciol (2011) was developed from a cross between the mutant low-linolenic linseed line NLN248 and the linseed cultivar Areco (Tejklová *et al*. 2011). The contents of linoleic (~40%) and α-linolenic (~30%) acids are medium. It is notable for a very low level of cyanogenic glycosides and high resistance to diseases. The brown seeded cultivar Recital (2004) bred by Laboulet Semences (France) is characterised by a high content of lignan secoisolariciresinol, low content of cyanogenic glycosides and high resistance to diseases (Sarajlija *et al*. 2012). Libra (2013) bred

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by Limagrain Advanta Nederland, B.V. (Holland) is another brown seeded cultivar with very high yield of seeds and high amount of oil. Both these brown seeded cultivars have high content of α-linolenic acid (~50–60%), but low content of linoleic acid (~15%) (Smykalova *et al*. 2013).

The varieties of flax are different in size, shape and colour of seeds that have been evaluated by image analysis in combination with multivariate statistics (Wiesnerová & Wiesner 2008; Smykalova *et al*. 2013; Nôžková *et al*. 2014). Seed colour is affected mainly by the amount, composition and polymerisation degree of tannins in the outer seed hulls (Troszynska & Ciska 2002). Discrimination of flax varieties based on seed colour thus reflects diversity in amount, structure and distribution of these compounds in seed hulls. There are two main market types of flax according to seed colour: brown and gold (yellow) seeded (Mittapalli & Rowland 2003). However, slight differences in seed colour of common flax varieties is difficultly classified because of continuous transitions in brown hue. Brown hue is the most common in flax seed colour, but the modern low linolenic flaxseed varieties are often yellow seeded (Smykalova *et al*. 2013).

Colour determination of seeds can be carried out by visual evaluation and instrumental analysis (Pathare *et al*. 2013). In the latter case colour parameters can be obtained by image analysis or reflectance spectroscopy in visible (Vis) region (380–770 nm). Colour determination by image analysis needs equipment for transformation of colour spaces and therefore cannot be as precise like determination based on spectroscopy. However, the subjectivity can be diminishing by standardisation. Vis reflection spectrophotometry possesses results similar to visual sensation. Firstly, reflectance R (%) as the ratio between reflected and impinging monochromatic visible light in dependence on wavelength λ (nm) is recorded from the surface of seeds; then the reflectance spectrum obtained is transformed into the colour parameters.

This work is devoted to discrimination of flax varieties based on Vis diffusion reflectance spectra and colour parameters of seeds.

MATERIAL AND METHODS

Flaxseed samples. Brown (Libra, Recital) and golden (Amon, Raciol) cultivars of flaxseed (*Linum usitatissimum*) were obtained from the local producer AGRITEC, Research, Breeding & Services Ltd. (Czech Republic). These cultivars were grown at Rapotín locality in the Czech Republic, at 49°58'21.213"N altitude, 16°58'0.341"E longitude and 329 m a.s.l. The crops were conducted according to standard methods for linseed growing. The air-dried, clean, whole and undamaged seeds were used for the measurements. Specification of flax cultivars analysed in this study is represented in Table 1.

Flaxseed composition. Contents of fats in milled flaxseeds were evaluated gravimetrically using Soxhlet extraction. Protein contents were determined by Keldahl method. Total dietary fibre (TDF) was determined on duplicate samples of dried and defatted flaxseeds using Megazyme kit TDF K-TDFR-100A/200A 04/17 (Megazyme, Ireland). Total phenolics in dried and defatted flaxseed samples were determined according to the method of Singleton and Rossi (1965). The total phenolic content was expressed as gallic acid equivalents (GAE) in μg per mg of the sample. The composition of individual flaxseed samples is summarised in Table 2.

Spectroscopic methods. Diffuse reflectance Vis spectra (range 380–800 nm, slot width 4 nm, scan speed 240 nm/min, resolution 2 nm, 5 scans) were recorded on UV-Vis spectrophotometer UV4 (Unicam, UK) using Labsphere holder (Labsphere Inc., USA) and Vision 3.0 software (Unicam, UK). The white spectralon standards were used for measuring of background on both spectrometers. Each sample was measured five times. All the spectra were converted to ASCII format and exported to Origin 6.0 software (OriginLab, USA) for further processing (smoothing, baseline correction and/or normalisation) and creation of graphical outputs.

