Journal of Animal and Veterinary Advances 10 (15): 2051-2057, 2011
ISSN: 1680-5593


# Determination of Nitrates, Nitrites and Oxalates in Kale and Sultana Pea by Capillary Electrophoresis

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**Abstract:** The objectives of this research were to determine the quantities of nitrate, nitrite and oxalate in two vegetables those are popular at Black Sea coast namely kale and sultana pea. The results were compared with the contents of spinach and chard. Simultaneous determination of nitrate, nitrite and oxalate was conducted with capillary electrophoresis by using an acidic run buffer to reverse the electroosmotic flow. Full factorial design with three variables was applied to decide the optimum capillary electrophoretic experimental conditions. The limit of detection was improved by large volume sample stacking. The average amount of nitrate in fresh kale leaves (2016±519 mg $kg^{-1}$) was greater than spinach and lower than chard. The nitrite ion concentrations were much more in the leaves of kale (111±4 mg $kg^{-1}$) than in stalks on the contrary of chard. Oxalate content was lower in kale (2970±672 mg $kg^{-1}$) than in spinach and chard.

**Key words:** Nitrate, nitrite, oxalate, electroosmotic flow reversal, kale, sultana pea

## INTRODUCTION

Nitrate and nitrite are ubiquitous in vegetables due to the nitrogen intake by green plants which is essential for protein synthesis. Some vegetables, particularly leafy vegetables such as lettuce and spinach have been shown to have relatively high levels of nitrate (MAFF, 1987). The level of nitrate is influenced by plant species and variety, geographical region, light intensity, air temperature, duration of the growth period, harvesting time and the use of nitrogen containing fertilizers (Walters, 1991; Gonzalez *et al*., 2010).

The significance of nitrate to human health derives from the fact that it can be converted to nitrite, nitric oxide and N-nitroso compounds those have potentially adverse health implications (Walker, 1990; McKnight *et al*., 1999). On the other hand it is also reported that dietary nitrates and nitrites may help heart attack survival and recovery (Bryan *et al*., 2007).

The first international evaluation of the risks associated with the ingestion of nitrate and nitrite was conducted by the Joint FAO/WHO. The Scientific Committee for Food reviewed the toxicological effects of nitrate and nitrite and established an Acceptable Daily Intake (ADI) of 0-3.7 mg $kg^{-1}$ body weight for nitrate and derived an ADI of 0-0.07 mg $kg^{-1}$ for nitrite.

Oxalates are also widely distributed in plants as readily water-soluble form of potassium, sodium and ammonium oxalates and as insoluble calcium oxalates (Holloway *et al*., 1989). High consumption of food containing oxalates has been demonstrated to be involved in urinary tract stone formation (Porena *et al*., 2007) and nephrolithiasis (Khan *et al*., 2007).

There is a great concern about the content of nitrates and nitrites in vegetables, a very large number of analyses have been performed in different geographical areas (Fytianos and Zarogiannis, 1999; Merino *et al*., 2006; Ayaz *et al*., 2007; Ozdestan and Uren, 2010; Mor *et al*., 2010; Pardo-Marin *et al*., 2010). There are also many studies those conducted to determine soluble and total oxalate contents in common vegetables (Judprasong *et al*., 2006; Radek and Savage, 2008; Moreau and Savage, 2009). Santamaria *et al*. (1999) had carried out a survey of both nitrate and oxalate content in fresh vegetables in Bari (Italy). Only a few studies are interested with simultaneous determination of nitrates, nitrites and oxalate concentrations in edible parts of vegetables (Kmiecik *et al*., 2004; Jaworska, 2005 a, b; Merusi *et al*., 2010).

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Kale, *B. oleracea* var. *acephala*, a dark green, leafy vegetable from the cabbage family has numerous nutritional benefits. It contains vitamin C, vitamin B6, carotenes, manganese, iron, dietary fiber, calcium, minerals and many other nutrients. Kale is mainly popular in the Black Sea coast including the Northern Turkey. Kale is frequently consumed in this region as soup, saute, pickles and dolma (stuffed vegetables).

