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Relationships between nitrate, chlorophyll and chromaticity values in rocket salad and parsley

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The relationships between nitrate concentration, total chlorophyll, chlorophyll a and b concentrations, chromaticity values L*, a*, b*, hue and chroma in parsley (*Petroselinum crispum* Mill.) and rocket salad (*Eruca sativa* Mill.) were investigated. Every month, during 2004 and 2005, three samples for each leafy vegetable were obtained from the market, and after the measurement of colour with a tristimulus colorimeter as Commission Internationale De L'eclairage L*a*b*, nitrate, total chlorophyll, chlorophyll a and b concentrations were determined analytically. Correlation analysis showed that in both vegetables, there were highly significant correlations (P<0.001) between nitrate, total chlorophyll, chlorophyll a, L* and a* values, and it was possible to estimate total chlorophyll and nitrate concentrations by L* or a* values.

Key words: *Eruca sativa* Mill., *Petroselinum crispum* Mill., nitrate concentration, leaf colour.

INTRODUCTION

Green leafy vegetables are important elements of a balanced diet with their mineral, vitamin and dietary fibre contents, and low carbohydrate content (Žnidarčič et al., 2011). However, leafy vegetables may accumulate high amounts of nitrate, and usually they are the major contributor of the daily nitrate intake changing between 30 to 90% (Santamaria, 2006). Although the potential health risks of nitrate intake was showed to be exaggerated recently, (L'hirondel and L'hirondel, 2002; Addiscott, 2005; Powlson et al., 2008), reducing dietary nitrate intake is advised (Santamaria, 2006) and European Union legislation impose limits on the maximum acceptable nitrate concentrations of lettuce and spinach, which is likely to be extended to other leafy vegetables in the future (Burns et al., 2011).

Nitrate accumulation in leafy vegetables is affected by many factors such as genetics, environmental conditions (temperature, light intensity, relative humidity and water content of the soil), and cultural practices (the form and amount of applied nitrogen and other nutrients) (Maynard et al, 1976). Santamaria (2006) classified vegetables according to their nitrate content, and vegetables which belong to Brassicaceae, Chenopodiaceae, Amarantaceae, Asteraceae and Apiaceae families, tend to accumulate nitrate more compared to others.

Since chlorophyll mostly consisted of nitrogen containing enzymes and other organic compounds (Chapman and Barreto, 1997), there is a positive correlation between the nitrogen and chlorophyll concentrations of leaves. Several researchers studied the possibility of using this relationship to determine the nitrogen status of plants (Wood et al., 1992; Sandoval-Villa et al., 1999, 2000; Madeira and Varennes, 2005) and instead of analytical methods, recent research mostly focused on chlorophyll meters since they are portable and chlorophyll concentration can be estimated non-destructively. Another possibility of estimating chlorophyll concentration is tristimulus colorimeters. Several studies showed that there is a correlation between the dominant pigment concentration and one or more chromatic parameters such as carotenoids in apricots (Ruiz et al., 2005) and orange juice (Melendez-Martinez et al., 2003), lycopene in tomatoes (Arias et al., 2000; Hyman et al., 2004), anthocyanins in Acer leaves (Schmitzer et al., 2009) and chlorophyll in pepper leaves (Madeira et al., 2003).

The aim of this study was to determine the correlations between nitrate concentration, chlorophyll concentration and chromaticity values in rocket salad and parsley, which are very high (>2500 $mg \cdot kg^{-1}$) and high (1000 to

Table 1. Summary of parsley leaf sample nitrate, chlorophyll and colour values.

Parameter	Nitrate ($mg \cdot kg^{-1}$)	Total Chlorophyll ($mg \cdot g^{-1}$)	Chl - a ($mg \cdot g^{-1}$)	Chl - b (mg·g-1)	L*	a*	b*	Hueº	Chroma
Mean	419.44	1.17	1.02	0.15	42.71	-17.27	21.45	128.81	27.57
SD	145.92	0.06	0.05	0.03	0.48	1.31	0.93	2.74	0.91
Min.	203.73	1.06	0.95	0.10	42.02	-19.90	19.86	123.75	25.31
Max.	1030.53	1.28	1.10	0.19	43.50	-15.05	22.98	134.96	29.73
Number	72	72	72	72	72	72	72	72	72

2500 $mg \cdot kg^{-1}$) nitrate containing leafy vegetables (Santamaria, 2006), in order to determine the possibility of using tristimulus colorimeters for estimating the nitrate content.