Colour determination. Flaxseed colour was determined by calculation of CIE $L^*a^*b^*$ parameters, i.e. L^* (lightness), a^* (red–green spectral axis) and b^* (yellow–blue spectral axis) values, from the modified

Table 1. Specification of flaxseed samples

Sample	Colour of seeds	Cultivar	Harvesting year
A1	yellow	Amon	2015
A2			2016
R1		Raciol	2015
R2			2016
R3		Raciol bio	2015
Rt	brown	Recital	2015
L		Libra bio	2017

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Table 2. Contents of dietary fibres and total phenols in flaxseed samples

Sample	Fat (% w/w)	Protein (% w/w)	Total dietary fibre (% of dry matter)	Total phenols (mg GAE/g of dry mater)
A1	41.5	22.6	31.6	3.46 ± 0.24
A2	40.8	22.4	30.2	2.60 ± 0.15
R1	38.6	23.7	33.7	2.58 ± 0.33
R2	39.8	23.3	33.2	2.39 ± 0.40
R3	39.3	23.9	33.9	2.27 ± 0.16
Rt	41.1	21.8	33.3	2.09 ± 0.22
L	44.7	20.5	31.6	2.09 ± 0.20

diffuse reflectance Vis spectra (180–770 nm, data interval 10 nm, smoothed by 5 ppt) using Microsoft Excel 2010 software.

Multivariate statistics. Diffuse reflectance Vis (% *R*) spectra in ASCII format and normalised values of colour parameters (L^*, a^* and b^*) were exported to Statistica 12.0 (Statsoft, USA) software for multivariate statistical evaluation. Hierarchy cluster analysis (HCA; Ward method of clustering, Euclidian distances) and principal component analysis (PCA; covariation matrix) of the Vis and FT-NIR data were made and graphical outputs, i.e. dendrograms of similarity and component score graphs, were created using Statistica 12.0 and Origin 6.0 software.

RESULTS AND DISCUSSION

Diffuse reflectance Vis spectra. Average diffuse reflectance Vis spectra of seven flaxseed samples are shown in Figure 1. It is evident that the spectra of brown flaxseeds differed significantly from those of yellow cultivars. Moreover, marked differences were found between darker (Libra) and lighter (Recital) brown seed cultivars, whereas the spectral differences between the yellow seed cultivars were not so pronounced.

Colour diagram. The CIE $L^*a^*b^*$ colour diagram for seven flaxseed samples is presented in Figure 2. Yellow and brown flaxseeds showed significant differences in all three colour space parameters. The values of L^* were in the range of 48.8–53.6 and 62.6–66.0% for brown and yellow flaxseeds, respectively. This parameter was significantly higher for light brown Recital (52.4–53.6%) in comparison with that of dark brown Libra (48.8–49.6%). The difference between L^* values of yellow flaxseeds was evident, but not so pronounced like in the case of brown cultivars. Chromatic parameters a^* and b^* also showed differences for yellow and brown flaxseeds. The values of a^* (b^*) were in the range of 2.6–4.9 (–2.8–3.4) and 7.9–9.4% (11.9–16.4) for brown and yellow flaxseeds, respectively. The evident difference in these parameters were found between Libra and Recital; the

