Project title:	Development and promotion of a rapid test for measurement of nitrate in lettuce and spinach at UK production and produce distribution centres by an 'industry friendly' method
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Grower Summary

Headline

The new Plant Nitrate Protocol can significantly reduce the costs of nitrate analyses on lettuce and spinach crops, can help demonstrate compliance with EC regulations and can provide evidence of a continuing duty of care to consumers.

Background and expected deliverables

As part of its programme on agricultural contaminants in food, the European Commission has put forward a series of Regulations that set maximum permissible limits for nitrate in lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea)*. For lettuce, these limits are 4500 ppm fresh weight in winter (harvested between October and March inclusive) and 3500 ppm in summer (harvested between April and September). Corresponding limits for nitrate in spinach are 3000 ppm when harvested in November to March inclusive, or 2500 ppm in April to October. Until recently, UK growers were granted a derogation which allowed them to exceed the limits, provided they could demonstrate that they followed an agreed Code of Good Practice, and that nitrate levels in their produce were not so high as to risk endangering the health of consumers. For lettuce growers, this derogation was due to be withdrawn on 1 January 2005, but a final decision will be made in February 2005. The derogation for spinach is also expected to be reviewed early in 2005.
[Post project note; UK lettuce will continue to receive a derogation from EU limits but this shall be subject to annual reviews and is most unlikely to continue in the medium term].

In order to help ensure compliance it will be necessary for producers to have regular analyses of nitrate concentration carried out on lettuce and spinach crops. The costs of these analyses in commercial laboratories can be expensive, and it can often take between 7 and 10 days before the results are available. The ability to assess the nitrate content of lettuce and spinach quickly in a reliable way on grower holdings or produce/retail distribution centres would clearly be very useful to the industry. Procedures for nitrate analysis that could be used in a rapid test were evaluated by Nottingham Trent University as part of the LINK project *Development of a decision*

support system for nitrogen fertiliser application in soil-grown lettuce crops, which was led by HRI. This showed that the use of Merckoquant® nitrate test strips, together with the Nitrachek® 404 meter

- gave results comparable to those of standard laboratory methods of analysis
- was reliable and reproducible, but the nitrate concentrations assayed were highly dependent on the conditions used to extract nitrate from the plant tissue prior to analysis

In addition, evidence showed that hot water extraction released more nitrate from fresh lettuce tissues than cold water methods. It was concluded that the Nitrachek meter has considerable potential for use on industry premises. However, as the method was only tested with a limited number of butterhead lettuce samples, and no tests were carried out using spinach or other types of lettuce, it was also concluded that further work was needed to validate it.

The conclusions about the efficiency of the hot water extraction procedure were subsequently confirmed in a follow-up project *A comparison of the accuracy of extraction methods used in the nitrate residues monitoring programme*, carried out by Direct Laboratories for the Food Standards Agency. This also showed that this extraction procedure was reliable and reproducible when used with standard analytical laboratory procedures, but some concerns were expressed about using it with colorimetric methods of analysis.

As the Nitrachek meter uses a colorimetric approach, it follows, therefore, that the next steps leading to commercial adoption of a reliable procedure for the measurement of nitrate in lettuce and spinach samples using the Nitrachek meter will involve

- refining the extraction and associated sample cleanup procedures for use with the Nitrachek meter
- validating the new methods with *both* lettuce and spinach samples
- preparing a ‘grower friendly’ protocol for use in production and/or retail distribution centres
- identifying outlets for supplying the necessary equipment and consumables

Assuming the new methods are validated, a protocol will be produced to describe the procedures in full. There will also be a need for a series of workshops to demonstrate and instruct key representatives of the industry in their use.

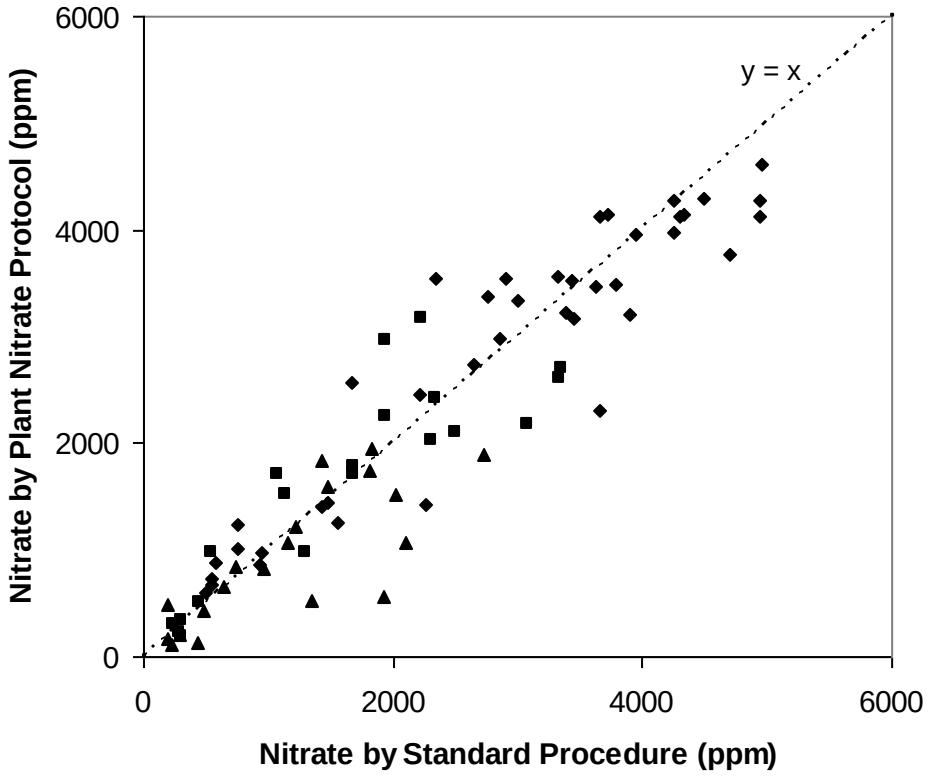
Summary of the project and main conclusions

The new studies of the extraction and analysis of nitrate in plant material showed that

- either frozen or unfrozen plant samples can be analysed
- the weight of plant material extracted into 1 litre of water can vary between 50 and 200g
- the plant material should be blended in water as an integral part of the extraction process
- hot water treatment during extraction of plant material is preferred (but it may be possible to relax this requirement if further evidence becomes available)
- simple filtration of the extract (using filter paper) is essential before analysis
- use of Merckoquant nitrate test strips and the Nitrachek 404 meter are suitable for determining the nitrate concentration in plant extracts provided that:
    - the meter is calibrated with a full range of nitrate standards (typically 50 to 500ppm)
    - only predominantly green or (at most) pale pink plant material is used *and not that which has a strong red colour*
    - the nitrate in the extracts is measured on the same day as the extraction
    - adjustments for the small bias (which varies between plant types) are built into the calculation of plant tissue nitrate concentration

A new method based on these observations was tested by comparing its performance against that of a standard laboratory procedure (similar to one recommended by the British Standards Institute) on a common set of lettuce and spinach samples. The measurements by the standard method were made independently by a commercial analytical laboratory. The tests showed that the new method was less variable than the standard procedure for the main types of material tested. The results were used to define the scope of the method, and to derive correction factors needed to adjust for the small biases introduced by the new method for the different plant materials

analysed. After making these corrections there was a good correlation between the results from the two methods, as shown by the graph below.



Graph showing agreement between the estimated nitrate concentrations from the new Plant Nitrate Protocol and those from a standard laboratory procedure for green lettuce (♦), pale pink lettuce (■) and spinach (▲), where y = x is the line of perfect agreement.

These conclusions were used to refine the method, and define a Plant Nitrate Protocol and a Plant Nitrate Calculator, which together provide: instructions on how to extract and measure nitrate in selected types of plant material; and a method for automatically calculating its original concentration in plant tissue on a fresh weight basis. The main steps in the Plant Nitrate Protocol are illustrated in the Appendix to the Grower Summary.

Workshops held to promote and demonstrate the new methods were well attended, and feedback indicated that the methods were well received.

Financial benefits

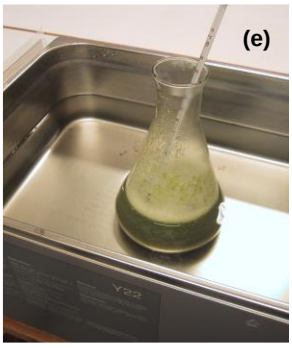
The ability of growers to test lettuce and spinach rapidly for nitrate will assist them in complying with the EC limits. Such rapid tests would give further support to the measures outlined in the Industry Code of Good Practice, and the Crop Specific Protocol for Protected Lettuce of the Assured Produce Scheme with respect to monitoring nitrate concentrations in salad crops. In economic terms, a reliable rapid method of analysis for use by growers or their representatives may help to prevent rejection of produce by multiple retailers. Records of analysis may enable growers to link nitrate levels with management practices such as fertiliser application and adjust these accordingly. In addition, environmental benefits may accrue if decisions are made to lower nitrogen levels in soils, particularly for winter crops. Finally, knowledge that growers are regularly monitoring the nitrate status of their crops can only enhance the public image of the lettuce and spinach production industries.

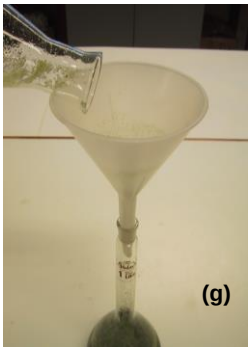
Although the rapid test would not completely remove all need for fully certified analyses, its adoption could rapidly produce a considerable saving in analysis costs to the industry. Based on both the fixed costs of the equipment needed and the additional recurrent costs of doing the analyses, it would take only 14 analyses at £40 per sample from a commercial laboratory to recover the outlay for the new test.

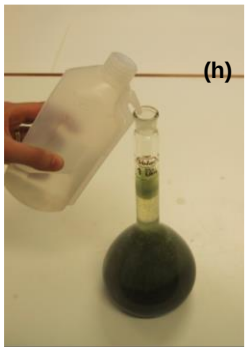
Action points for growers

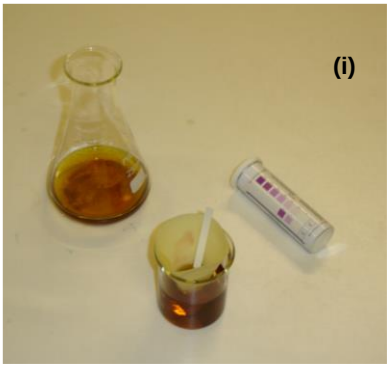
- Use the new Plant Nitrate Protocol to get rapid answers on the nitrate contents of lettuce and spinach crops, and to save on the costs of commercial analyses. *Please contact the HDC office for the full protocol and accompanying Excel file.*

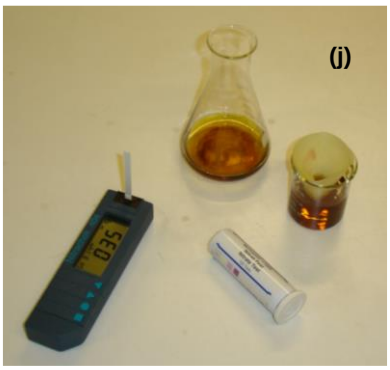
- Use the new Protocol to compile data on the nitrate content of successive crops
  - to demonstrate compliance with EC regulations and a continuing duty of care to consumers
  - to link nitrate contents with N fertiliser use in order to confirm decisions on future N management practices











Illustrated guide to the steps in the Plant Nitrate Protocol: (a) chopping the plant tissue; (b) weighing the plant tissue; (c) blending the plant tissue in water; (d) transferring the extract to a conical flask; (e) hot water treatment of the extract; (f) cooling the extract to room temperature in cold water; (g) transferring the extract to a graduated flask; (h) making up the extract to a known volume; (i) reverse filtration of the extract and immersing the test strip; (j) measuring the colour developed on the test strip in the Nitrachek meter; and (k) calibrating the Nitrachek meter with standard nitrate solutions.

SCIENCE SECTION

INTRODUCTION

As part of its programme on agricultural contaminants in food, the European Commission put forward a Regulation that set maximum limits for nitrate in lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea)* (Anonymous, 1997a). This came into force in the EU on 15 February 1997, and was amended in April 1999 (Anonymous, 1999a) and in March 2001 (Anonymous, 2001). Together, these regulations set maximum nitrate levels in lettuce of 4500 ppm fresh weight in winter (harvested between October and March inclusive) and 3500 ppm in summer (harvested between April and September). Corresponding limits for nitrate in spinach are 3000 ppm when harvested in November to March inclusive, or 2500 ppm in April to October. Until recently, UK growers were granted a derogation which allowed them to exceed the limits, provided they could demonstrate that they followed an agreed Code of Practice (Anonymous, 1999b), and that nitrate levels in their produce were not so high as to risk endangering the health of consumers. For lettuce growers, this derogation was due to be withdrawn on 1 January 2005, but a final decision will be made in February 2005. The derogation for spinach is also expected to be reviewed early in 2005.

In order to demonstrate compliance it will be necessary for producers to have regular analyses of nitrate concentration carried out on lettuce and spinach crops. The costs of these analyses in commercial laboratories can be expensive, and it can often take between 7 and 10 days before the results are available. The ability to assess the nitrate content of lettuce and spinach quickly in a reliable way on grower holdings or retail distribution centres would clearly be very useful to the industry. The primary objective of this project is to develop a simple, safe and inexpensive method for use in such centres, and to evaluate its performance on lettuce and spinach samples containing a range of different nitrate concentrations. Assuming the new method proves satisfactory, a secondary objective is to promote and demonstrate the new method to potential users from the industry.

Analyses of nitrate in whole plants are made in two steps:

1) extraction of the nitrate from the plant tissue into a suitable solution; and

2) measurement of the nitrate concentration in the resulting extract.

A range of alternative options for both steps have been proposed in the literature, but because some of these reports are contradictory, there is no general concensus over which methods are acceptable. As a result, different extraction and measurement procedures can be used by commercial laboratories, and these often produce quite different results.