MATERIALS AND METHODS

Plant material

Rocket salad (*Eruca sativa* Mill.) and parsley (*Petroselinum crispum* Mill.) samples were obtained from local markets in Izmir during year 2004 and 2005. Starting from January, every month three 1 kg samples of rocket salad or parsley were obtained. Samples were immediately brought to the laboratory, where they were washed first with tap water, then twice with deionized water. Excess water was removed with a domestic salad spinner.

Colour measurement

Leaf colour was measured with a Minolta CR 300 Colorimeter (Minolta, Japan) as Commission Internationale De L'eclairage (CIE) L*a*b*. Colorimeter was calibrated with the standard white tile prior to measurements (Žnidarčič et al., 2010). A minimum of 20 plants were measured twice for each sample, and chroma and Hue angle values were calculated from a* and b* values with the following formulae;

$$Chroma = \sqrt{a^2 + b^2}$$

$$Hue^\circ = tan^{-1} \cdot (b/a)$$

Chlorophyll analysis

Total chlorophyll, chlorophyll a and chlorophyll b were determined according to the standard method of AOAC (1995). Briefly, 2 g of leaf sample was disintegrated with circa 0.1 g $CaCO_3$ and 85% aqueous solution of acetone; filtered through quantitative filter paper, and washed with acetone until no visible pigment remained. The filtrate was made up to volume with the aqueous solution of acetone, and 25 ml of pigment solution was poured into a separation funnel and 50 ml of diethyl ether was added. After all the colour was transferred to diethyl ether layer, water was discarded. Diethyl ether solution was washed with distilled water until all the acetone was removed. Acetone free solution was transferred to a volumetric flask and made up to volume with diethyl ether, and a teaspoonful of Na_2SO_4 added to obtain a clear solution. Then, this solution was diluted to give an absorbance value of circa 0.6 at 660 nm, and the absorbance values at 642.5 and 660 nm were determined. Total chlorophyll, chlorophyll a and chlorophyll b were calculated with the following formulae, and expressed as $mg \cdot g^{-1}$ fresh weight (FW);

$$\text{Total chlorophyll} = 7.12 \cdot A_{660} + 16.8 \cdot A_{642.5}$$

$$\text{Chlorophyll a} = 9.93 \cdot A_{660} - 0.777 \cdot A_{642.5}$$

$$\text{Chlorophyll b} = 17.6 \cdot A_{660} - 2.81 \cdot A_{642.5}$$

Nitrate analysis

Nitrate concentration of fresh leaves was determined colorimetrically according to the method of Balk and Reekers (1955). 5 g of fresh leaves were homogenized in a Waring Blender with 50 ml of distilled water, transferred to 100 ml volumetric flasks, made up to volume and filtered through white ribbon filter paper. After centrifuging at 7500 rpm for 7 min, 1 ml of salicylic acid (5%, 5 g salicylic acid in sulphuric acid-both from Sigma-Aldrich Chemie, Germany) was added to 0.5 ml of the filtrate, and vortexed. After the solution was cooled, 10 ml of 5% NaOH (Sigma-Aldrich Chemie, Germany) was added, vortexed for 1 min and the absorbance value of the coloured phase was measured at 410 nm. Nitrate ($mg \cdot kg^{-1}$ FW) concentration was calculated against the standard curve constructed by known concentrations of potassium nitrate (Sigma-Aldrich Chemie, Germany).

Statistical analysis

All the chemical analyses were carried out in triplicate for each sample and average values of chemical analyses and colour measurements for each sample were used in statistical analysis. The data were analyzed statistically with SPSS v. 11 for Windows (SPSS Inc., USA). Pooled data were subjected to correlation and linear regression analyses to determine the relationships between chromaticity, chlorophyll and nitrate concentrations.

RESULTS

Parsley

The changes in colour, total chlorophyll, chlorophyll a and b, and nitrate concentration of 72 parsley samples are given in Tables 1 and 2. The nitrate concentrations of the leaves were below the acceptable daily intake of 3.7 mg nitrate per kg bodyweight (Scientific Committee on Food, 1995). The monthly averages of nitrate concentrations changed between 354.53 in March, and 445.66 $mg \cdot kg^{-1}$ in June. In general, nitrate concentrations of the leaves

Table 2. Changes in parsley leaf sample nitrate, chlorophyll and colour values during the study (data are the mean of year 2004 and 2005).