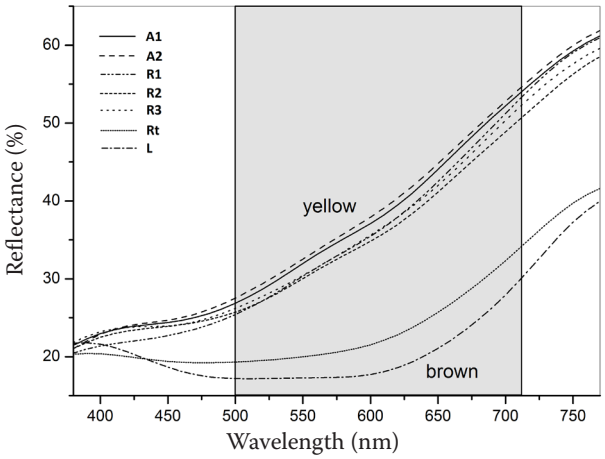

Figure 1. Average diffuse reflectance Vis spectra of flaxseed samples

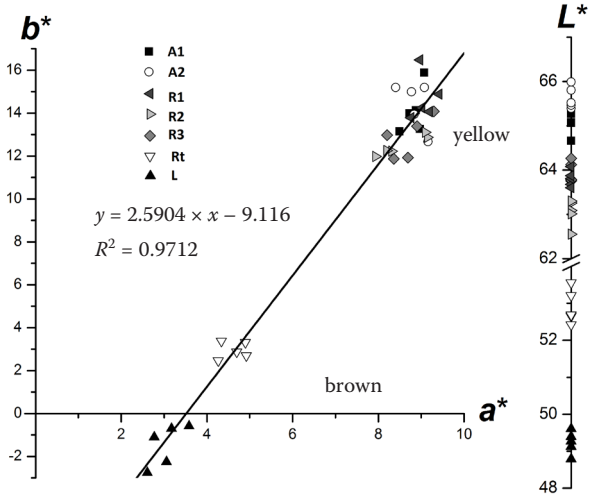

Figure 2. CIE $L^*a^*b^*$ colour diagram for flaxseed samples

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cluster corresponding to former cultivar is located in the second red-blue quadrant due to negative values of b^* (from –2.8 to –0.6%), while all yellow seed samples and the latter one are situated in the red-yellow quadrant. In addition to this, a strong linear correlation (R_2 = 0.9712) was found between a^* and b^* parameters for all the samples. It is known that the pigment cells in inner layer of flaxseed coat contain tannins responsible for brown colour of flaxseeds (Boesewinkel 1980). Tannins were found in *n*-butanol extract obtained from defatted flaxseed meal (Kasote *et al*. 2011). In opposite to this, light (yellow) flaxseeds contain no tannins, but the yellow cotyledons, which contain carotenoids, are visible through the transparent seed coat (Tammes 1928). Similarly, yellow seeded forms of common bean (*Phaseolus vulgaris* L.) had the lowest amounts of tannins, while the highest level of tannins was observed for the darkest seeds (Caldas & Blair 2009). Therefore, these compounds contribute to seed coat colour, distribution or intensity of pigments. It can be assumed that the correlation between chromatic parameters mentioned above could be characteristic for the flaxseed tannins on the carotenoid background, so the observed colour of seeds depends on amount and distribution of these pigments. Colour parameters showed no correlation with contents of total phenols in flaxseed samples (Table 2), probably amounts of tannins might demonstrate dependence on the colour of whole flaxseed.

Condensed tannins (proanthocyanidins) are oligo- and polymers of phenolics, namely flavan-3-ols or flavan-3,4-diols. These astringent, bitter-tested and coloured compounds are able to bind or even precipitate proteins during digestion and thus demonstrate various antinutritional effects (Butler 1992; Bele *et al*. 2010). On the other hand, tannins, as well as other phenolics, and carotenoids are known as antioxidants that may positively affect human health (Rao & Rao 2007; Koleckar *et al*. 2008; Kyselka *et al*. 2017). For example, condensed tannins in coloured hulls of pea (*Pisum sativum* L.) seeds were characterised as heat-stable antioxidants that can be applied in foods (Troszynska & Ciska 2002). Therefore, colour estimation might have nutritional importance in characterisation of flaxseeds. Saedi and Rowland (1999) reported that seed vigour is reduced in yellow flaxseed in comparison with brown ones, so soil microorganisms or other constituents may affect negatively the germination of yellow seeded flax. By contrast, tannins should improve seed vigour in brown seeded flax due to their antioxidant and antibacterial properties.

The colour of flax seeds is determined by multiple genes, and the entire colour ranges from very dark brown to light yellow (Coşkuner & Karababa 2007). Each point at this palette could thus be assigned as natural colour of flax seeds in the gene bank. High content of α-linolenic acid is natural for flaxseeds notwithstanding the colour of the seeds, and materials with altered fatty acid content were created in the 1960s by mutation breeding via radiation or chemomutagenesis. Today's varieties, which have differently reduced α-linolenic acid content from moderate to very low levels like Raciol and Amon, respectively, have the origin in materials produced by mutation breeding. Among each of low, medium and high α-linolenic cultivars there are representatives of both yellow and brown seeded flax, for example yellow seeded Amon and brown seeded Lola as low α-linolenic flaxseeds. Therefore, being a nutritionally important phenotypical characteristic of flax cultivar, seed colour should not be tightly connected with fatty acid composition.

Discrimination of flax cultivars based on Vis data. The values of Vis reflectance (%) in spectral region of 500–720 nm (data interval 10 nm) was used for discrimination of flax cultivars using multivariate statistical methods (HCA and PCA). Resulting graphical outputs are represented in Figure 3. Both HCA and PCA of Vis spectra led to satisfactory discrimination of all flaxseed cultivars of this study. Indeed, the dendrogram of similarity first of all illustrate clustering of brown and yellow seeded cultivars; then the pairs of individual cultivars of the same seed colour, i.e. Amon versus Raciol (yellow seeds) or Libra versus Recital (brown seeds), formed own clusters at similar distances (Figure 3A). On the other hand, PCA of Vis data resulted in the first principal component (PC1), which covers the overwhelming majority of all scattering (99.78%). The flaxseed cultivars were separated by the sign of PC1 according to seed colour: negative for yellow seeded and positive of brown seeded forms (Figure 3B). In addition, like in the case of HCA, the individual cultivars were also well separated from each other by the value of PC1.