Most of the extraction methods proposed rely on the use of water to extract the nitrate (*eg* Sen and Donaldson, 1978; Lox and Okabe, 1982; Hertz and Baltensperger, 1984; Lyons *et al*., 1991). This appears to be satisfactory when measurements of nitrate are needed, provided the extracts are analysed immediately (Sen and Donaldson, 1978; Beljaars *et al*., 1994). However, where nitrite concentrations are also required, then alkaline extracts containing either sodium hydroxide (pH 8·0) (Sen and Donaldson, 1978; Lyons *et al*., 1991) or ammonium chloride buffer (pH 9·0) (Beljaars *et al*., 1994) are considered preferable. The extraction process is often conducted by blending the plant sample directly in the extractant (Sen and Donaldson, 1978; Lox and Okable, 1982; Lyons *et al*., 1991, Baljaars *et al*., 1994), although this is not always practical when bulk samples of large numbers of plants are analysed. For example, to meet EC recommendations, a sub-sample from ten different lettuce heads should be taken (Anonymous, 1997b). Under these circumstances, it is often more convenient to homogenise the whole sample first using a macerator, in which case subsequent blending in water during extraction may be unnecessary (BSI, 1997; Farrington, 2002). However, separate maceration relies on the plant tissue being thoroughly homogenised before the extraction step to ensure any sub-sample is representative, and that its nitrate content is readily released into the extractant without further agitation.

There is some uncertainty about whether the extraction should be carried out either hot or cold. Sen and Donaldson (1978), Lox and Okabe (1982) and the BSI (1997) method recommended the use of a hot water treatment (above 50˚C), whereas Lyons *et al.* (1991) found no difference between boiling water extraction and blending in water at room temperature. Beljaars *et al*., (1994) also obtained satisfactory results by blending in water at room temperature, provided that it was carried out on a frozen

sample. This would suggest that the fracturing of plant cells caused by expansion of tissue water as the sample thaws enhances the release of nitrate, ensuring an efficient extraction at low temperature. However, other reports suggest that an aqueous methanol extractant is equally effective (Salomez and Hofman, 2002).

Most of the methods use a simple filtration process (through filter paper) to cleanup the extract before analysis. However, membrane filters or solid-phase extraction columns are also recommended, especially where colloidal or fine particulate contaminants could compromise the subsequent analysis (BSI, 1997). Chemical cleanup procedures using either zinc sulphate (Sen and Donaldson, 1978) or Carrez Solutions (a mixture of zinc acetate and potassium hexacyanoferrate) (Lox and Okabe, 1982; BSI, 1997) in an attempt to decolorise or deproteinate the extract can also be used, but were generally not considered necessary by Farrington (2002).

Once the extract has been prepared, there are two main options for the measurement of its nitrate content in the laboratory: colorimetric or chromatographic techniques. Colorimetric methods normally rely on the use of cadmium reduction columns to convert nitrate to nitrite, followed by diazotisation and coupling with sulphanilamide and N-[1-naphthyl] ethylenediamine respectively, to form a red-violet colour. The intensity of this colour is related to the concentration of nitrate in the extract. This method has been used successfully for the measurement of nitrate in foodstuffs (Sen and Donaldson, 1978; Lyons *et al.*, 1991), with the latter authors finding the results essentially equivalent to chromatographic measurements in comparative tests. However, concerns have been expressed about the reliability of this as a suitable laboratory method (Lox and Okabe, 1982; Schuster and Lee, 1987), because the reduction step is highly pH-dependent. For example, some tests showed that it was more variable and detected 63% less nitrate than a chromatographic method of analyis (Schuster and Lee, 1987). As a result, the current view seems to be that analyses using either high performance liquid chromatography (HPLC) or ion chromatography (IC) are likely to be superior (BSI, 1997; Farrington, 2002). For the purposes of this project, however, the main problem with chromatographic methods is that they require expensive equipment and skilled operators, so they cannot readily be incorporated into a relatively simple method for use by non-specialist analysts.

In contrast, the colorimetric approach does offer opportunities for being developed into a simple streamlined method, particularly as measurements could be made using Merckoquant nitrate test strips (which develop the red-violet colour automatically when dipped in solutions containing nitrate), without having to handle the colour-developing reagents directly. Furthermore, despite the above concerns about the reliability of the colorimetric approach, preliminary studies using these test strips in conjunction with the Nitrachek 404 meter (which reads the colour intensity on the strips more accurately than he naked eye), showed this method had considerable potential as the basis of a simple test for use on growers holdings or at retail distribution centres (Burns, 2000). In particular, Nitrachek measurements on extracts from butterhead lettuce were found to be comparable to those from chromatographic and specific ion electrode methods. However, whilst the Nitrachek meter proved to be both reliable and reproducible, the accuracy of the resulting tissue nitrate concentrations was highly dependent on the extraction procedure used. Under the conditions of that study, substantially more nitrate was released by extracting with hot water than at room temperature.

In the light of these preliminary investigations, the current project has been designed to carry out further scientific studies in an attempt

- to develop a simple, safe and reliable procedure for the extraction of nitrate from plant tissue and its analysis using Merckoquant nitrate test strips and the Nitrochek meter
- to compare the performance of this method against that of a standard laboratory procedure (used by independent operators) on a common set of lettuce and spinach samples
- to use these results to validate and refine the method, and to draw up a protocol to describe it.

Assuming the final version of the method proves to be robust, the new protocol will be promoted to relevant sectors of the industry and demonstrated at a series of workshops to encourage its take-up.

MATERIALS AND METHODS

General Procedures

Nitrate standards

A stock nitrate standard of 1000ppm nitrate was made up using 1·6307g of oven dried potassium nitrate (KNO_3) in 1000ml of deionised water. Other standards were made up by diluting this stock standard using pipettes and graduation flasks, as shown in Table 1. All standards were made up fresh every two weeks and kept in the fridge in the interim. Different subsets of these standards were used in each of the studies described below.

Table 1. Details of nitrate standards used for calibration of the Nitrachek meter. All were made up from a 1000 ppm stock solution in 100ml graduated flasks.

Concentration (ppm)	Volume of 1000ppm standard required (ml)
5	0.5
10	1.0
50	5.0
100	10.0
200	20.0
300	30.0
400	40.0
500	50.0

In the latter stages of the investigations the 1000ppm stock standard solution was purchased from either VWR International Limited or Fisher Scientific UK Limited. This was contained sodium nitrate dissolved in deionised water.

Using the Nitrachek meter

About 5 to 10ml of each standard was transferred to a separate 50ml beaker and allowed to reach room temperature before use. Solutions of any extracted plant material were treated similarly. Measurements were then made by dipping a new Merck nitrate test strip into each solution and measuring the colour which developed

after 1 minute using a Nitrachek 404 meter, ignoring any units displayed with the meter reading. The meter had previously been set at Lot 9. The batch number and expiry date of the test strips was always recorded for each set of measurements.

The following procedure was used for all analyses. First a colour measurement was made for each standard in increasing order of concentration. A minimum of two (and up to six) colour measurements were then made for each sample of plant extract solution in turn followed by a repeat measurement on each of the standards. Where there were a large number of plant extracts being analysed, the standards were also re-measured at intervals (after every ten to twenty extracts).

Nitrate concentrations in the plant extracts were calculated from calibration graphs of colour measurement against nitrate concentration for the standards. Corresponding nitrate concentrations in the original plant tissue were then calculated from the weight of tissue used and the nitrate concentration in the extract.

Perfecting the Nitrate Measurement Procedure

Checking the calibration of the Nitrachek meter

Colour readings for a full set of nitrate standard solutions were measured six times. These data were used to investigate the shape of the calibration graph of mean colour reading against nitrate concentration (using simple curve-fitting procedures in Excel), and the accuracy of nitrate estimation in the different regions of the calibration graph.

Optimising the sensitivity of the nitrate measurements

Tissue nitrate concentrations in most glasshouse salad crops greatly exceed the range which can be measured using the Nitrachek meter (which operates up to 500 ppm). An experiment was therefore conducted to examine the effects of different dilution ratios (which could be used in the extraction process) on the sensitivity of the subsequent nitrate measurements. In order to standardise the conditions of this study, the work was carried out using an independent set of standard solutions of different nitrate concentration (representative of those in plant tissues) prepared as follows. A 5000ppm nitrate solution was made up by dissolving 8·1535g of dried KNO_3 in 1 litre

of water in a graduated flask. This was used to make up a series of other standards with lower concentrations, see Table 2.

Table 2. Nitrate solutions made up from a 5000ppm stock solution to simulate the range of concentrations found in fresh tissue of glasshouse lettuce. All were made up in 250ml graduated flasks.

Nitrate Concentration (ppm)	Volume of 5000ppm required (ml)
4000	200
3000	150
2000	100
1000	50
500	25

Each of these solutions was diluted with water by 50, 25 or 12·5 times to represent three different dilution ratios during extraction of fresh plant tissue. These are equivalent to extracting 20g, 40g or 80g of fresh plant material into 1 litre of water (assuming a plant tissue density of 1·0). Each of these diluted solutions was measured a total of four times using the Nitrachek meter. The nitrate concentrations corresponding to the colour measurements were calculated using a mean calibration graph prepared from independent standards (see Table 1), each measured four times. The results were used to determine a suitable dilution ratio for the extraction process.

Carry-over effects between successive samples

Possible effects on the measurement of nitrate which might occur from switching from low to high concentrations and vice versa were investigated by alternating between five successive measurements of a 5ppm standard followed by five successive measurements of a 500ppm standard, over two complete measurement cycles. The results were analysed graphically for evidence of any carry-over between successive solutions caused by possible contamination of the meter sensor from any bleeding of the colour developed on the test strips.

Perfecting the Plant Extraction Procedure

Preparation and sub-sampling plant material

All plant material was analysed fresh (*ie* without drying). Because of the size of most plants, each was normally sub-sampled before extraction. The following procedure was used in all studies where identical representative sub-samples from the same plant were required to compare the effects of different sample storage or extraction methods. Each plant was cut into either four or eight equal portions longitudinally down its stem, and either opposite quarters or opposite eighths combined to provide either two or four sub-samples. In most studies, the sub-samples were used immediately. However, where studies were made on frozen sub-samples, these were kept in a freezer for at least 24 hours to ensure they were thoroughly frozen before use.

Comparison of extraction methods

Basic extraction procedure. All plant material was extracted into deionised water. The plant sub-sample (between 50 and 200 g) was weighed to one decimal place immediately before extraction. This was chopped coarsely into small pieces using a knife on a plastic tray with a rim, which helped to contain the sample and avoid any losses. The sample was added to a blender in 30 to 50 g batches. 200 ml of water was added to the first batch, and the blender switched on in short bursts to break up any lumps of stem etc. Further batches of plant tissue and an additional 150 ml of water were added gradually as the plant tissue was broken down. Care was taken to avoid any loss of liquid or plant pulp when removing the lid of the blender. Once all of the sample had been added, the blender was left on for a full minute, rocking it gently during operation. The lid and bowl of the blender were carefully rinsed down with water, the lid replaced and the blender run for another minute as before.

The entire liquid pulp was then transferred to a 1 litre conical flask, using further rinse water as necessary. The flask was then partially immersed in a hot water bath (at > 90^{o}C) for between 20 and 30 minutes, swirling it occasionally to equalise the temperature of the flask contents. The flask was removed when the temperature of the latter had reached at least 70 ^{o}C, and was placed in a bowl of cold water to allow it to cool to room temperature.

The contents of the flask were then quantitatively transferred to a 1 litre graduated flask (again using extra wash water as necessary) and made up to the mark. The sample was thoroughly shaken and filtered through a Whatman No 2 filter paper, and approximately 50 ml collected for analysis.

Variations on the basic procedure. A number of modifications to the above method were tested in order to determine whether the basic procedure could be simplified, or whether alternative steps were needed to improve the reliability of the method. These included comparing:

- extracting frozen or unfrozen plant material
- extracting with the hot water treatment or at room temperature

Comparison of cleanup methods. The effects of additional cleanup steps on the final extract were also considered, including the use of:

- microfilters (0.45 μm) to help clarify the extract further
- dilution to reduce colour interference from natural plant pigments in the extract
- Carrez solutions to remove proteins and other plant constituents from the extract which might otherwise interfere with the method

The Carrez solutions were prepared as follows. Carrez Solution No 1 was made up by dissolving 37.5 g of potassium hexacyanoferrate (II) ($K_4[Fe(CN)_6].3H_2O$) in water and diluting to 250 ml. This was stored in a brown bottle in a dark cupboard, and replaced every week. Carrez Solution No 2 was made up by dissolving 55.0 g of zinc acetate ($Zn(CH_3COO)_2.2H_2O$) in water, adding 7.5 ml of glacial acetic acid and diluting to 250 ml with water.

The two Carrez Solutions were used as follows. 40 ml of plant extract was pipetted into a 50 ml graduated flask, followed by 2 ml of each of Carrez Solutions 1 and 2, before being made up to the mark with water. After shaking, the contents of the flask were filtered through a microfilter (0.45 μm). Nitrate concentrations in the extract were measured before and after the microfiltration step. The results were compared with a 'control' in which the Carrez Solutions were replaced with deionised water.

Range of plant material tested. The above procedures were tested using tissue from a range of different plant types which included: iceberg lettuce, pale pink lettuce (cv Lollo Rossa), dark red lettuce (cv Crest) and dark red cabbage, as appropriate. Further details of how these materials were used to test modifications to the extraction process are given in the Results and Discussion section.

These tests were used to devise the extraction procedure for the Nitrachek method.

Testing the Nitrachek Method

Production of plant material

A selection of plant material consisting of a butterhead lettuce (cv Vegus), a pale pink lettuce (cv Lollo Rossa) and spinach (cv Samish) were grown specifically for these investigations. Seeds of each crop were sown into 4 cm cube rockwool blocks on the 12 January 2004 and left for 14 days in trays with a shallow layer of tap water. Blocks containing the most uniform seedlings were then transferred to four gullies (spaced at 20 cm centres) of a recirculating NFT system continuing nutrient solution of composition: 4mM $Ca(NO_3)_2$; 2mM K_2SO4; 1mM KH_2PO_4; 0·8mM $MgSO_4$; 0·1mM Fe Na EDTA; and adequate micronutrients; pH was maintained between 6 and 7. Plants of each crop were arranged in lines of twenty, initially at a spacing of 7·5cm within the gully. The relative positions of the group of twenty plants of each crop was varied randomly between gullies to even out any effects of local differences in aerial environment in the glasshouse. Daytime supplementary lighting was used to the same end. The composition of the nutrient solution was checked regularly, and adjusted as necessary. On the 8 March the concentration of the nutrient solution was doubled and maintained at this level until the 24 March when it was totally replaced with a Zero-N solution in which all of the $Ca(NO_3)_2$ was replaced with $CaSO_4$. At the same time the within-gully spacing was increased to 15 cm by transferring alternate plants of each crop to the same relative position in a new gully. The plants were allowed to continue to grow over the next 12 days and, because no nitrate was available from the nutrient solution, they gradually depleted the excess nitrate in their tissues. Plate 1 shows the size of the plants at this time.