Month	Nitrate ($mg \cdot kg^{-1}$)	Total Chlorophyll ($mg \cdot g^{-1}$)	Chl-a ($mg \cdot g^{-1}$)	Chl- b ($mg \cdot g^{-1}$)	L*	a*	b*	Hueº	Chroma
Jan	412.96	1.18	1.03	0.15	42.66	-17.31	21.20	129.22	27.38
Feb	432.04	1.18	1.02	0.16	42.58	-17.54	20.98	129.86	27.36
Mar	354.53	1.15	1.00	0.15	42.92	-16.72	21.45	127.93	27.23
Apr	401.95	1.17	1.01	0.16	42.70	-17.23	22.04	128.00	28.01
May	363.18	1.15	1.01	0.14	42.87	-16.75	22.42	126.73	28.02
Jun	445.66	1.19	1.04	0.15	42.55	-17.59	22.09	128.52	28.27
Jul	407.22	1.17	1.02	0.15	42.73	-17.18	21.82	128.21	27.80
Aug	410.27	1.17	1.02	0.15	42.74	-17.21	21.95	128.07	27.91
Sep	396.00	1.17	1.02	0.15	42.81	-17.07	21.69	128.18	27.62
Oct	427.99	1.18	1.04	0.14	42.65	-17.41	21.49	128.98	27.68
Nov	433.05	1.18	1.03	0.15	42.62	-17.49	21.61	129.01	27.82
Dec	399.77	1.17	1.01	0.15	42.72	-17.16	21.97	128.00	27.90

Table 3. Correlation coefficients between nitrate, chlorophyll and colour parameters in parsley leaves.

	Nitrate	Total Chl.	Chl-a	Chl-b	L*	a*	b*	Hueº
Total Chlorophyll	0.938***	1.000						
Chl-a	0.801***	0.865***	1.000					
Chl-b	0.431***	0.446***	-0.064^{NS}	1.000				
L*	-0.899***	-0.979***	-0.869***	-0.396***	1.000			
a*	-0.945***	-0.995***	-0.864***	-0.437***	0.981***	1.000		
b*	-0.352**	-0.316**	-0.296*	-0.100^{NS}	0.277*	0.320**	1.000	
Hueº	0.881***	0.906***	0.195***	0.383**	-0.880***	-0.911***	-0.682***	1.000
Chroma	0.589***	0.654***	0.550***	0.320**	-0.670***	-0.658***	0.502***	0.290**

NS, Not significant; *, ** and *** significant at P<0.05, P<0.01 and P<0.001 levels, respectively.

were slightly higher during the colder months (Table 2). The average chlorophyll concentration of the leaves differed slightly during the study. Highest total chlorophyll (1.19 $mg \cdot g^{-1}$) concentration was observed in June, whereas lowest total chlorophyll concentrations were obtained in March and May (1.15 $mg \cdot g^{-1}$). Similarly, leaf colour values showed that the darkest coloured leaves (lower lightness and higher hueº) were obtained during June.

There were statistically significant correlations ($p<0.01$) between total chlorophyll, chlorophyll a concentrations, and lightness, a*, b* Hueº and chroma values (Table 3). Chlorophyll a concentration also correlated significantly with all the chromaticity parameters except b*. Negative correlations between total chlorophyll concentration and L*, a* and b* values indicate that leaves containing more total chlorophyll have lower lightness values (darker colour), lower a* values (greener) and lower b* values (bluer). Positive correlations between Hueº and chroma, indicate that higher total chlorophyll concentrations result in brighter (chroma) and darker green (Hueº) leaves.

Nitrate concentration also correlated significantly with chlorophyll concentration and chromaticity values (Table 3). There were positive correlations between nitrate concentration and total chlorophyll, chlorophyll a and b, Hueº and chroma values; and negative correlations with L*, a* and b* values. Highest statistical significance of correlation coefficients for nitrate were observed from a* (-0.922), total chlorophyll concentration (0.915) and L* (-0.865).