There is also a pea cultivar that is endemic in Black Sea region, it is named sultana pea in Turkey, a more specifically variety of pea eaten as whole with flat pods like snow pea (*Pisum sativum* var. *saccharatum*). It can be found in local markets of Black Sea region in early autumn for only 3 or 4 weeks. In contrast to limited cultivation it is well known in the region. There is no available data about nitrate, nitrite and oxalate contents of these commonly consumed vegetables in Black Sea coast. Here in this research we aimed to measure the nitrate, nitrite and oxalate quantities in kale and sultana pea. In order to compare the data of these vegetables samples of spinach (*Spinacia olearia L.*) and chard (*Beta Vulgaris* var. *Cicla*) were also studied.

Simultaneous determination of nitrate, nitrite and oxalate anions was conducted with a capillary electrophoretic method that was developed by Merusi *et al.* (2010) with modifications. Full factorial experimental design with three variables (pH, temperature, applied voltage) was applied to decide the optimum capillary electrophoretic experimental conditions. In order to improve the limit of detection sample stacking was employed by carrying out the separation on an uncoated capillary and reversing the electroosmotic flow by employing an acidic buffer at pH 3.5.

## MATERIALS AND METHODS

**Reagents and materials:** All chemicals were analytical reagent grade and used without further purification. Chemicals used were $H_3PO_4$ (96% w/w Merck), HCl (36-38% w/w, Merck), sodium hydroxide (Merck), sodium nitrate (Carlo Erba), sodium nitrite (Carlo Erba) and sodium oxalate (Carlo Erba). Mesityl oxide was purchased from Fluka and used as neutral marker.

Stock standard solutions (5 g $L^{-1}$) were prepared by weighing the salts of nitrate, nitrite and oxalate separately and by diluting with water. Working standard solutions were prepared daily by appropriate aqueous dilutions. All solutions were filtered through a 0.45 μm membrane filter (Syringe filters Millipore) and were kept under refrigeration (4°C).

The water employed in all procedures was ultrapure water obtained from a Millipore (Simplycity) purification system.

Four types of vegetables, all fresh were purchased from local market places in Samsun, Turkey. Ten samples of each kale, sultana pea, spinach, chard were examined. All samples were grown in the open field and purchased through March-July 2010. All vegetables were processed within 6 h after purchase.

**Sample preparation:** Fresh vegetables were washed with water and dried with adsorbent paper. The leaves of spinach, chard and kale were separated from stalks. Sultana peas were processed wholly.

The extraction process for nitrate, nitrite and soluble oxalate was performed according to Farrington *et al.* (2006). These soluble ions were extracted from 5 g of fresh chopped stalks and leaves with hot water. The extracts were brought to 200 mL volume with water after filtration.

Total oxalates were extracted from aliquots of 2 g of washed and chopped sample with 2 mol $L^{-1}$ hydrochloric acid (Honow and Hesse, 2002).

The extracts of nitrate, nitrite and soluble oxalate were diluted for three times before subjecting to capillary electrophoretic analysis while total oxalate extracts were diluted ten times with water.

Representative 2-10 g samples of fresh vegetables were dried to constant weight in oven set at 60°C for overnight.

**Apparatus and analytical methodologies:** This research was performed with an Agilent CE capillary electrophoresis system equipped with diode-array detector that was set at 200 and 214 nm. The electropherograms were recorded and processed using Agilent Chemstation software for capillary electrophoresis.

Separations were conducted using an untreated fused silica capillary (Polymicro Technologies) of 50 μm inner diameter and with an effective length of 35 cm (total length 42 cm).

The background electrolyte system consisted of 0.050 mol $L^{-1}$ phosphate buffer solution. The pH of the background buffer electrolyte, applied voltage and the temperature were chosen as a result of designed 16 experiments. The pH of the phosphate buffer was selected as 3.5. The polarity was reversed by applying -20 kV voltage. Capillary and the background electrolyte temperatures were maintained at 30°C.

Samples were loaded by hydrodynamic injection into the capillary by applying 50 mBar pressure. Injection times were manipulated for signal enhancement and injection time of 50 sec was decided.

The capillary was rinsed daily with water for 15 min to prevent capillary clogging due to the electrolyte precipitation in the capillary. Prior to each run capillary was purged with 0.10 mol $L^{-1}$ HCl for 3 min, water for 3 min and then background electrolyte for 8 min.

Calibration graphs were prepared by using synthetic standard mixtures. For recovery experiments, known amounts of nitrate, nitrite and oxalate standard solution mixtures were added to the portions of previously analyzed vegetable extracts.

Each sample was analyzed in triplicate and data were presented as mean±standard deviation mg $kg^{-1}$ for fresh and dry matter.