A total of eight plants of each crop (one from each gully) were destructively sampled by cutting their stems at the junction with the rockwool blocks at the time the nutrient solution was switched (day 0 after withholding nitrate), and on day 1, 2, 3, 4, 5, 6, 8, 10 and 12 subsequently. The plants were sampled following a systematic pattern which was designed to keep the spacing between each group of ten plants of the same species in each gully as even as possible over the duration of sampling. Each plant was individually weighed, transferred to a separate polythene bag, and stored in a freezer with other bagged plants of the same type sampled on the same date.

Plant sub-sampling for comparative analysis

While still frozen, either two plants (for butterhead lettuce) or four plants (for pink lettuce and spinach) from the eight of each sampled on the same date were combined to create samples of roughly the same weight. This produced a total of 40 combined samples of butterhead lettuce and 20 combined samples of the other two crops from all samplings. Each plant in each of these combined samples was then individually subdivided (using procedures similar to those described above) to produce two identical sub-samples from each combined sample.

In addition, to provide samples of a contrasting deep red colour, twelve heads of a dark red lettuce (cv Crest) and eight heads of a dark red cabbage were purchased at three different supermarkets. The heads were sub-sampled individually using the procedures described above to produce two identical sub-samples of each of the 20 samples, and stored in a freezer until required for analysis.

Comparison of the Nitrachek method with the standard laboratory procedure

The above preparations created matching sub-samples from

- 40 bulked samples of butterhead lettuce (numbers 1 to 40)
- 20 bulked samples of a pale pink lettuce (numbers 41 to 60)
- 20 bulked samples of spinach (numbers 61 to 80)

that covered a range of nitrate concentrations depending on sampling date.

Plate 1. NFT system for growing the butterhead lettuce (green), Lollo Rossa lettuce (green with pale pink flush), and spinach (dark green) with different nitrate concentrations, used for comparing the Nitrachek method with the standard laboratory method of nitrate analysis.

In addition, there were further matching sub-samples from

- 12 individual heads of a dark red lettuce (numbers 81 to 92)
- 8 individual heads of a dark red round cabbage (numbers 93 to 100)

that were of unknown nitrate concentration.

One of each of the above matching sub-samples was chosen at random, and extracted and analysed for nitrate using the new Nitrachek method. All of the remaining sub-samples were packed in dry ice in an insulated container and transported while still frozen to Direct Laboratories (in Wolverhampton) for analysis by their standard laboratory procedures. This laboratory was chosen because they are FAPAS registered, they have considerable experience of measuring nitrate in lettuce, and they currently run the UK monitoring service for nitrate in lettuce on behalf of the Food Standards Agency.

Briefly, the method used by Direct Laboratories was based closely on the method recommended by the British Standards Institute (1997). The main differences from the new Nitrachek method were:

- plant samples are macerated for about 5 minutes *without* added water before extraction
- only a 10 g sub-sample of the macerated tissue is used for the extraction
- this is mixed with 400 ml of water and placed in a boiling water bath for 15 minutes
- after cooling the hot water extract is diluted to 500 ml
- nitrate concentrations are measured by HPLC using a UV detector
- a solid-phase cleanup procedure is included if needed to avoid interference in the analysis

RESULTS AND DISCUSSION

Perfecting the Nitrate Measurement Procedure

Calibrating the Nitrachek meter

Figure 1 shows typical calibration data for a full set of nitrate standards. The points and error bars represent the mean and standard deviations respectively from six independent colour measurements on each standard solution. Each graph shows the fit of a different equation to the same data. Although all of the fitted lines look similar (and all are a reasonable fit to the data) it is clear that the quadratic equations give a better overall fit than the linear equations. This is reflected in their slightly

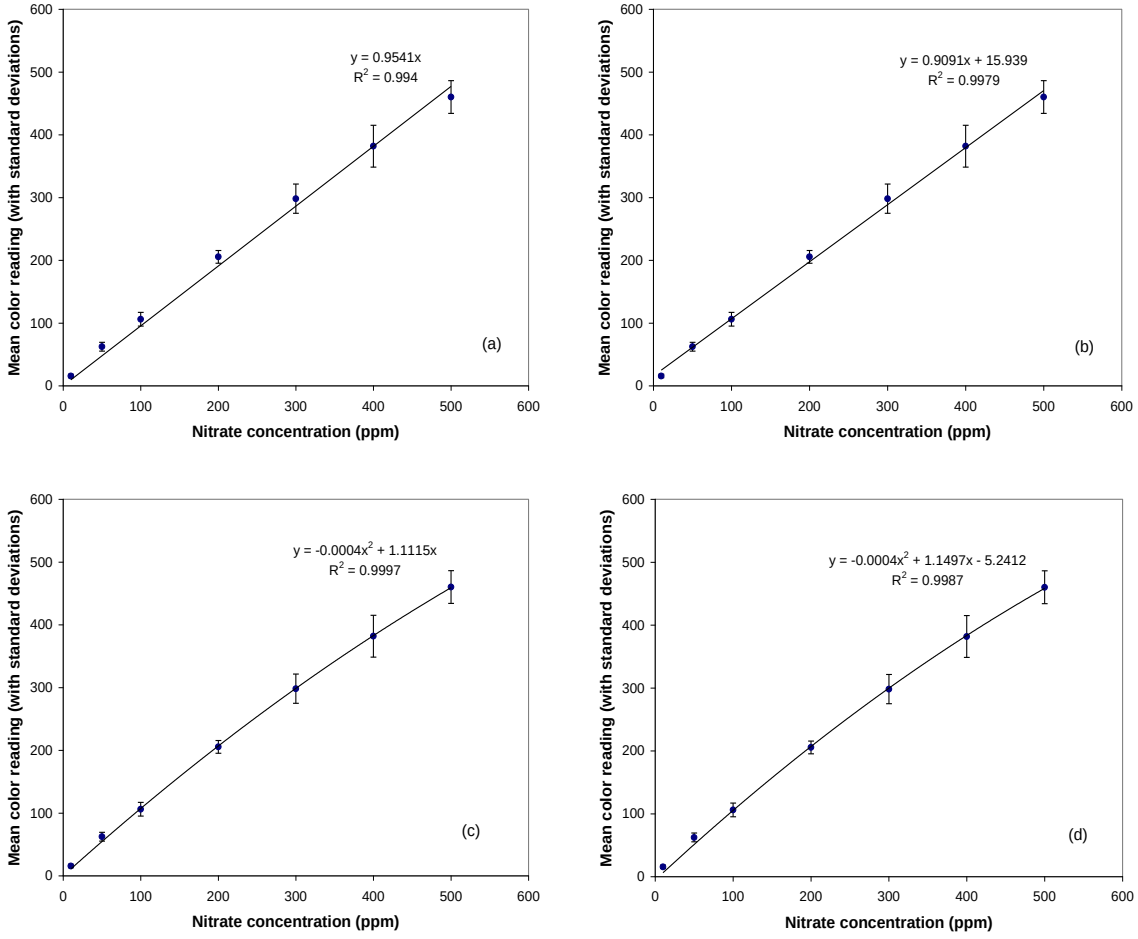


Figure 1. Calibration graphs for the Nitrachek meter using different fitted equations to interpolate between the same experimental data: (a) straight line through the origin; (b) straight line with constant; (c) quadratic curve through the origin; and (d) quadratic curve with constant. All points represent the mean of six separate measurements and the vertical bars are their corresponding standard deviations.

higher R^2 values, which represent the variance accounted for by the equations. The highest R^2 value was obtained for the quadratic curve constrained through the origin (Figure 1c).

The accuracy of each equation in predicting the estimated nitrate concentration in a solution from its Nitrachek colour measurement was then tested using exactly the same dataset as follows. The mean colour reading for each of the above standards was substituted (as the y value) in each of the fitted equations shown on the graphs (in Figure 1) in order to calculate the corresponding estimated nitrate concentration (x value). The differences between the estimated and actual concentration for each equation are plotted as deviations in Figure 2. These show that use of the linear calibration equations consistently overestimated nitrate levels in the mid-concentration range (100 to 400ppm) and consistently underestimated them at higher concentrations (400 to 500ppm). The estimates from the quadratic curves were much closer to expectation, especially above 100ppm nitrate, where deviations were seldom more than 2ppm. On the strength of these observations it was decided to use the quadratic calibration line constrained to pass through the origin in all further work.

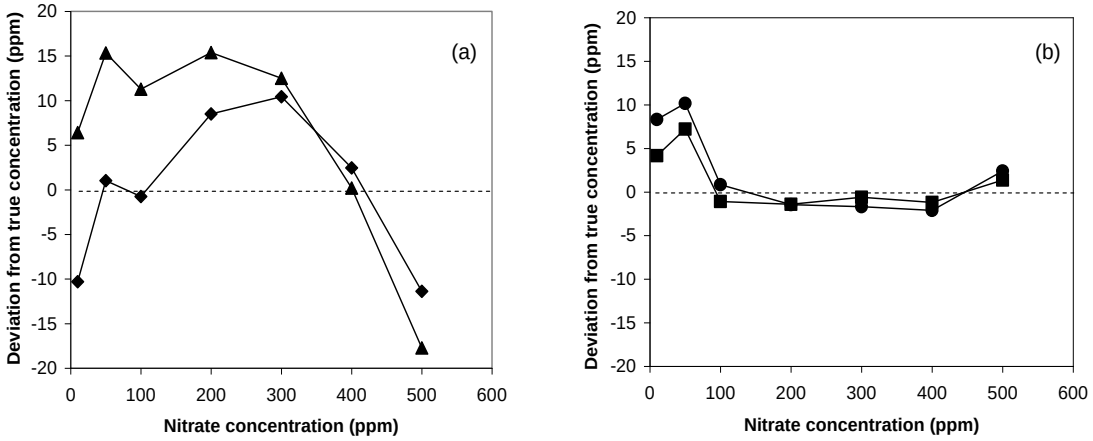


Figure 2. Graph of deviations of the estimated nitrate concentrations from their true values. The estimated concentrations were calculated from their original colour measurements using the different fitted calibration equations given in Figure 1: (a) straight line through the origin (▲), or straight line plus constant (◆); (b) quadratic curve through the origin (■), or quadratic curve plus constant (●). Note that the scale of the vertical axis on both graphs is the same.

Optimising the sensitivity of the nitrate measurements

Most plants sampled are likely to have a nitrate concentration in the range between 2000 and 5000 ppm on a fresh weight basis. These nitrate concentrations need to be diluted during the extraction process to bring them down to below the maximum 500 ppm measured by the Nitrachek meter. However, it is also important to ensure that an appropriate dilution factor is chosen to avoid any loss of accuracy in measurement.

Tests of the accuracy of estimates of nitrate concentration were carried out using the set of nitrate solutions given in Table 2. Different volumes of each of these solutions (20, 40 or 80 ml) were diluted to 1 litre to produce dilution factors of 50, 25 and 12·5 respectively. Four colour measurements for each of the resulting diluted solutions were then taken with the Nitrachek meter. Nitrate concentrations were estimated from these data using a quadratic calibration curve fitted to the mean of four separate measurements on independent standards (made up according to Table 1). The resulting pattern of estimated nitrate concentrations was similar for all of the dilution ratios (see Figure 3a), with all tending to overestimate the true nitrate concentration slightly, and with the largest relative errors generally occurring at the low and high extremes of nitrate concentration. However, although the pattern of results was similar for each of the dilution factors, there were distinct differences in their standard deviation (see Figure 3b), with measurements using a 50-fold dilution factor generally

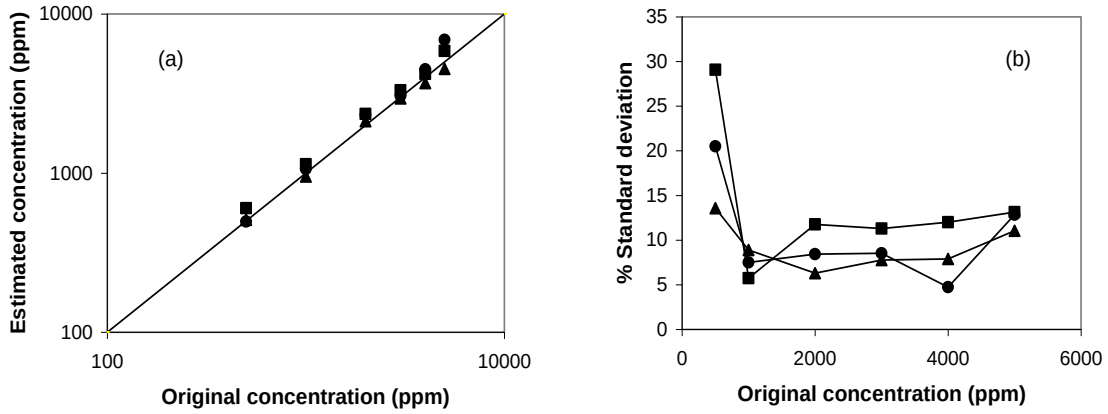


Figure 3. Effects of dilution factor on nitrate analysis: (a) graph of estimated vs true nitrate concentration (log scales); and (b) graph of standard deviations for the different dilution factors. Key: ■ 50 fold; ● 25 fold; ▲ 12·5 fold.

more variable than the other two. This was caused by relatively larger errors of estimation in the lower concentration region of the calibration curve (see Figure 2b). Although % standard deviations for the 25 and 12·5 dilution factors were generally smaller, they also tended to increase slightly at low and high ends of the concentration range.

These results suggest that dilution factors in excess of 25 generally gave more reliable results. This would be equivalent to extracting 40 g or more of plant sample into 1 litre of water. However, the accuracy of the results will be further enhanced by using a dilution factor which avoids measuring nitrate concentrations in extracts at the extremes of the calibration curve, *ie* below 100ppm or above 400ppm.

Carry-over effects between successive samples

Figure 4 shows Nitrachek colour measurements for cycles of 5 repeated determinations on a 5ppm nitrate standard, followed by 5 determinations on a 500ppm standard. The graph shows there were no detectable carry-over effects from switching from a low to high concentration solution, or vice versa. This indicates that calibration data for the Nitrachek meter is unlikely to be affected by the order in which standard solutions of contrasting nitrate concentration are measured. Similarly there will be no bias introduced if plant extracts containing high and low nitrate concentrations are measured in succession.