Discrimination of flax cultivars based on seed colour parameters. The normalised CIE $L^*a^*b^*$ colour parameters were used for discrimination of flax cultivars by the use of HCA. It was found that two of them, i.e. L^* and a^*, are sufficient for clustering of the

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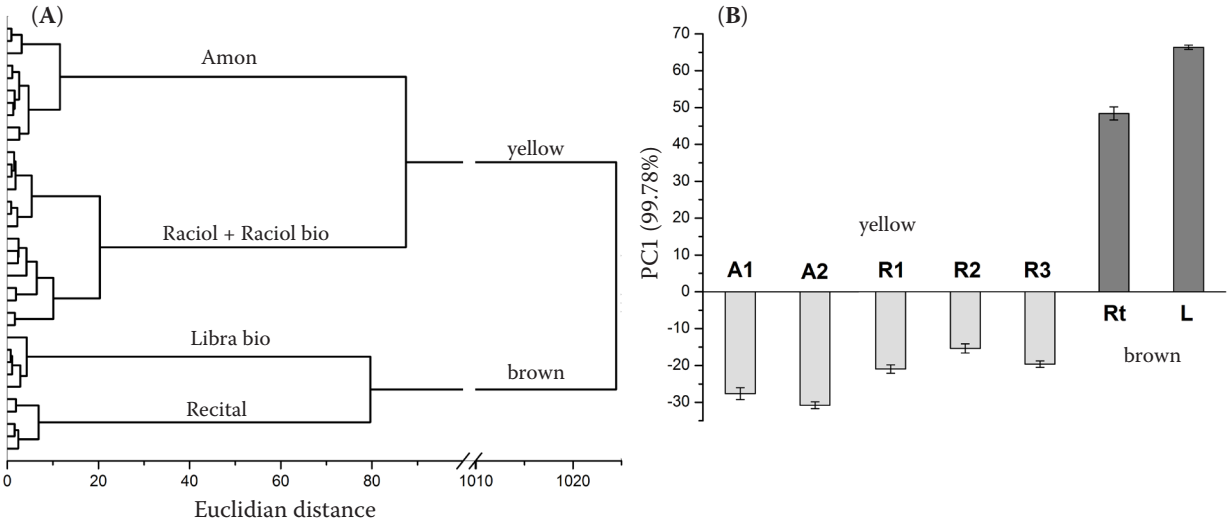

Figure 3. Dendrogram of similarity (**A**) and PC1 values (**B**) for flaxseed samples obtained by multivariate analyses of Vis data

individual cultivars, as it is seen in the dendrogram of similarity obtained by Ward method of clustering using Manhattan block distances (Figure 4). It was not necessary to use parameter b^* because, as it was mentioned above, it highly correlated with b^* for flaxseed cultivars of this study.

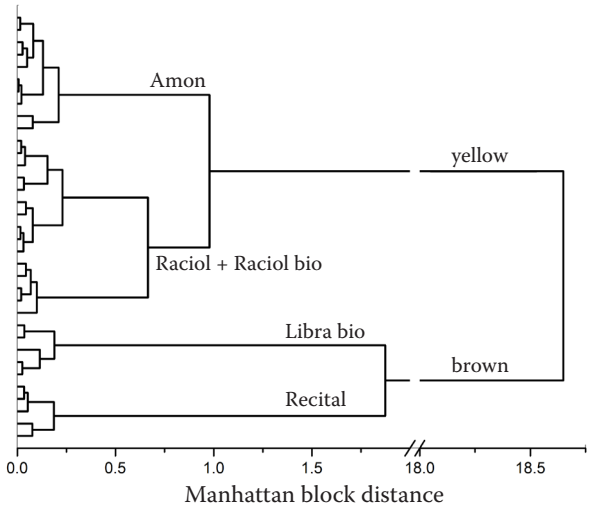

Figure 4. Dendrogram of similarity for flaxseed samples obtained by HCA of normalised parameters L^* and a^*

CONCLUSIONS

Diffusion reflectance Vis spectra were successfully used for discrimination of several flax cultivars according to seed colour. The Vis spectra processing by HCA and PCA at the specific sections demonstrated well separation of the clusters corresponding to individual cultivars. Obtained results also indicate that two colour parameters L^* and a^* calculated from Vis spectroscopic data are sufficient for the clustering of the individual flax cultivars using HCA. These results contribute to characterisation of crop cultivars and estimation of their quality that is important for food processing.

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