## RESULTS AND DISCUSSION

**Method development and optimization:** As a high-speed separation method, capillary electrophoresis has been increasingly utilized to analyze anionic substances. In the conventional capillary electrophoresis configuration, the detector is located near the cathodic outlet, electroosmotic flow is directed to the cathode and the direction from the anodic inlet toward the cathodic outlet is assumed to be positive voltage. Anions injected into the capillary move towards the anode because of their negative charge. So it is possible to analyze the anionic substances faster when Electroosmotic Flow (EOF) and the voltage is reversed. The EOF reversal can be performed by coating the capillary with electroosmotic flow modifiers, by adding these additives into the background electrolyte. EOF is strongly influenced by the pH. Luckas and Jorgenson (1985) pointed out that EOF is reduced significantly with pH in the range from 8-3 due to the suppression of ionization of silanol groups at low pH. Appropriate reduction in the pH of the background buffer electrolyte will reverse EOF so that the EOF is slower than the electrophoretic mobility of small organic and inorganic anions.

In the majority of previously developed capillary electrophoretic methods for nitrate and nitrite determination, high pH separation buffers were used (Doble and Haddad, 1999) where both nitrite and nitrate possess high but similar electrophoretic mobilities. Amran *et al.* (1993) studied the effect of pH from 2-8 on the migration behavior of anions and optimized the analysis of anions in a pH 3 phosphate buffer. Separation of nitrate and nitrite was also improved by providing a partial protonation of nitrous acid at low pH. Janini *et al.* (1994) developed a capillary zone electrophoretic method for the separation and analysis of nitrate and nitrite in water and urine by using a polyacrylamide-coated column with a modified phosphate buffer at pH 3. The low pH migrating solutions without adding EOF modifiers were used for the separation of nitrate and nitrite in different matrices (Takayanagi *et al.*, 1996; Budanova *et al.*, 2009).

Melanson and Lucy (2000) reported that the resolution of nitrate and nitrite is increased on lowering the pH from 3.5-2.5. Merusi *et al.* (2010) simultaneously analyzed nitrate, nitrite and oxalate in food by capillary electrophoresis using phosphate buffer at pH 2.5 within only 4 min. On the contrary of these two reports, Gaspar *et al.* (2005) indicated that at pH 2.5 while using phosphate buffer (25 mmol $L^{-1}$) and fused silica capillary of 48 cm total length, it is not possible to detect nitrite within 25 min. Gaspar *et al.* (2005) preferred to use phosphate buffer at pH 6.8 to determine nitrite, nitrate and thiocyanate in saliva.

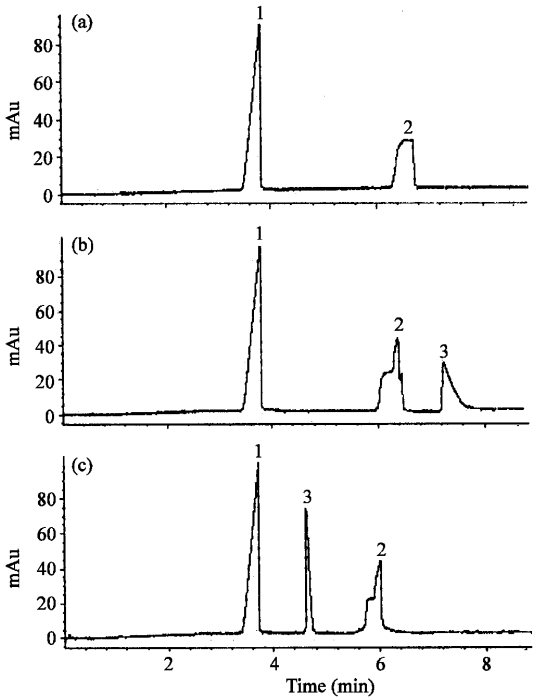


Fig. 1: Effect of pH on migration times of nitrate, nitrite and oxalate anions in standard solutions: (a) pH 2.5. (b) pH 3.0. (c) pH 3.5. Peak Identification: (1) Nitrate, (2) Oxalate, (3) Nitrite

In this research, at pH 2.5 while using phosphate buffer (50 mmol $L^{-1}$) as background electrolyte, nitrite ion did not appear within 10 min of analysis time as shown in Fig. 1. On the other hand oxalate ion migrated faster than nitrite at pH 3; nitrite peak emerged in front of oxalate peak at pH 3.5. These results are in accordance with Gaspar *et al.* (2005).