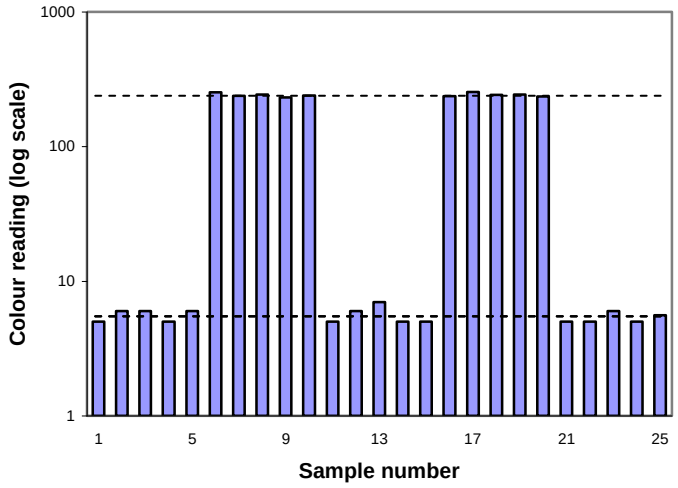


Figure 4. Colour readings from two cycles of five successive solutions at 5ppm and five successive solutions at 500ppm to test for carry-over effects.

Perfecting the Plant Extraction Procedure

Comparison of extraction methods

An experiment with sub-samples taken from each of five different heads of iceberg lettuce was carried out to compare the effects of

- storage conditions, using unfrozen vs frozen plant samples
- extraction temperature, using cold (room temperature) vs hot water extraction
- unfiltered vs filtered extract solutions, using Whatman No 2 filter papers

with the extracts from each of the above treatments measured before and after filtration.

Although the lettuce samples were prepared from different heads of the same type of (iceberg) lettuce purchased at the same supermarket on the same date, there was some head-to-head variability in their nitrate concentrations. Despite this, an analysis of variance was used to evaluate the effects of the different sample storage and extraction treatments. The results showed there were no significant effects of any of the treatments (probably because of the relatively small size of the dataset), although there were clear trends which are worth recording. These can be seen in Table 3, which summarises the treatment means for the five lettuce heads. This shows that, on average, estimated nitrate concentrations were slightly larger with previously frozen samples when extracted at room temperature, especially where measurements were made on unfiltered extracts. Possible reasons for these effects are discussed below, after more detailed comparisons of the different treatment effects.

Table 3. Effects of sample storage temperature, extraction temperature and extract filtration on mean estimates of nitrate concentration in five iceberg lettuce heads.

Storage Condition	Hot Water Extraction		Room Temperature Extraction	
	Filtered Extract	Unfiltered Extract	Filtered Extract	Unfiltered Extract
Unfrozen	1106	1098	1132	1125*
Frozen	1129	1158	1132	1224*

* these values were significantly different ($0.05 < P < 0{\cdot}01$)

The effects of either freezing the samples before extraction, or increasing the temperature of the water during extraction are compared in Figures 5 and 6 respectively, using the individual sample data. Given the relatively narrow range of nitrate concentrations for the plant samples used, there were reasonable relationships between the contrasting methods compared. However, there were a few notable outliers which tended to reduce the correlation coefficient in each case. Most notably, these were more prominent amongst the unfiltered samples, which generally produced larger estimates for the frozen samples compared with the unfrozen ones (see Figure 5) and, to a lesser extent, for the samples extracted at room temperature rather than into hot water (see Figure 6).

Figure 7 compares the effects of filtering the extracts, and shows that there was quite a strong relationship between the estimates of nitrate in filtered and unfiltered samples. The few outliers observed occurred predominantly with unfiltered solutions extracted at room temperature from frozen samples, which generally produced higher estimates than the unfrozen samples, or samples extracted with hot water.

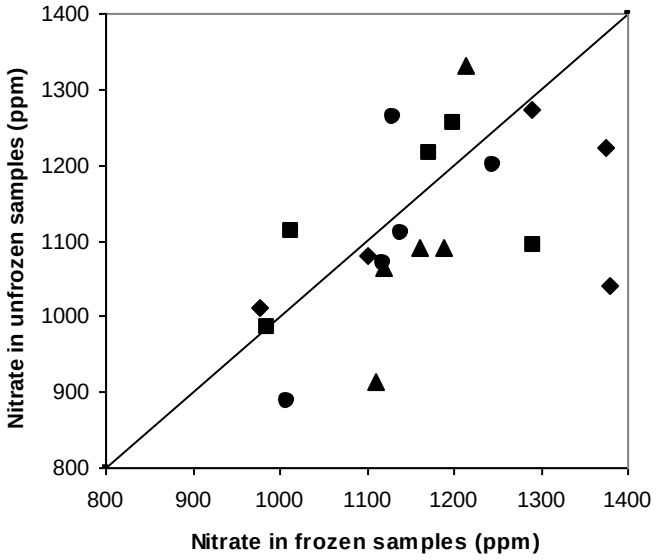


Figure 5. Estimated nitrate concentration from unfrozen samples of iceberg lettuce vs corresponding data from frozen samples. Key: ■, ♦ room temperature extraction with and without filtering respectively; ●, ▲ hot water extraction with and without filtering respectively.

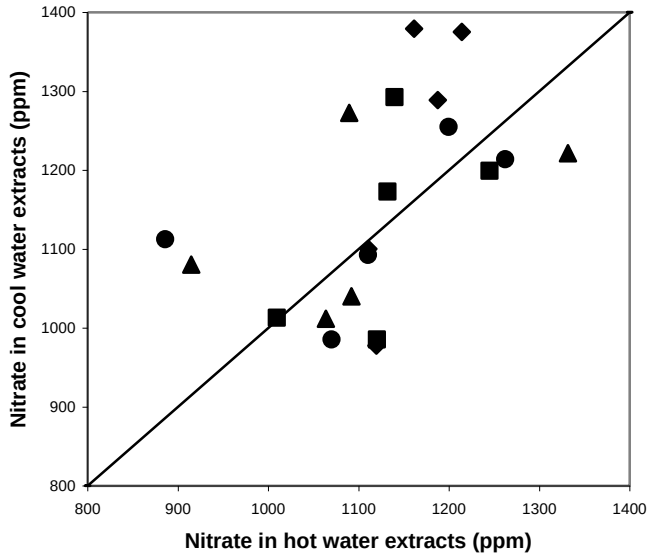


Figure 6. Estimated nitrate concentration from iceberg lettuce samples after extraction at room temperature vs corresponding data from hot water extraction. Key: ■, ♦ frozen samples extracted with and without filtering respectively; ●, ▲ unfrozen samples extracted with and without filtering respectively.

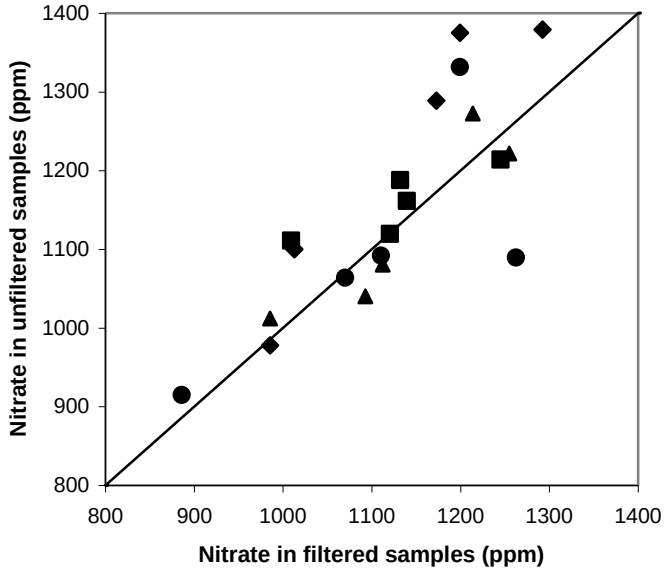


Figure 7. Estimated nitrate concentration from filtered extracts of iceberg lettuce vs corresponding data from unfiltered extracts. Key: ■, ♦ frozen samples extracted into hot water or at room temperature respectively; ●, ▲ unfrozen samples extracted into hot water or at room temperature respectively.

The reasons for these effects appear to be related to colour interference on the nitrate test strips used with the Nitrachek meter because

- the cold water extracts generally appeared to be duller and darker green in colour than the hot water extracts which were darker brown
- traces of dark green pulped tissue were present on the test strips from the unfiltered samples

However, without independent analyses it is impossible to say whether the enhanced estimates of nitrate concentration in cold water extracts (particularly with the frozen samples) was caused by interference from the increased green colour of the extracts, or (possibly) from some denaturing of nitrate in hot water extracts, although evidence from other sources suggest that the latter is less likely to be the case (Burns, 2000). These results also suggest that it is important to filter all samples to avoid the risk of biasing the results (and possibly contaminating the sensor surface of the Nitrachek meter). Making measurements on filtered extracts would also avoid introducing additional variability with the use of frozen samples, especially if they were extracted at room temperature. It is noteworthy that the small effect of extraction temperature in these results agrees with the data of Lyons *et al*. (1991), but contradicts those reported previously by Burns (2000), who found much larger differences. Farrington (2002) also found that extracting at room temperature produced more variable estimates of nitrate concentration. These apparent differences might suggest that use of a hot water treatment is less important when the plant material is blended directly in the water extractant as an integral part of the extraction process (as in the new method), rather than being macerated separately before extraction (as in the BSI (1997) method). However, in view of the small size of the dataset in this current study, further work would be needed to confirm this hypothesis.

Comparison of cleanup methods

Filtration treatments. Because of the benefits of simple filtration as a preliminary cleanup procedure with iceberg lettuce (see above), further investigations were carried out on a wider range of sample types with increasing levels of red pigmentation. These included iceberg lettuce, a pale pink lettuce (cv Lollo Rossa), a dark red lettuce (cv Crest), and a dark red cabbage. Each of the plants was frozen before being extracted using the hot water method. The cleanup treatments compared were:

filtration (using Whatman No 2 filter paper); microfiltration (0·45 μm); a combination of these two methods; and simple filtration followed by a two-fold dilution with water (to reduce colour interference effects). The resulting extracts ranged in colour from a pale green-brown (iceberg lettuce), through shades of mid to dark brown (pink and red lettuce types) to deep maroon (red cabbage), see Plate 2. The colour of the extracts was not significantly affected by the different cleanup treatments, except for that which included the two-fold dilution, which was paler in each case. The means of between three and five colour readings from the Nitrachek meter were converted into nitrate concentrations using a quadratic calibration curve through the origin and the results adjusted for the dilution were necessary.

During the measurement of nitrate on the red cabbage extracts, it was immediately obvious that, unlike those for the different types of lettuce, colour development on the test strips was virtually instantaneous, with both the nitrate and nitrite sensitive zones turning a pink-purple colour. This suggested that the red cabbage extracts contained a significant concentration of nitrite (which invalidated the measurements). Similar, although somewhat less pronounced effects, were also observed when extracts from iceberg lettuce were left overnight before measuring nitrate concentration, suggesting that nitrite had been generated in those samples in the interim.

Examination of the results shows that the effects of the different cleanup methods on the estimated nitrate concentrations were generally small, see Figure 8. The only consistent effect was for the filtered and diluted extracts which were slightly greater than for the other treatments. This probably originated from a small positive bias introduced because the Nitrachek colour measurements for these diluted extracts were at the lower end of the calibration curve, see Figure 2b.

These results show that normal filtration with Whatman filter papers was as effective as microfiltration in removing the pulped plant material from the extracts prior to analysis. They also confirmed the need to maintain the colour readings in the mid-range of the calibration curve for maximum accuracy. Colour measurement should be made as soon as possible after extraction and, in all cases, on the same day as the extraction, to avoid generation of nitrite in the extracts. However, the method should not be used with red cabbage, unless some method can be found for avoiding the

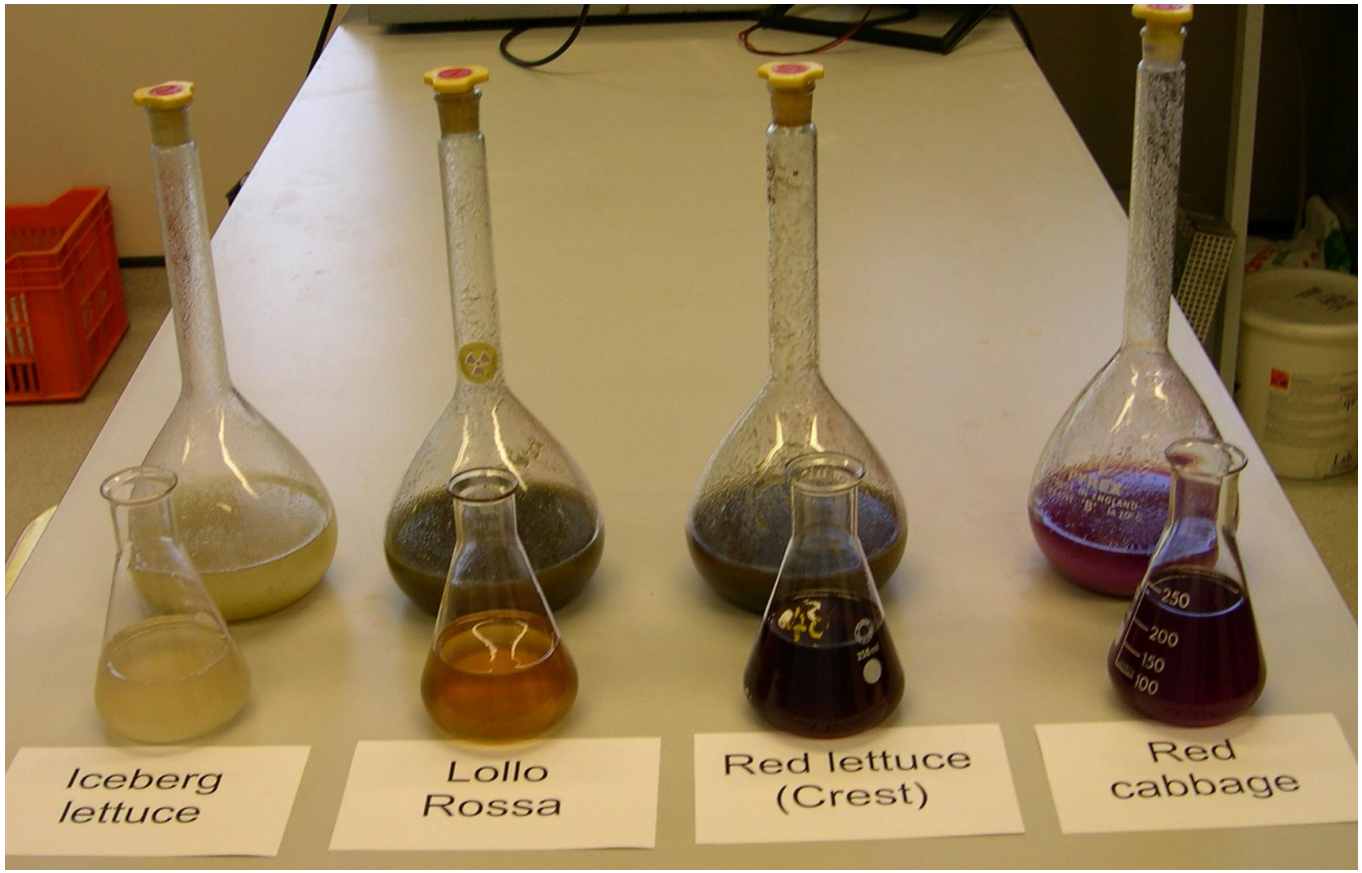


Plate 2. Effect of natural pigments in iceberg lettuce, pale pink lettuce (cv Lollo Rossa), dark red lettuce (cv Crest) and red cabbage samples on the colour of their hot water extracts.