Regression analysis was performed on total chlorophyll and lightness and a* values, which were highly correlated. For both relationships, high determination coefficients (R^2) were found (Figure 1a and b). The coefficient of determination for L* was found to be rather high (R^2 = 0.958), and was even higher for a* (R^2 = 0.989), which showed that L* and a* values can be used to estimate total chlorophyll concentration. For total chlorophyll estimation, the following equations could be used;

Lightness = -8.571 Total chlorophyll + 52.76; or

a* = -23.48 Total chlorophyll x + 10.29

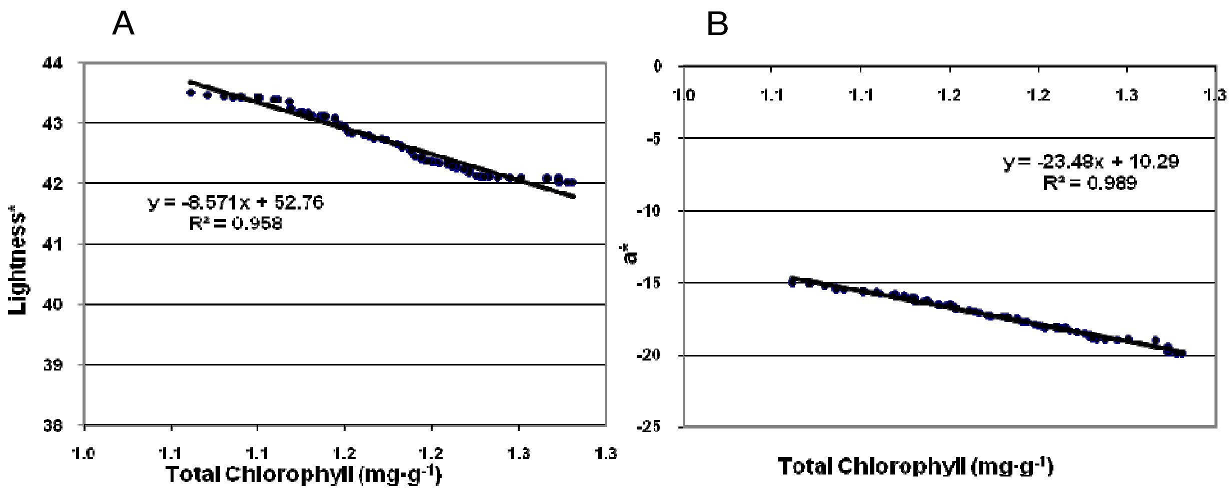

Figure 1. Relationship between total chlorophyll concentration (mg·g^{-1}) and (A) L* and (B) a* values in parsley.

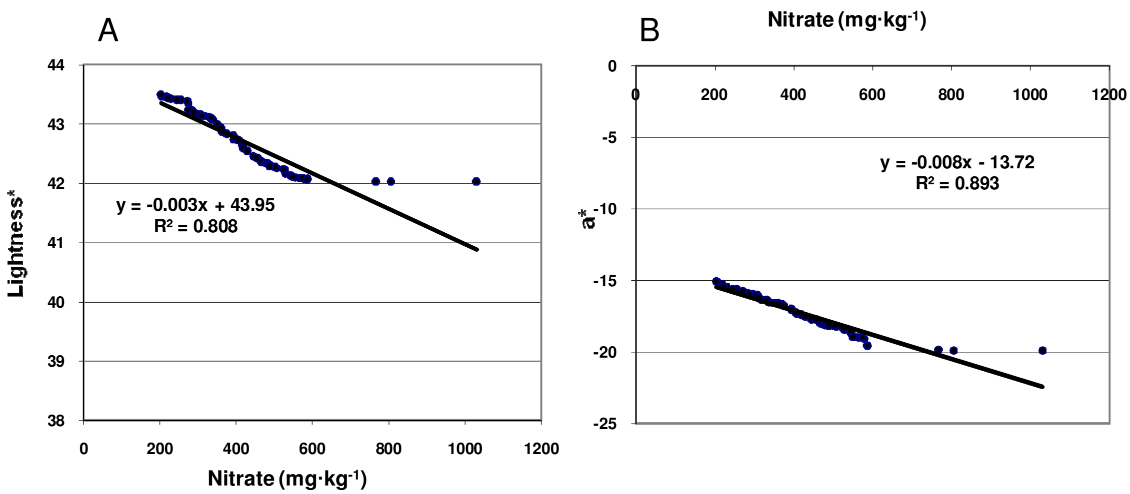

Figure 2. Relationship between nitrate concentration (mg·kg^{-1}) and (A) L* and (B) a* values in parsley.