In order to optimize the capillary electrophoretic separation of nitrate, nitrite and oxalate chemometric approach was used with full factorial experimental design. Randomized experiments were conducted using three variables with low and high levels as; pH (2.5-3.5), temperature (23-30°C) and applied voltage (-15-20 kV). These ranges were chosen depending on preliminary experiments. High levels of temperature and applied voltage have been avoided in order to apply sample

Table 1: Chemometric models as a result of full factorial experimental design and verification of models

| Analyte | Chemometric model | $R^2$ |
|---|---|---|
| Nitrate | $Y = 762.80-4.717x_1+43.79\ x_2+1.63\ x_1\ x_2-158.34x_3+45.84\ x_1\ x_3-20.12x_2x_3+3.69x_1x_2x_3$ | 0.967 |
| Nitrite | $Y = 2912.11+226.57x_1+166.80x_2-15.02\ x_1\ x_2+2789.96\ x_3+195.86\ x_1\ x_3+151.72\ x_2x_3-25.58x_1x_2x_3$ | 0.922 |
| Oxalate | $Y = 2026.84+41.16x_1+146.80\ x_2+21.44\ x_1\ x_2+7.145\ x_3+135.99\ x_1\ x_3+39.47\ x_2x_3+17.61328x_1x_2x_3$ | 0.972 |

Y = Corrected peak area, $X_1$ = Applied potential, $X_2$ = Temperature, $X_3$ = pH, $R^2$, Regression coefficients of logarithmic probability plots using residuals

Table 2: Performance characteristics of calibration curves

| Analyte | Linear concentration range (μm $L^{-1}$) | Slope | Intercept | $S_a^*$ | $S_b^{**}$ | LOD (μm $L^{-1}$) | LOQ (μm $L^{-1}$) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| Nitrate | 1-5000 | 1.42E07 | -430.31 | 496.84 | 28.33E04 | 1.11 | 3.71 | 0.99 |
| Nitrite | 10-150 | 2.62E06 | 30.15 | 11.47 | 12.44E04 | 13.20 | 43.80 | 0.99 |
| Oxalate | 150-450 | 1.82E06 | 45.75 | 14.88 | 43.19E03 | 25.00 | 81.80 | 0.99 |

$S_a^*$ Standard deviation of intercept, $S_b^{**}$ Standard deviation of slope, $R^2$ Correlation coefficient, LOD Limit of Detection, LOQ Limit of Quantification

stacking which would produce heat in the capillary. Chemometric models for nitrate, nitrite and oxalate peaks were evaluated using the migration time corrected peak areas (area/migration time) of anions as data of the experiments and shown in Table 1. The data of the experiments were processed with Microsoft Excel by matrix approach. Verification of models were performed by plotting logarithmic probability plot with residuals and the regression coefficients of the plots representing the fit of the models to the experimental data were also placed in Table 1. High regression coefficients verify models, since the residuals lie approximately along a straight line (Montgomery and Runger, 1994).

From the equations in Table 1, it can be inferred that the main effect is pH for nitrate and nitrite peaks. While increasing the pH, the peak area of nitrite increases profoundly and although less affected, the peak of nitrate decreases due to the negative sign of the effect. The main effect for oxalate ion seems to be the temperature. The second main effect for oxalate and nitrate is the interaction between applied potential and the pH. Nitrite ion is affected by applied potential in the second order. Optimum experimental conditions were chosen as: pH 3.5; temperature 30°C; applied potential (-20 kV), also considering the best separation and the shape of peaks (Fig. 2).

Linear regression data was established by considering the migration time corrected peak areas of each anion as a function of at least six standard concentration levels. The statistic parameters calculated from least-square regression and the performance characteristics are shown in Table 2. Limit Of Detection (LOD) and Limit of Quantification (LOQ) values were estimated using three and ten times the standard deviation of the y estimate obtained from the regression line, respectively. Owing to sample enrichment by large volume stacking the detectability was superior to that of previously reported capillary electrophoretic method by Merusi *et al*. (2010).

Recovery tests were performed for leaves and stalks of kale by using the method of standard additions. Standard solutions of nitrate, nitrite and oxalate anions were added to aliquots of sample extracts and the analysis was completed by using the developed procedure. Table 3 lists the results of recovery study. Recoveries were satisfactory varying from 90.3-110.0%.