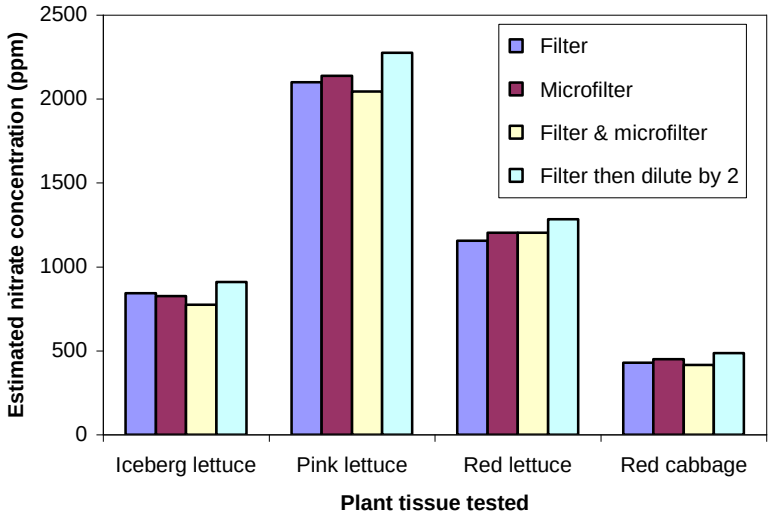


Figure 8. Estimated nitrate concentrations in plant tissue from iceberg lettuce, pink lettuce, dark red lettuce and dark red cabbage after various cleanup procedures on the extracts. Note, the estimates for the red cabbage sample were unreliable because of probable interference from nitrite (see text).

apparent effects of nitrite interference, which invalidates its use for this type of plant material.

Colour interference from natural plant pigments in the extracts. Separate studies of the effects of pigmentation in the plant extracts used in the above study were made using dummy test strips made up with squares of untreated filter paper stuck to the test strips in place of those impregnated with colour reagent. These were used in exactly the same way as normal test strips, but as there was no colour reagent present, any colour measured on the strips by the Nitrachek meter originated entirely from the natural pigments in the extracts. Means of five separate measurements on filtered extracts of each of the different plant types were converted into estimated nitrite concentrations as before, to give a measure of the amount of colour interference from each of the extracts. The results, given in Figure 9, show that colour interference ranged from an equivalent of 3 ppm of nitrate in iceberg lettuce to 63 ppm in red cabbage, with the pink and dark red lettuce types containing an equivalent of 34 and 56 ppm nitrate respectively. These 'apparent' nitrate concentrations are relatively small compared with the normal range of nitrate in lettuce and spinach (2000 to 5000 ppm) so are unlikely to present a problem with the analysis method in most situations, even after adjustment for dilution during extraction.

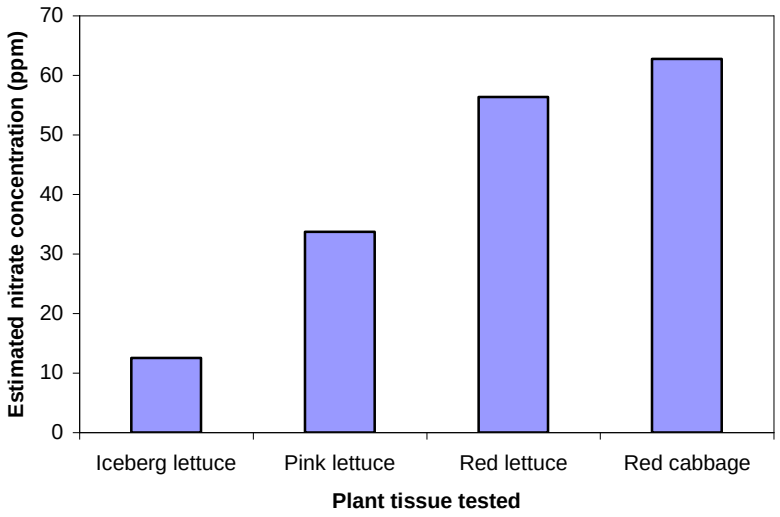


Figure 9. Apparent nitrate concentrations in plant tissue from iceberg lettuce, pink lettuce, dark red lettuce and dark red cabbage measured using dummy test strips on filtered extracts. These data give a measure of the effects of colour interference with the measurement procedure.

Use of Carrez Solutions. Possible benefits from using a mixture of Carrez Solutions 1 and 2 as a cleanup method were investigated using two identical sub-samples from an individual head of iceberg lettuce and red cabbage. The latter was included in the test to determine whether this chemical cleanup procedure could remove the apparent nitrite interference problems encountered previously. The plant material was frozen before use and the sub-samples extracted into either 1 or 2 litres of hot water, with all extracts filtered before further cleanup procedures were employed. The latter included the addition of the mixed Carrez Solutions, or an equivalent volume of water, both with or without subsequent microfiltration to remove any precipitation produced. Addition of the Carrez Solutions had little visible effect on the iceberg lettuce extracts, but turned those for red cabbage from deep maroon to a powdery blue colour. However, the original maroon colour was re-established (albeit a little less intensely) after subsequent microfiltration. Microfiltration did not change the colour intensity of the red cabbage extracts when no Carrez Solutions were added. Unfortunately, however, treating the red cabbage extracts had no apparent effect on the interference problems (probably from nitrite) observed earlier.

Nitrate concentrations in the original plant sub-samples were calculated from the mean of between three and five Nitrachek colour measurements (using a quadratic calibration curve through the origin) after adjusting for the different dilution factors in the various treatments. The effects on the estimated nitrate concentrations are summarised in Figure 10. This shows that addition of the mixed Carrez Solutions increased the estimated concentrations (particularly for the iceberg lettuce samples), but subsequent microfiltration reduced the estimates again, possibly to below those for the simple filtered extracts. The effects of replacing the Carrez Solutions with an equivalent volume of water was somewhat inconsistent between the cleanup treatments for each sub-sample of plant material, largely because differences in dilution ratio were small (40 ml diluted to 50 ml) compared with the undiluted treatment in each case. However, the two-fold difference in dilution between sub-samples A and B of red cabbage (which contained relatively low levels of nitrate compared with the lettuce), tended to increase the estimated nitrate concentrations slightly in sub-sample B, in line with the effects of the two-fold dilution in the first set of cleanup experiments above.

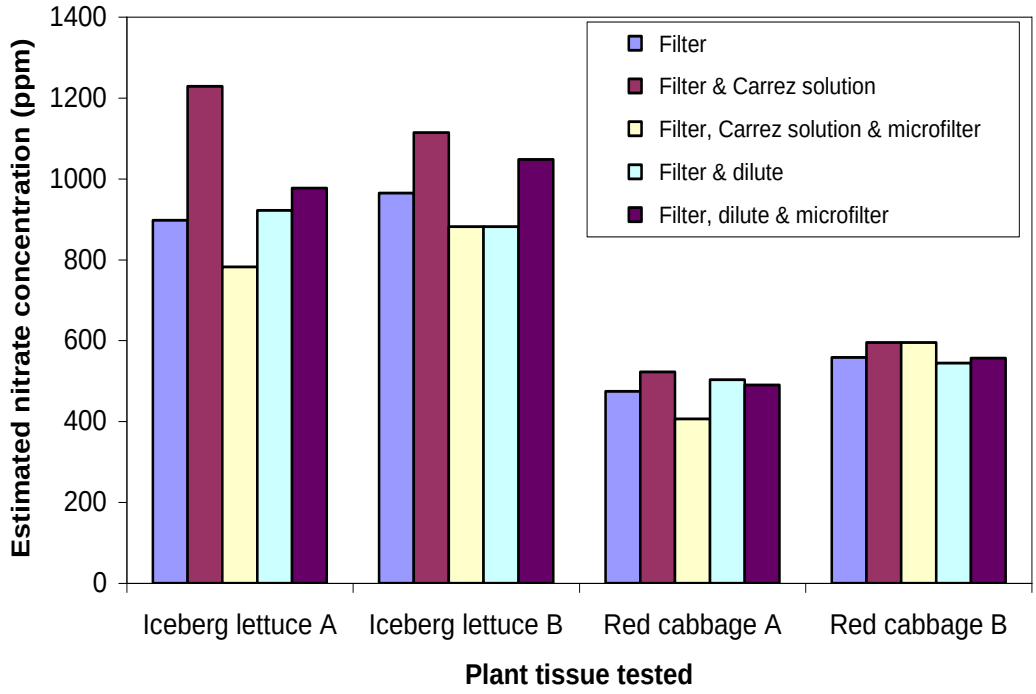


Figure 10. Estimated nitrate concentrations in two identical sub-samples of iceberg lettuce and red cabbage after hot water extraction into either 1 litre (sub-sample A) or 2 litres (sub-sample B) of water and subsequent use of Carrez Solutions No 1 and 2 to cleanup the extracts. Note, the estimates for the red cabbage samples were unreliable because of probable interference from nitrite (see text).

These results show that use of the mixed Carrez Solutions (as recommended in the British Standards (1997) method of nitrate analysis) did not appear to improve the reliability of the method. It also had no effect on removing the apparent nitrite interference effects in the red cabbage extracts.

Defining the Nitrachek Method

On the basis of the above results it was concluded that the Basic Extraction Procedure

- provided a suitable basis for the new Nitrachek method
- either frozen or unfrozen plant samples could be used
- the method should involve hot water treatment during extraction, largely because further work would be needed to verify that extraction at room temperature gave truly comparable results
- filtration through Whatman No 2 filter papers was an essential cleanup step
- no other cleanup procedures were necessary

Details of recommended procedures for calibrating the Nitrachek meter, for measuring nitrate in the plant extracts, and for calculating nitrate concentrations in the plant tissues were included in a draft protocol for the new Nitrachek method. The importance of measuring the plant extracts on the same day as the extraction to avoid compromising the results was emphasised.

Testing the Nitrachek method

General Observations

Nitrate concentrations in butterhead lettuce, pale pink lettuce, young spinach, dark red lettuce and dark red cabbage were estimated on sub-samples prepared and assembled as described in the Materials and Methods section. Measurements using the Nitrachek method were carried out independently at Warwick HRI by a skilled technician who had not previously been involved in the method development. No problems were experienced in following the new method, other than those from the probable nitrite interference with the red cabbage measurements, as described above. Although this interference would normally invalidate the Nitrachek measurements for red cabbage samples, the resulting data were still included in the subsequent statistical analyses to provide a full comparison of the two methods. Corresponding matching sub-samples

of all of the plant materials were independently analysed by Direct Laboratories in Wolverhampton using their standard (FAPAS approved) laboratory procedure. A preliminary comparison of data revealed that a few samples exhibited extremely large differences between the two methods and, where possible, these samples were reanalysed by Direct Laboratories, and their data adjusted accordingly. It was not possible to reanalyse samples used for the Nitrachek method because all of the sub-samples were normally used for the original measurements.

Raw data from the resulting independent measurements of nitrate concentration in the different types of plant material for the two methods are listed by sample number in Appendix 1. Preliminary graphical comparisons of the estimated nitrate concentrations for the two methods are illustrated in Figure 11. Examination of the latter shows that there was broad agreement between the two methods for most samples, although some isolated ones still showed significant differences. As a general rule, the deviations were more pronounced at higher nitrate concentrations and, where these occurred, estimates from the Nitrachek method tended to be lower than those from the standard method for butterhead lettuce, and the reverse for pink lettuce and (especially) for spinach. Estimated nitrate concentrations for the dark red lettuce samples showed no consistent pattern, other than the estimates from the Nitrachek method were lower than those from the standard method for the first five samples (numbers 81 to 85), and much higher for the subsequent ones (numbers 86 to 92), largely because of a big increase in the size of the data from the standard laboratory method. The reasons for this change are unclear. Nitrate concentrations in the red cabbage samples were all quite small, with the estimates from the two methods in reasonable general agreement, despite the considerable uncertainty caused by the likely nitrite interference with the Nitrachek measurements. However, the estimates from the latter were generally slightly larger than those from the standard method.

Butterhead lettuce, pink lettuce and spinach data

The dataset for each type of plant material was analysed separately. All data were transformed to their square roots to equalise the residuals before an analysis of variance was carried out. Table 4 shows that the resulting coefficients of variation for both methods were lowest for the butterhead lettuce and highest for the spinach, with pink lettuce intermediate.