When the regression analysis was performed for the relationships between nitrate and chromaticity values L* and a*, quite high determination coefficients were obtained (Figure 2a and b). However, for high nitrate concentrations (>800 mg·kg^{-1}) both chromaticity values showed no changes, thus implicating that they would be inadequate for estimating high nitrate concentrations.

Rocket salad

The changes in colour, total chlorophyll, chlorophyll a, b, and nitrate concentration of 72 rocket salad samples are given in Tables 4 and 5. Maximum nitrate concentrations never exceeded the limit of acceptable daily intake of 3.7 mg nitrate per kg bodyweight (Scientific Committee on Food, 1995). The highest nitrate concentration was observed in December with 468.20 mg·kg^{-1}, while the lowest concentration was observed in July with 353.62 mg·kg^{-1} (Table 5). The chlorophyll concentrations differed slightly, and highest and lowest total chlorophyll and chlorophyll a concentrations were obtained in December and July, respectively. The differences in chlorophyll b concentrations were smaller, and while the lowest concentration was observed in July (0.13 mg·g^{-1}), the highest (0.17 mg·g^{-1}) were obtained in June, September, November and December. The lightness values, which represents the relative lightness of colours and range between 0 (black) and 100 (white), and a* which represents the greenness when negative, showed similar tendencies with chlorophyll concentrations. The highest L* and a* values, which indicates lighter green leaves, were observed in July, and lowest L* and a* values, which indicates darker green leaves were

Table 4. Summary of salad rocket leaf sample nitrate, chlorophyll and colour values.

Parameter	Nitrate ($mg \cdot kg^{-1}$)	Total Chlorophyll ($mg \cdot g^{-1}$)	Chl-a ($mg \cdot g^{-1}$)	Chl-b ($mg \cdot g^{-1}$)	L*	a*	b*	Hueº	Chroma
Mean	426.24	1.25	1.10	0.16	42.59	-17.45	20.63	130.18	27.05
SD	128.84	0.07	0.06	0.03	1.29	1.44	0.87	2.62	1.14
Min.	206.13	1.13	1.00	0.12	40.17	-19.88	19.01	124.70	24.48
Max.	876.33	1.39	1.20	0.20	44.99	-15.00	22.00	135.15	29.27
Number	72	72	72	72	72	72	72	72	72

Table 5. Changes in salad rocket leaf sample nitrate, chlorophyll and colour values during the study (data are the mean of year 2004 and 2005).

Month	Nitrate ($mg \cdot kg^{-1}$)	Total chlorophyll ($mg \cdot g^{-1}$)	Chl- a ($mg \cdot g^{-1}$)	Chl-b ($mg \cdot g^{-1}$)	L*	a*	b*	Hueº	Chroma
Jan	451.16	1.27	1.11	0.16	42.39	-17.73	20.07	131.42	26.79
Feb	473.05	1.26	1.11	0.15	42.60	-17.41	20.81	129.87	27.14
Mar	411.36	1.24	1.10	0.14	42.82	-17.27	20.74	129.75	26.99
Apr	447.41	1.26	1.11	0.16	42.29	-17.69	20.73	130.47	27.29
May	387.28	1.23	1.09	0.15	42.92	-17.05	20.41	129.85	26.61
Jun	445.48	1.26	1.09	0.17	42.37	-17.77	21.07	130.11	27.57
Jul	353.62	1.22	1.07	0.13	43.26	-16.77	20.48	129.24	26.53
Aug	381.13	1.23	1.09	0.15	42.97	-17.04	20.83	129.21	26.95
Sep	409.15	1.25	1.08	0.17	42.73	-17.34	20.53	130.15	26.91
Oct	433.29	1.26	1.11	0.15	42.47	-17.56	20.99	129.83	27.39
Nov	453.73	1.27	1.10	0.17	42.21	-17.78	20.96	130.26	27.52
Dec	468.20	1.29	1.12	0.17	42.00	-18.05	19.98	131.99	26.95

Table 6. Correlation coefficients between nitrate, chlorophyll and colour parameters in salad rocket leaves.