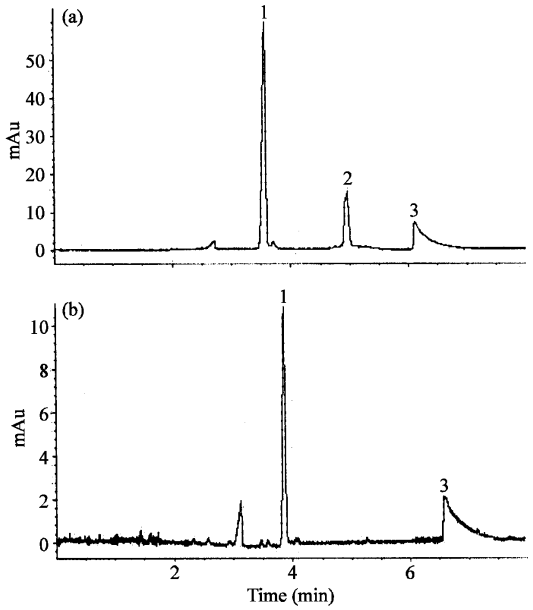


Fig. 2: Electropherograms of vegetable extracts: (a) Kale, (b) Sultana pea. Peak Identification: (1)Nitrate, (2) Nitrite, (3) Oxalate. Experimental conditions: 42 cm capillary (35 to detector), -20 kV voltage, 30°C temperature, UV detection at 200 nm, hydrodinamic injection of 50s with 50 mbar pressure, 50 mM phosphate buffer at pH 3.5

Table 3: Recovery results for nitrate, nitrite and oxalate tested in kale matrix

| Analyte | Recovery (%) | RSD (%) |
|---|---|---|
| Nitrate (leaves) | 110.0 | 1.5 |
| Nitrate (stalks) | 106.7 | 1.8 |
| Nitrite (leaves) | 107.0 | 2.1 |
| Nitrite (stalks) | 102.0 | 2.5 |
| Oxalate (leaves) | 106.3 | 2.0 |
| Oxalate (stalks) | 90.3 | 2.9 |

RSD: Relative Standard Deviation of six samples

The separation method was also evaluated by calculating the Relative Standard Deviation (RSD) of migration times and corrected peak areas of the

components. Very similar migration times and peak areas were obtained for twelve repeated measurements of nitrate, nitrite and oxalate ions. The RSDs for peak area were below 3% for nitrate and oxalate and below 5% for nitrite. The precision of migration times was better than that of peak areas as expected (Table 4).

In vegetables, nitrate and oxalate present from mid to low mg $L^{-1}$ level and thereby can be determined by capillary electrophoresis directly without preconcentation (Santamaria *et al*., 1999). On the other hand, in most vegetables nitrite ion occurs in much lower concentrations and their accurate quantification is only feasible after substantial sample enrichment. A straightforward way of improving detection limits in capillary electrophoresis is sample stacking (Baidoo *et al*., 2003) which is based on conductivity differences between the sample and the electrolyte solution. In sample stacking, injection of sample solution which has lower ionic strength than the running buffer results a narrow stacked zone of solute ions after application of the voltage. This improves both sensitivity and peak shape. In this research, a phosphate run buffer solution of 50 mmol $L^{-1}$ was used and the extracts were diluted at least three times to fulfill the sample stacking requirements.

The results for nitrate, nitrite and soluble oxalate contents of studied vegetables are presented as fresh and dry matter (mg $kg^{-1}$) in Table 5. There is a high variability in the published data of spinach and chard nitrate concentrations and most of them are related with the whole vegetable without discriminating the stalks and leaves. In Table 5, it can be seen that the content of nitrate is much greater in stalks than in leaves for spinach, chard and kale, as stated by Jaworska (2005a) for spinach. The content of nitrate found in this study for spinach and chard are compatible with the results of Merusi *et al.* (2010) and Jaworska (2005a). The amount of nitrate in kale leaves is greater than spinach and lower than chard.

Nitrite ion was determined in chard and kale in this study. On the contrary of chard, the nitrite concentrations were much more in the leaves of kale than in stalks.