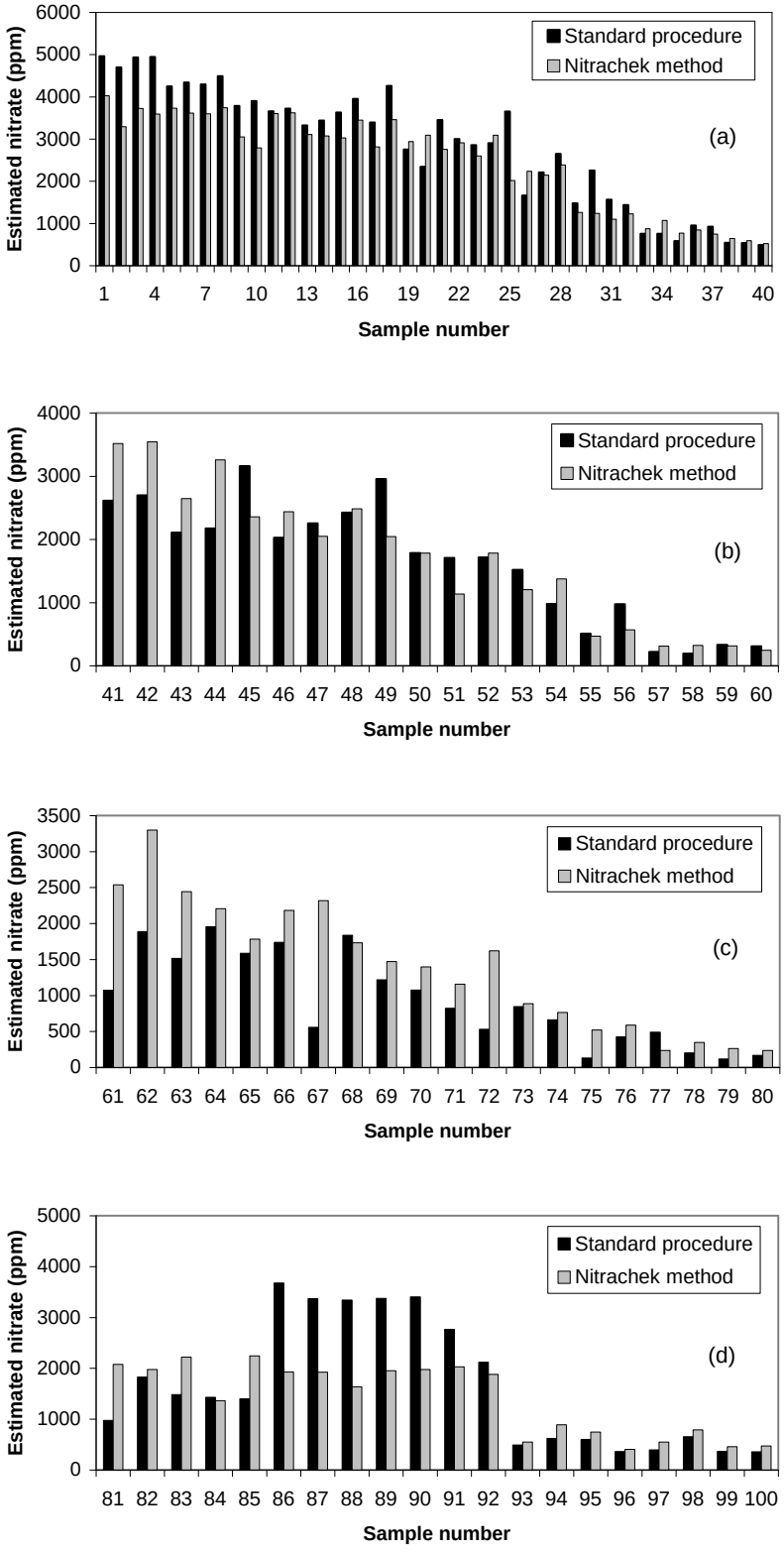


Figure 11. Comparison of the original estimates of nitrate concentration from the Nitrachek method and the standard procedure on individual samples of: (a) butterhead lettuce; (b) pale pink lettuce; (c) young spinach; and (d) dark red lettuce (samples 81 to 92) and dark red cabbage (samples 93 to 100).

Table 4. Coefficients of variation for nitrate data measured by the Nitrachek method and the standard laboratory procedure for different types of plant material. All nitrate data were square root transformed before analysis.

Plant Material	Coefficient of Variation (%)	
	Nitrachek method	Standard Method
Butterhead lettuce	4·4	8·2
Pale pink lettuce	7·1	11·9
Young spinach	8·5	20·8

Coefficients of variations for the Nitrachek method were also consistently less than the standard laboratory method, typically by as much as a half. This is likely to be caused by additional sub-sampling errors in the standard method, which only used about 10 g of each of the samples provided, whereas the Nitrachek method generally used all of the plant material available (*ie* up to about 200 g).

Figure 12 shows the estimated changes in mean nitrate concentration (from the time at which nitrate was withheld from the nutrient supply) in each type of plant material. The mean values were calculated on the square root transformed data and then back-transformed to normal concentration units before being plotted. The results confirm earlier observations on the raw data that the biggest differences between the two methods mainly occurred shortly after the nitrate was withheld, when tissue nitrate concentrations were still high. In butterhead lettuce, estimates from the Nitrachek method were greater than those for the standard method at this time, whereas the reverse occurred with young spinach and (to a lesser extent) with pink lettuce.

The relationships between the square root transformed data from the two methods are compared directly for each type of plant material in Figure 13, and the results of the corresponding regression analyses given in Table 5. These show that estimates from both methods were highly correlated, with data for each plant type fitting a linear regression line through the origin. The slopes of these lines are given in Table 5, and are all close to unity (*ie* to the line of perfect agreement). For butterhead lettuce, the slope is slightly greater than one, whereas for the other two plant materials, they are

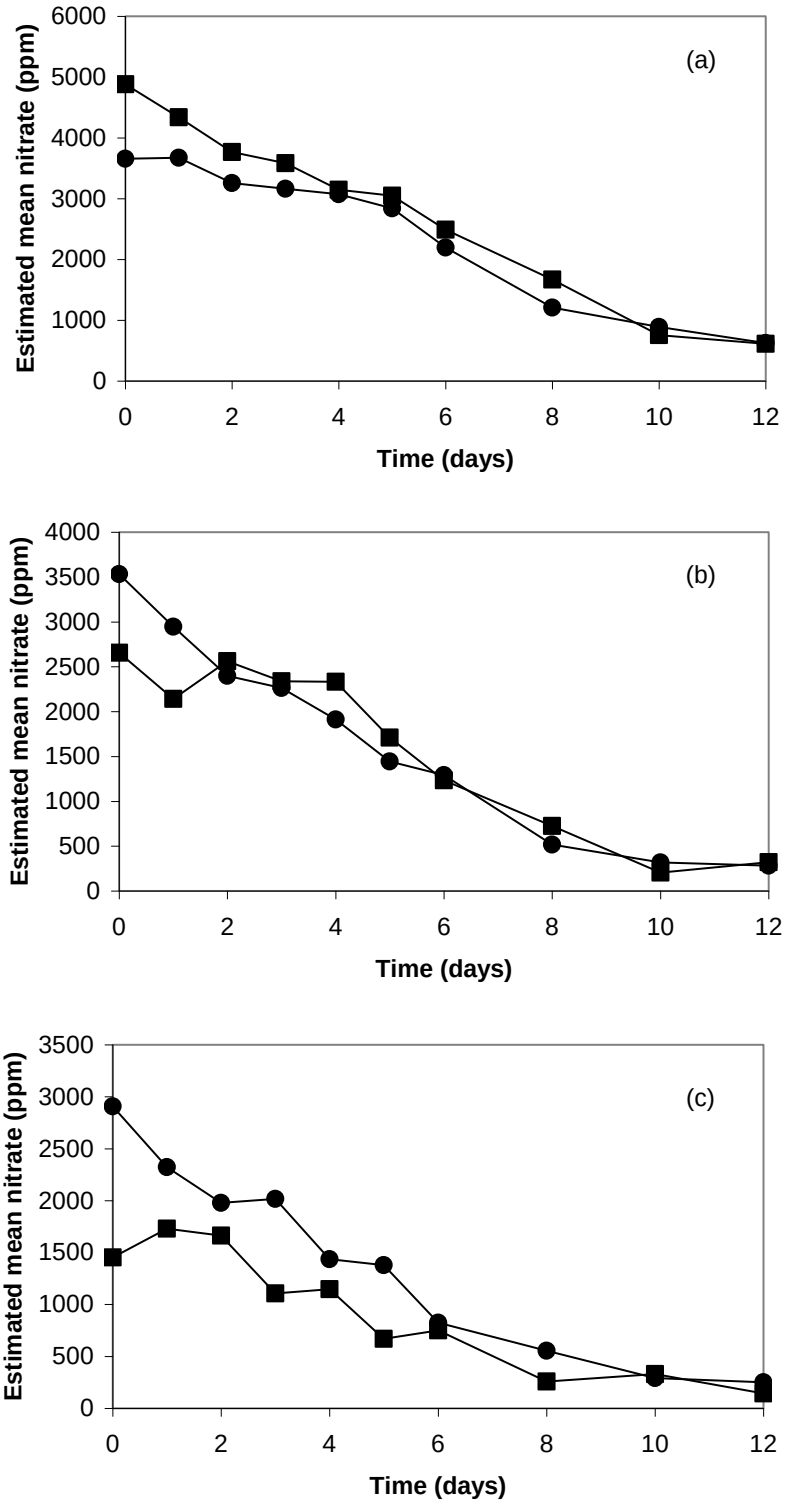


Figure 12. Changes in estimated mean nitrate concentration from the Nitrachek method (●) and the standard method (■) over time after withholding nitrate from the nutrient supply for: (a) butterhead lettuce; (b) pale pink lettuce; and (c) young spinach. The means were calculated from square roots of the original data and back-transformed to show normal concentration units.

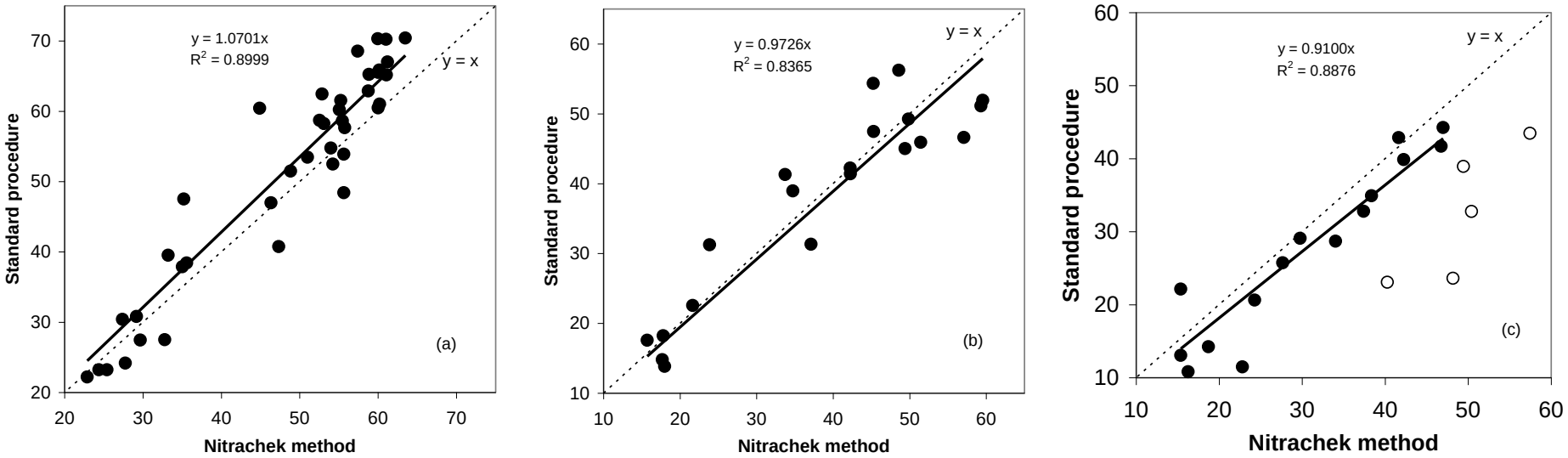


Figure 13. Graphs of estimated nitrate concentration (square root transforms) from the standard method (y) against those from the Nitrachek method (x) for: (a) butterhead lettuce; (b) pale pink lettuce; and (c) young spinach. The bold line on each graph is for the regression of y on x through the origin for all data except for spinach where the identified outliers (shown as open circles) have been excluded to avoid biasing the result. The (dotted) line of perfect agreement (y = x) is also shown on each graph.

both less than one, with the slope of the line for the pale pink lettuce not significantly different from it.

Table 5. Regression coefficients for estimates of nitrate concentration measured by the standard laboratory method vs the Nitrachek method for different types of plant material. All nitrate data were square foot transformed before analysis.

Plant material	Number of data	% Variance accounted for	Regression Line through origin: slope ± se
Butterhead lettuce	40	90·0	1·0701 ± 0·0154
Pink lettuce	20	83·7	0·9727 ± 0·0305
Young spinach	20	67·2	0·8034 ± 0·0383
Young spinach	15*	88·8	0·9100 ± 0.0328

* sub-set of the whole dataset, but excluding five identified outliers which otherwise bias the relationship.

Dark red lettuce and red cabbage

The heads of the red lettuce and cabbage were purchased from three local supermarkets in an attempt to vary their source as much as possible. However, as these were produced commercially, all samples of the same plant type were expected to have similar nitrate concentrations in their tissues. The red lettuce heads were all individually packaged and labelled with their cultivar (cv Crest) in a similar way; it is, therefore, possible that they were all produced by the same grower for different retail outlets. The red cabbage heads were sold unpackaged, but all were of similar size and appearance, and there was no way of identifying the cultivar or their site of production.

Estimated nitrate concentrations (square root transformed) from the standard laboratory method are plotted against those from the Nitrachek method in Figure 14 for both types of produce. This shows that agreement between the two methods was very poor for the red lettuce samples, with the Nitrachek method showing only small differences between samples, whereas the estimates from the standard method varied from about 1000ppm to 4000ppm. Although this variability was larger than expected

for samples of a commercially produced crop, there is no way of determining which method was more accurate without an independent analysis using a completely different method. This was not possible in the current trial. Thus with the information available, it was not possible to recommend the use of the Nitrachek method with dark red lettuce samples.

Surprisingly, agreement between the two methods was much closer for the red cabbage samples, despite the questions about the reliability of the Nitrachek method with these samples due to the probable nitrite interference. Both methods were highly correlated, and the square root transformed data fitted a regression line (of slope 0·8909) through the origin, which accounted for 87·2% of the variance. On the basis of these rather limited results, it would appear that the Nitrachek method is capable of giving sensible results for red cabbage, provided a correction factor was used to adjust the data for the average bias for this type of plant material. However, given the uncertainty over the reliability of the nitrate test strips with red cabbage samples, it is impossible to recommend its use with this type of plant material.

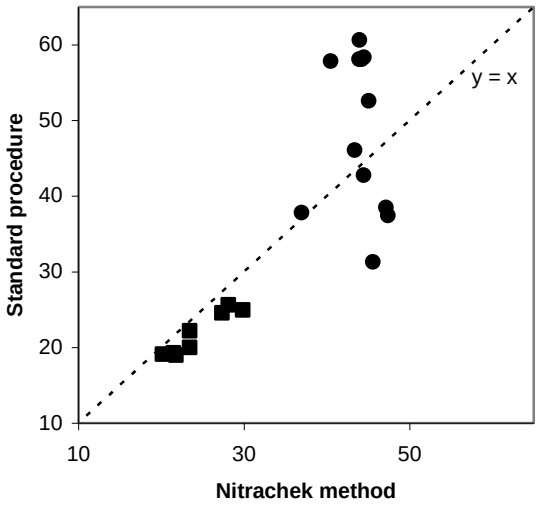


Figure 14. Graph of estimated nitrate concentration (square root transforms) from the standard method (y) against those from the Nitrachek method (x) for red lettuce (●) and red cabbage (■). The dotted line of perfect agreement (y = x) is also shown.