	Nitrate	Total Chl.	Chl-a	Chl-b	L*	a*	b*	Hueº
Total Chlorophyll	0,977***	1,000						
Chl-a	0,887***	0,891***	1,000					
Chl-b	0,405***	0,401***	-0,000^{NS}	1,000				
L*	-0,971***	-0,996***	-0,892***	-0,387***	1,000			
a*	-0,970***	-0,993***	-0,892***	-0,379***	0,995***	1,000		
b*	-0,024^{NS}	-0,008NS	0,098^{NS}	-0,085^{NS}	0,006^{NS}	0,001^{NS}	1,000	
Hueº	0,851***	0,878***	0,746***	0,375***	-0,881***	-0,889***	-0,457***	1,000
Chroma	0,800***	0,810***	0,780***	0,258**	-0,811***	-0,811***	0,585***	0,453***

observed in December. In general, in colder months, rocket salad leaves contained more chlorophyll and were darker green (Table 5).

Statistically significant correlations were observed for most of the parameters (Table 6). Except for b* values, high statistical significance ($P<0.001$) was observed between nitrate and chlorophylls and chromaticity values. Only L* and a* values showed negative correlations with nitrate concentration. Total chlorophyll was also highly correlated ($P<0.001$) with all chromaticity values. L* and a* values had negative correlation with total chlorophyll and chlorophyll a, while Hueº and chroma had positive correlations. Similar to parsley samples regression analysis performed on total chlorophyll concentration and L* and a* values, showed that there were strong relationships (Figure 3a and 3b). Although determination coefficient for a* value was higher for parsley, determination coefficient of L* value was higher (0.991) in rocket salad, and a similar coefficient of determination was obtained for a* value (0.986) (Figure 3a and b). The following equations can be used to estimate total chlorophyll concentration;

Lightness = -19.32 Total chlorophyll + 66.83

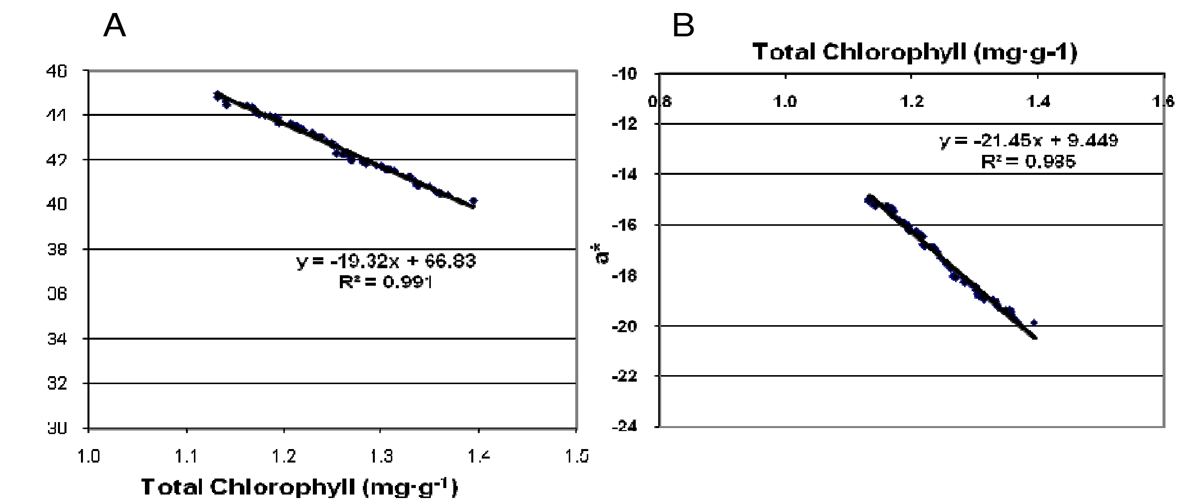

Figure 3. Relationship between total chlorophyll concentration (mg·g^{-1}) and (A) L* and (B) a* values in rocket salad.