Opposite to nitrates, oxalates are known to accumulate chiefly in leaves of vegetables (Jaworska, 2005a; Libert and Franceschi, 1987). Although, total oxalates were calculated, only the data of soluble oxalates were shown in Table 5. There was no distinction between total and soluble oxalate results in this study. Although, Jaworska (2005a) presented both total and soluble oxalate data, the examination will reveal the similarity of them which is supporting the findings of this study. In Fig. 3, an electropherogram was presented for kale extract of total oxalate. The first and huge peak is the chloride ion and the migration time of oxalate ion is moved to about 8 min. Oxalate content is apparently lower in kale than in spinach and chard.

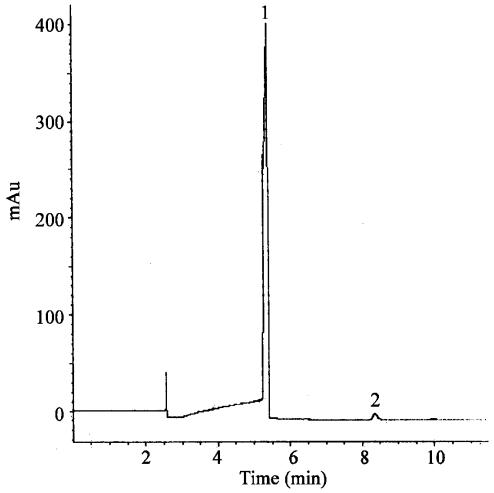


Fig. 3: An electropherogram of kale total oxalate extract. Peak Identification: (1) Chloride, (2) Oxalate

Table 4: Precision of of the capillary electrophoresis method using migration time and corrected peak area

| Analyte | Migration time (min) | RSD (%) | Corrected peak area* | RSD (%) |
|---|---|---|---|---|
| Nitrate | 3.67 | 0.46 | 124.58 | 2.27 |
| Nitrite | 4.82 | 1.58 | 555.77 | 4.73 |
| Oxalate | 5.68 | 1.34 | 653.90 | 2.58 |

*The concentration of standard solutions: nitrate (2.5 μm $L^{-1}$), nitrite (70 μm $L^{-1}$), oxalate (300 μm $L^{-1}$) RSD:Relative Standard Deviation of six samples

Table 5: Contents of nitrate, nitrite and oxalate in vegetables

| | Nitrate (mg $kg^{-1}$±SD) | | Nitrite (mg $kg^{-1}$±SD) | | Oxalate (mg $kg^{-1}$±SD) | |
|---|---|---|---|---|---|---|
| Vegetable samples | FM[a] | DM[b] | FM[a] | DM[b] | FM[a] | DM[b] |
| Kale (leaves) | 2016±519 | 15630±4030 | 111±4 | 857±32 | 2970±672 | 23020±5210 |
| Kale (stalks) | 6395±977 | 77990±11920 | 42±4 | 511±48 | ND | ND |
| sultana pea | 159±26 | 1280±210 | ND | ND | 218±47 | 1760±380 |
| Chard (leaves) | 5064±729 | 50640±7290 | 80±30 | 800±300 | 11248±1645 | 112480±16450 |
| Chard (stalks) | 5910±656 | 84430±9380 | 809±492 | 11560±7030 | 3543±662 | 50620±9460 |
| spinach (leaves) | 1395±205 | 14680±2160 | ND | ND | 12228±1812 | 128720±19080 |
| spinach (stalks) | 3647±400 | 72940±8010 | ND | ND | 5281±546 | 105610±10920 |

[a]Fresh Matter; [b]Dry Matter; ND: Not Determined; SD: Standart Deviation

In sultana pea samples, nitrate and oxalate contents were very low and the nitrite ion concentration was under limit of detection.

## CONCLUSION

In this research simultaneous determination of nitrate, nitrite and oxalate anions were conducted with a capillary electrophoretic method for the analysis of vegetables cultivated in Black Sea coasts namely; kale and sultana pea. The results were compared with the contents of spinach and chard.

The amount of nitrate in kale leaves was found to be in between spinach and chard contents. On the other hand oxalate content was lower in kale than in spinach and chard. The concentrations of nitrate and oxalate in sultana pea were very low. Soluble and total oxalate extracts of vegetables gave similar results.

Phosphate acidic run buffer at pH 3.5 gave reasonable electrophoregrams for separation and determination of nitrate, nitrite and oxalate anions by capillary electrophoresis. The limit of detection and the limit of quantification were improved by employing sample stacking method which had critical importance for especially nitrite ion in vegetables.

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