Refining the Nitrachek Method

Following further investigations a simplification was made to the filtration step used to cleanup a sample of extract immediately before its nitrate concentration was measured. Previously this had been carried out by the conventional filtration method, using a filter paper folded into a cone shape and inserted into a funnel, and a conical flask to collect the filtrate. This was replaced with a reverse filtration method in which the filter paper cone was inserted point downwards directly into a 50 or 100ml beaker already containing the unfiltered extract. The extract passes through the filter paper in the opposite direction to normal (from the outside to the inside of the filter paper cone). The nitrate test strips are then dipped directly into the clarified extract on the inside the filter paper cone. Use of this reverse filtration method is simpler and often quicker than conventional filtration, and it removes the need for separate funnels and conical flasks for each of the extracts.

The tests of the Nitrachek method (detailed above) showed that it gave good relationships with the standard laboratory procedure for samples of butterhead lettuce, pale pink lettuce and spinach although, on average, there were small differences between the estimates from the two methods. By definition, the standard laboratory procedure must be presumed to give the definitive measure of nitrate concentration (albeit with some variability), so this method must provide the 'standard' against which others are compared. On this basis, the estimates of nitrate concentration from the Nitrachek method must all be considered slightly biased, by an amount which depends on the type of plant material being analysed. The magnitude of this bias can be calculated from the slopes of the regression lines given in Table 5, and these can be used as a correction factor to counteract the bias. Allowing for the square root transformation to the original data, the equation of each regression line is

$$y^{½} = a.x^{½} \qquad (1)$$

where y and x are the original estimates of nitrate concentration from the standard procedure and the Nitrachek method respectively, and a is the slope of the regression line. From this it follows that

$$y = a^2.x \qquad (2)$$

Thus a^2 represents the multiplication factor for correcting the estimates of nitrate concentration from the Nitrachek method into 'bias-free' estimates, equivalent to

those for the standard laboratory method. Values of these correction factors are given in Table 6. These show that, on average, the Nitrachek method underestimates the standard laboratory method by 14·5% for butterhead lettuce, and overestimates it by 5·4% and 17·2% for the pale pink lettuce and spinach respectively. On the basis of these results it was decided to build these correction factors into the procedure for calculating the results (see below).

Table 6. Multiplication factors for correcting for bias in estimates of nitrate concentration from the Nitrachek method.

Plant Material	Correction Factor
Butterhead lettuce	1·1451
Pink lettuce	0·9461
Young spinach	0·8281*

* excluding outliers

A further modification to the Nitrachek method was made by specifically excluding the measurement of nitrate in samples of dark red lettuce or dark red cabbage.

The final version of the Nitrachek method was released under the name of Plant Nitrate Protocol and is reproduced in full in Appendix 2.

Procedure for Calculating the Results

A simple set of procedures were developed in the form of data-entry tables in a Template with embedded equations (constructed in an Excel worksheet) to streamline the calculation of results. A summary of its use is given below.

Once all of the measurements have been collected, the user first completes a table in the Template giving details of the calibration data (the concentration of each standard solution and its associated colour measurements). The Template automatically calculates the equation of the calibration graph, assuming a quadratic curve through the origin, and the curve and data points are displayed on the right hand side of the worksheet. After the user is satisfied that the calibration curve is satisfactory, he/she

copies the two parameters of the calibration equation (displayed on the graph) into a predefined area of the worksheet so they can be used for the subsequent calculations.

The user then fills the details of the measurements on the plant samples into a separate table in the Template. The data include the type of plant material analysed, the weight of tissue extracted and the Nitrachek colour measurements. The Template then automatically calculates the concentrations of nitrate in the extract and in the original plant tissue. These results are automatically adjusted for the average bias expected for each type of plant material, using equation (2) together with the correction factors given in Table 6. It is assumed that these correction factors are applicable to all types of green lettuce (*ie* butterhead, iceberg, cos, etc.,), pale pink lollo rosso types of lettuce, and spinach respectively.

The Template also checks to see if the mean colour measurement for each plant sample is within the range of those for the standards, and gives a message 'within range' or 'out of range' accordingly. If the sample is 'out of range', the user is encouraged to dilute the offending extract and re-measure it without delay. A separate table is also provided for calculating the nitrate concentration of any diluted sample, which automatically adjusts the result for the appropriate dilution factor.

The Template allows data to be entered only into predefined areas of the various tables, to minimise the risk of errors; other areas of the Template are protected against data entry, to prevent users accidentally overwriting the embedded equations. The Template is designed so that it is, by default, printed out on a single page, so that hard copies can be filed conveniently for future reference. The Template comprises a single worksheet and is provided in an Excel workbook, together with two other worksheets containing Instructions and an Example illustrating the use of the Template respectively. The workbook file is supplied under the name Plant Nitrate Calculator. Printouts of the three worksheets are given in Appendix 3.

Estimated Costs of Analysis by the Plant Nitrate Protocol

The costs of essential items needed to carry out the tests are estimated in Table 7.

Table 7. Estimates of fixed and recurrent costs of analysis by the Plant Nitrate Protocol.

Item	Number recommended	Total cost (£)
One-off purchases:		
Balance (weighing to 0.1g with 200g capacity)	1	71
Kitchen blender	1	34
Water bath (max 100^{o}C)*	1	60
Nitrachek 404 meter	1	150
Knife	1	10
Chopping board	1	5
Plastic wash bottle (with jet)	1	10
Measuring cylinder (1 litre)	1	15
Conical flask (1 litre)	4	25
Graduated flask (1 litre)	2	35
Funnel (14 cm diameter, plastic)	2	20
Beaker (50 ml)	10	10
Bottle (100 ml)	10	20
Pippete (25 ml graduated, piston type)	2	45
Sub-total		**510**
Repeat purchases (per 100 tests):		
Merckoquant nitrate test strips	5 tubes	195
Filter paper (Whatman No 2, 185 mm diameter)	1 box	20
Sodium nitrate standard solution (1000 ppm)		60
Nitrate-free water		50
Sub-total		**325**
Overall Total		**835**

* based on the cost of a large deep fat fryer with thermostat controller

The fixed costs for the basic equipment amount to an outlay of about £510, with the recurrent costs of the consumables for the analysis of 100 plant samples of about £325. The latter are dominated by the cost of the test strips. The requirement for numbers of test strips is based on the assumption that six standard solutions will be used for every ten samples measured, with each standard and sample measured three times. This gives a requirement of 48 test strips for every ten samples, or 480 test strips (*ie* just under five tubes) per 100 samples. The purchase cost of the deionised

water is also relatively expensive, but this outlay could be reduced if a supply of nitrate-free water is already available.

On the basis of this cost structure, the additional (recurrent) cost of carrying out one test is £3.25, once the basic equipment has been purchased. Thus the total cost (T) of using the analysis on n plant samples can be calculated from the equation:

$$T = 3.25\ n + 510 \quad (3)$$

If both fixed and additional (recurrent) costs are included in the unit cost (U) of each analysis, the latter will decline with the number of samples analysed:

$$U = T / n \quad (4)$$

Combining equations (3) and (4) gives:

$$U = 3.25 + (510 / n) \quad (5)$$

Table 8 uses equation (5) to show how this unit cost declines with the number of plant samples analysed.

Table 8. Variation in the unit cost of analysis with numbers of plant samples analysed.

Number of samples analysed (n)	Unit cost per sample analysed (U) in £
100	8.35
200	5.80
300	4.95
400	4.53
500	4.27

Furthermore, a rearranged version of equation (5) may also be used to estimate the number of plant samples that would need to be analysed by the Plant Nitrate Protocol to cover the cost of an equivalent number of commercial analyses:

$$n = 510 / (U_c - 3.25) \quad (6)$$

where U_c is the unit cost of an analysis in the commercial laboratory. Thus if the commercial cost per plant sample is £40.00, then a grower or his representative would only need to analyse 14 samples by the Plant Nitrate Protocol to start saving money. At this price for a commercial analysis, using the new Protocol would save £3165 over 100 samples, and £17865 over 500 samples.

TECHNOLOGY TRANSFER

A series of six separate workshops were organised and managed by Stockbridge Technology Centre (STC) to promote and demonstrate the Plant Nitrate Protocol. These were held at five different locations (chosen to span the North, Midlands and South England) on four different dates in November and December 2004. The workshops were designed to appeal to producers or distributors of both glasshouse lettuce and spinach. A total of 58 people attended the workshops, including a representative from the Food Standards Agency, the organisation with overall responsibility for the UK Lettuce Nitrates Monitoring Programme. All those attending were Members or Corporate Members of the HDC, or were affiliated with HDC in various ways.

Every attendee was given a handout containing a brief description of the Background to the Method and why it was needed; a full copy of the Plant Nitrate Protocol; a copy of the Plant Nitrate Calculator Excel file on a floppy disk; a laminated Method Summary Sheet (suitable for reference purposes whilst using the Protocol); and a list of websites for purchasing second-hand equipment. Copies of the handouts on the Background and the Method Summary Sheet are reproduced in Appendices 4 and 5 respectively. A brief report of the workshops (prepared by Julian Davies of STC) is given in Appendix 6.

SUMMARY AND CONCLUSIONS

Studies of the extraction and analysis of nitrate in plant material showed that

- either frozen or unfrozen plant samples can be analysed
- the weight of plant material extracted into 1 litre of water can be varied between 50 and 200g
- the plant material should be blended in water as an integral part of the extraction process
- hot water treatment during extraction of plant material is preferred (but it may be possible to relax this requirement if further evidence becomes available)
- simple filtration of the extract (using filter paper) is essential before analysis
- use of Merckoquant nitrate test strips and the Nitrachek 404 meter are suitable for determining the nitrate concentration in plant extracts provided that:

- the meter is calibrated with a full range of nitrate standards (typically 50 to 500ppm)
- only predominantly green or (at most) pale pink plant material is used *and not that which has a strong red colour*
- the nitrate in the extracts is measured on the same day as the extraction
- adjustments for the small bias (which varies between plant types) are built into the calculation of plant tissue nitrate concentration

A new method based on these conclusions was tested by comparing its performance against that of a standard laboratory procedure (similar to one recommended by the British Standards Institute) on a common set of lettuce and spinach samples. The measurements by the standard method were made independently by a commercial analytical laboratory. The tests showed that the new method was less variable than the standard procedure for the main types of material tested. The results were used to define the scope of the method, and to derive correction factors needed to adjust for the small biases introduced by the new method for the different plant materials analysed.

These results were used to refine the method, and define a Plant Nitrate Protocol and a Plant Nitrate Calculator which together provide instructions on how to extract and measure nitrate in selected types of plant material, and provide assistance in the calculation of its original concentration on a fresh weight basis.

Workshops held to promote and demonstrate the new methods were well attended, and feedback indicated that they were well received.

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(Warwick HRI, Wellesbourne) who carried out the statistical analyses; and Julian Davies (STC, Stockbridge House) who organised and managed the workshops, and provided useful practical suggestions for refining the Plant Nitrate Protocol.

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Appendix 1

Data from the Nitrachek Method and the Standard Laboratory Procedure used for Comparing the Two Methods

Sample no	Sample type	Days after witholding nitrate	Standard procedure	Nitrachek method
1	Green lettuce	0	4959	4029
2	Green lettuce	0	4697	3294
3	Green lettuce	0	4935	3725
4	Green lettuce	0	4945	3596
5	Green lettuce	1	4245	3730
6	Green lettuce	1	4336	3620
7	Green lettuce	1	4292	3602
8	Green lettuce	1	4491	3747
9	Green lettuce	2	3786	3053
10	Green lettuce	2	3899	2794
11	Green lettuce	2	3657	3603
12	Green lettuce	2	3724	3624
13	Green lettuce	3	3324	3109
14	Green lettuce	3	3438	3074
15	Green lettuce	3	3627	3031
16	Green lettuce	3	3953	3452
17	Green lettuce	4	3392	2818
18	Green lettuce	4	4256	3464
19	Green lettuce	4	2754	2942
20	Green lettuce	4	2344	3095
21	Green lettuce	5	3447	2759
22	Green lettuce	5	2998	2914
23	Green lettuce	5	2859	2602
24	Green lettuce	5	2903	3095
25	Green lettuce	6	3651	2016
26	Green lettuce	6	1661	2240
27	Green lettuce	6	2208	2148
28	Green lettuce	6	2651	2386
29	Green lettuce	8	1476	1265
30	Green lettuce	8	2258	1241
31	Green lettuce	8	1562	1104
32	Green lettuce	8	1434	1227
33	Green lettuce	10	753	880
34	Green lettuce	10	756	1074
35	Green lettuce	10	584	771
36	Green lettuce	10	950	851
37	Green lettuce	12	924	750
38	Green lettuce	12	540	646
39	Green lettuce	12	539	595
40	Green lettuce	12	493	524

Sample no	Sample type	Days after witholding nitrate	Standard procedure	Nitrachek method
41	Pink lettuce	0	2613	3519
42	Pink lettuce	0	2696	3546
43	Pink lettuce	1	2109	2647
44	Pink lettuce	1	2174	3259
45	Pink lettuce	2	3161	2359
46	Pink lettuce	2	2027	2440
47	Pink lettuce	3	2253	2051
48	Pink lettuce	3	2425	2484
49	Pink lettuce	4	2955	2046
50	Pink lettuce	4	1782	1783
51	Pink lettuce	5	1706	1138
52	Pink lettuce	5	1714	1785
53	Pink lettuce	6	1518	1207
54	Pink lettuce	6	981	1377
55	Pink lettuce	8	509	469
56	Pink lettuce	8	977	570
57	Pink lettuce	10	219	313
58	Pink lettuce	10	192	323
59	Pink lettuce	12	332	317
60	Pink lettuce	12	309	247
61	Young spinach	0	1073	2540
62	Young spinach	0	1890	3300
63	Young spinach	1	1515	2443
64	Young spinach	1	1958	2206
65	Young spinach	2	1589	1784
66	Young spinach	2	1739	2184
67	Young spinach	3	558	2320
68	Young spinach	3	1839	1733
69	Young spinach	4	1219	1471
70	Young spinach	4	1076	1399
71	Young spinach	5	824	1157
72	Young spinach	5	532	1621
73	Young spinach	6	846	885
74	Young spinach	6	662	765
75	Young spinach	8	132	520
76	Young spinach	8	425	589
77	Young spinach	10	490	236
78	Young spinach	10	203	350
79	Young spinach	12	117	264
80	Young spinach	12	170	236

Sample no	Sample type	Standard procedure	Nitrachek method
81	Red lettuce	979	2078
82	Red lettuce	1830	1976
83	Red lettuce	1483	2224
84	Red lettuce	1430	1367
85	Red lettuce	1401	2245
86	Red lettuce	3679	1932
87	Red lettuce	3373	1927
88	Red lettuce	3345	1638
89	Red lettuce	3378	1955
90	Red lettuce	3406	1980
91	Red lettuce	2768	2031
92	Red lettuce	2122	1882
93	Red cabbage	493	549
94	Red cabbage	621	892
95	Red cabbage	604	748
96	Red cabbage	365	406
97	Red cabbage	400	550
98	Red cabbage	657	789
99	Red cabbage	371	461
100	Red cabbage	361	475

Appendix 2

Plant Nitrate Protocol

The full document is reproduced on the following pages.