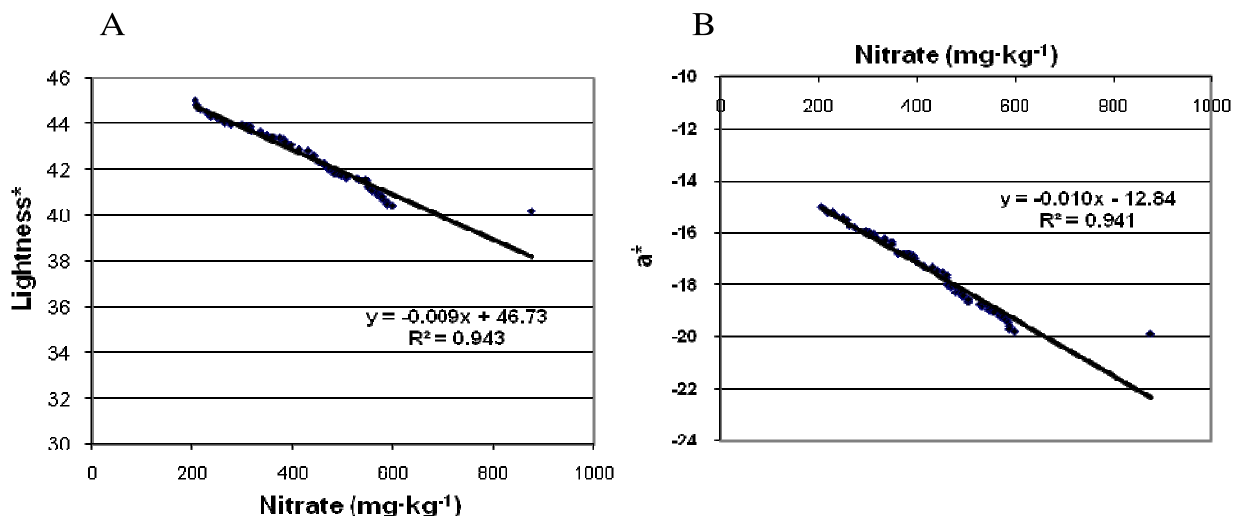

Figure 4. Relationship between nitrate concentration (mg·kg^{-1}) and (A) L* and (B) a* values in rocket salad.

a*= -21.45 Total chlorophyll + 9.449

The determination coefficients of rocket salad for the relationships between nitrate and L* and a* values were higher than those of parsley, however, for nitrate concentrations higher than 850 mg·kg^{-1}, the relationship weakened (Figure 4a and 4b).

DISCUSSION

Although there are seasonal differences, the nitrate concentrations of rocket salad and parsley in this study, were found to be lower than the limit values set by European Union for spinach and lettuce (European Commission, 2006). The seasonal differences of nitrate concentrations in rocket salad samples were more pronounced compared to parsley samples, and samples obtained during the winter months contained more nitrate with the exception of June (Table 5). Low light intensity and shorter days during the winter months is reported to increase nitrate accumulation in leaves (Umar and Iqbal, 2007).

In addition, there were statistically significant correlations between chlorophyll concentrations, nitrate concentrations and chromaticity values in both parsley and rocket salad. Different researchers also reported similar relationships between total nitrogen/nitrate concentrations, chlorophyll concentration and chromaticity parameters in green bean (Da Silveira et al., 2003; Soratto et al., 2004), soy bean seeds (Sinnecker et al., 2002), faba bean (Abdelhamid et al., 2003), pepper leaves (Madeira et al., 2003; Madeira and Varennes, 2005), spinach (Liu et al., 2006) and water cress (Meir et al., 1992). Madeira et al. (2003) reported that there were strong relationships between SPAD-502 chlorophyll meter and L*, b*, Hueº and chroma values, but the relationship with a* values were poor. Similarly, Leon et al. (2007) also found statistically significant relationships between L* and hueº values, however they did not observe a significant relationship with a* and chroma values, while b* values resulted in a low but significant coefficient of determination. More also, Sinnecker et al. (2002) reported that L*, a* and a*/b* values were significantly correlated with chlorophyll content of soy bean seeds dried at 25℃. It seems that L* values are better correlated with chlorophyll concentrations since there is always a significant correlation regardless of the species. However, other chromaticity values show different trends in different species. Although we found significant correlations with all the chromaticity values except b*, L* and a* values showed stronger correlations with total chlorophyll and nitrate concentrations.

In conclusion, it could be said that tristimulus colorimeters can be used to predict the total chlorophyll and nitrate concentrations in parsley and rocket salad, and could be an alternative to time consuming and destructive analytical methods since they are faster and easier.

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