Introduction

EC Regulation

European Commission (EC) Regulation No. 194/97 specifies the maximum levels of nitrate in lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea)*. The Regulation came into force in EU on 15 February 1997, and was amended in April 1999 by EC Regulation No. 864/99. The two regulations set maximum nitrate levels in protected lettuce of 4,500ppm fresh weight in winter (harvested between October and March inclusive) and 3,500ppm in summer (harvested between April and September). Corresponding limits for nitrate in spinach are 3,000ppm when harvested in November to March inclusive, or 2,500ppm from April to October. UK growers currently have a derogation provided they can demonstrate that they follow an agreed Code of Practice, but the derogation for protected (and outdoor) lettuce crops will be withdrawn on 1 January 2005, when they must comply with the EU limits. The derogation for spinach remains in place, at least in the short term.

Ensuring compliance

In order to help ensure compliance with the EU Regulations on nitrate concentrations, it is necessary for producers to have regular analyses of nitrate concentrations carried out on lettuce and spinach crops. At present, these tests need to be undertaken at a FAPAS accredited laboratory. The costs of these analyses can be expensive, and it can often take between 7 and 10 days before the results are available.

Development of the Nitrachek® meter

The ability to assess the nitrate content of lettuce and spinach quickly and in a reliable way on grower holdings or produce distribution centres would clearly be very useful to the industry. Procedures for nitrate analysis that could be used in a rapid test were initially evaluated by Nottingham Trent University as part of the LINK project '*Development of a decision support system for nitrogen fertiliser application in soil-grown lettuce crops'* (HDC project PC 88a). This work concluded that the portable Nitrachek® meter has considerable potential for use on industry premises provided a suitable extraction procedure could be developed. *Based on both the fixed costs of the equipment needed and the additional recurrent costs of doing the analyses, it would take only 14 analyses at £40 per sample from a commercial laboratory to recover the outlay for the new method.*

Protocol for nitrate extraction and measurement

Further work has now been completed at Warwick HRI to develop a complete protocol for the extraction and measurement of nitrate using the Nitrachek® meter for lettuce and spinach samples (HDC project PC 218). This rapid test method is suitable for samples of green lettuce and spinach and for lettuce with pale pink leaves. However, it must <u>not</u> be used for any plants with a strong red colour, as the latter can interfere with the analysis and bias the results. This document provides protocols on the sampling, extraction and the measurement of nitrate levels in spinach and lettuce using the Nitrachek® meter, together with a Plant Nitrate Calculator (provided as an Excel file) for converting the meter measurements into tissue nitrate concentrations.

Demonstrating due diligence

This rapid and cost-effective nitrate testing procedure will provide growers with further support to the measures outlined in the Industry Code of Good Practice, and the Crop
Specific Protocol for Protected Lettuce of the Assured Produce Scheme with respect to monitoring nitrate concentrations in salad crops. Although the rapid Nitrachek® meter test will not completely remove all need for fully certified analyses, its adoption should assist growers and suppliers in demonstrating due diligence regarding EU nitrate limits. Regular records of nitrate analysis may also enable growers to link nitrate levels with management practices such as fertiliser application and adjust these accordingly.

Protocol for the Extraction and Analysis of Lettuce and Spinach Samples

1. Overview of the method

The method is suitable for green lettuce and spinach samples. It may also be used satisfactorily with lollo rossa plants which have pale pink flushes on their leaves. *However, it must not be used for any plants with a strong red colour, as this can interfere with the analysis and bias the result.*

A weighed amount of plant tissue is homogenised in water using a blender and is then heat-treated to extract the nitrate. After further dilution, the extracts are reverse filtered and nitrate is measured by the colour developed on nitrate test strips using a Nitrachek 404 meter. The test strips and meter are calibrated with standard solutions of known concentration at the beginning and end of the analysis.

Summary of method

1) Take a representative sample of plant material (between 50 and 200 g) either from an individual plant or from several plants. (Note to conform to EC regulations, a bulk sample from ten plants should be used for each analysis.)

2) Weigh the plant tissue and then homogenise it with between 200 and 350 ml of pure water in a blender.

3) Transfer the homogenised pulp to a 1 litre conical flask and stand it in a boiling water bath for 20 to 30 minutes until its temperature is greater than 70^{o}C.

4) Cool the homogenised pulp to room temperature, transfer it to a 1 litre graduated flask and make it up to the mark with pure water.

5) Shake the flask thoroughly, and reverse filter a sub-sample of the contents in a beaker.

6) Dip the test strips in a series of standard solutions of known nitrate concentration and measure the colour developed using the Nitrachek meter.

7) Dip the test strips into the filtered sample extracts and measure the colour developed using the Nitrachek meter; measure each solution at least twice.

8) Repeat the calibration in step 6.

9) Enter the data into the blank *Template* worksheet in the Excel file*:* ***Plant Nitrate Calculator*** (supplied with this Protocol) to calculate the nitrate concentration in the original plant tissue. The Calculator adjusts the result for differences in the type of plant material analysed.

Units of nitrate concentration

There are different ways of expressing the *same* nitrate concentration in solution or in plant tissue. For instance:

- 1000 mg/l = 1000 µg/ml = 1000 ppm nitrate in solution
- 1000 mg/kg = 1000 µg/g = 1000 ppm nitrate in plant tissue

For simplicity, nitrate concentrations in this protocol are expressed as ppm for both.

2. What you need to carry out the analysis

Equipment

- Fridge to store nitrate standards and test strips
- Balance (weighing to 0.1 g with maximum capacity of at least 200 g)
- Kitchen blender (1 litre volume)
- Water bath (maximum temperature 100 ^{o}C)
- Nitrachek 404 meter

Laboratory apparatus

- Knife and chopping board or plastic tray
- Plastic wash bottle (with jet) for pure water
- Two 25 ml graduated pipettes (piston type, with 0.1 ml increments starting at 1 ml; these are available with the piston moulded to the pipette body)
- Measuring cylinder (500 ml or 1 litre)
- Graduated flask (1 litre) - at least one; preferably one for each tissue analysis
- Conical flask (1 litre) - one for each tissue analysis
- Plastic funnel (conical) – about 14 cm in diameter to fit graduated and conical flasks
- Thermometer (0-100 ^{o}C, mercury-free) - optional
- Beakers (50 or 100 ml) – one for each tissue analysis
- Bottles (50 or 100 ml, screw topped) – one for each standard

Disposables

- Filter paper (1 box of Whatman No. 1 or No. 2 grade, diameter approx 185 mm; preferably fluted or ready-folded)
- Merckoquant nitrate test strips
- Tissues or paper towels for drying apparatus -optional

Reagents

- Stock nitrate standard (sodium nitrate solution ***in water*** containing 1000 ppm of NO_3); see note on units of nitrate concentration in the section above)
- Pure water (distilled, de-ionised or equivalent) - ***not tap water***

3. Good Analytical Practice

Keep all standards, and the nitrate test strips in a refrigerator at all times, except on the day of use.

Always use clean apparatus for the extraction and analysis. Between samples, ensure all apparatus is rinsed twice in pure water *after* being washed thoroughly in tap water. All but the graduated flask and the pipettes should also be thoroughly dried (preferably in an oven) and kept under clean conditions between use. Do not use any apparatus while still warm.

The nitrate standard used in this protocol is not classed as toxic for Health and Safety purposes, but (as with all chemicals) it should be treated with care. Waste solutions may be flushed down sinks with excess tap water after use.

Note – if in doubt use *pure water* in the procedures below, unless tap water is specifically mentioned.

4. Details of the extraction and analysis procedure

Important: This method must not be used for any plants with a strong red colour, as the latter can interfere with the analysis and bias the results.

Preparation and storage of the samples

Make sure all plant material is clean. If necessary, wash off any contaminating soil with tap water first and shake or dab the plant material dry. Prepare a sample of plant material (between 50 and 200 g) for nitrate analysis using the following guidelines, which assume an average plant weight of about 300 g.

To analyse plants individually, slice each into quarters along the stem, and use two opposite quarters for the extraction and analysis. To analyse a bulk sample of ten plants, choose one of the quarters at random, slice it again into four approximately equal sections (also along the stem) and select all leaf and stem material from one of the sections. Repeat for each plant in turn, combining all selected sections into a single bulk sample. Make sure each sample or section is representative of all parts of the plant from which it was taken, because stems, inner leaves and outer leaves can have very different nitrate concentrations.

If the sample is not to be analysed immediately, transfer it to a polythene bag and store it either in a refrigerator (for up to 24 hours), or in a freezer (for longer periods) until required.

Before starting the extraction and analysis procedure

- Switch on the hot water bath so it is up to temperature when needed.
- Remove the nitrate test strips and the diluted nitrate standards from the refrigerator and allow them to warm to room temperature before use.

Extraction of the samples

Chop up the plant material coarsely on a chopping board and record its total weight to the nearest 0.1 g. Check this is between 50 and 200 g.

Add *all* of the chopped plant sample to the blender in about 50 g batches. Using the measuring cylinder, add about 200 ml of pure water to the first batch of plant material, and initially switch on the blender in short bursts to break up any lumps. *Make sure the blender lid is always tightly fitted before switching it on to avoid losing any sample.* Gradually add further batches of the sample and up to 150 ml more pure water as the lumps are broken down. Be especially careful not to spill any sample when removing the lid, which can accumulate both liquid and bits of plant tissue.

Once all of the large lumps in the whole sample have been broken up, leave the blender on for a full minute, rocking it gently during operation. Remove the lid carefully and rinse down the lid and sides of the blender with *a minimum amount* of pure water from the plastic wash bottle. Replace the lid and run the blender continuously for another minute, before visually checking that the pulp is fully homogenized. Should any sample or solution be accidentally lost during any part of the extraction procedure, abandon the process and start the extraction again with a new sample.

Carefully pour all of the liquid pulp into a 1 litre conical flask, and rinse with *a minimum amount* of pure water to transfer the last of the pulp. The final volume should be no more than about 800ml.
Place the conical flask securely in a hot water bath with the water level in the latter about half way up the level of liquid inside the flask. Note that the bath must be close to boiling, but not necessarily at 100°C. Do use a hotplate to heat the flask, it can denature the plant tissue. Leave the flask in the hot bath for 20 to 30 minutes, swirling the flask every 10 minutes to equalise the temperature of the contents. Check that the final temperature of the liquid pulp is at least 70 °C. Remove the flask, cover it loosely to prevent contamination, and sit it in cold water (*eg* in a sink) to cool it to room temperature as quickly as possible (1 to 2 hours). Take care that the flask does not overturn whilst cooling.

When cool, carefully transfer *all* of the liquid pulp to a 1 litre graduated flask through a funnel. Add the solution slowly tilting the flask if necessary to minimise frothing. Use additional pure rinse water as needed to help transfer all of the solution, then make up to the mark with more pure water. Shake the flask thoroughly and immediately pour about 30 ml of the liquid pulp into a clean dry beaker. Open up a fluted or folded filter paper to create a cone shape and insert it in the beaker point downwards to allow clear liquid to pass from the outer to the inner area by reverse filtration. Note that the final solution may be lightly coloured, but should not be unduly cloudy. Once the sample extract is filtering, the graduated flask and funnel may be washed and reused for the next extract.

Keep the beakers with the filtered extracts at room temperature (avoiding direct sunlight) until analysis, which should be on the same day as it was extracted. If you do not filter the extracts the colour of the pulp may interfere with the analysis. Debris from the pulp may also adhere to the colour sensitive zones on the nitrate test strips, contaminating the Nitrachek meter during measurement.

Preparation of the diluted standard nitrate solutions

Prepare a series of *at least five* standards of different nitrate concentration using graduated pipettes to dilute the stock 1000 ppm nitrate standard solution with pure water. Alternatively use the balance to weigh out the equivalent amounts (to 0.01 g) of the stock standard and water. If pipetting, use one pipette to measure out all volumes of stock solution, and a separate one to measure out the water. Make sure the stock solution is at room temperature before use. Mix the diluted standards thoroughly and store in labelled screw topped bottles. Keep these in a refrigerator when not required for analysis, and make up a completely fresh batch every week. Make sure the sample of stock solution is at room temperature before use.
Choose the standards so that they span the concentration range up to 500 ppm of nitrate reasonably evenly. The volumes required for a range of typical standards are given in the table below. Standards of higher nitrate concentration can be used, but may exceed the range of the Nitrachek meter.

Nitrate concentration of diluted standard (ppm)	Volume or weight of stock solution required (ml or g)	Volume or weight of pure water required (ml or g)
50	1.0	19.0
100	2.0	18.0
200	4.0	16.0
300	6.0	14.0

400	8.0	12.0
500	10.0	10.0

Analysing the extracts for nitrate

This must be completed on the day that the tissue samples are extracted, using the Merckoquant Nitrate Test Strips and the Nitrachek 404 meter. See below for instructions for use of the latter. Make sure all test strips used each day have the same Batch No. and Expiry Date.

Carry out the measurements in the following order. First measure each of the standards once in ascending order of concentration, recording the colour reading from the meter to provide initial calibration data. Then make at least two separate measurements on each of the filtered solutions (from the inside area of the filter paper cones) for the extracted plant tissue. Finally repeat the measurements on the standards once more to complete the calibration.

Use the *Template* worksheet of the ***Plant Nitrate Calculator*** Excel file to calculate the plant nitrate concentrations, as described below. This automatically adjusts the results for any difference in bias introduced by the lettuce, lollo rossa or spinach tissues.

Examine the mean calibration graph that is automatically plotted on the worksheet and check that the data points fall close to the fitted curve before discarding any solutions or standards. Make additional measurements on any standards or sample solutions if their original two replicate measurements differ significantly, or if the calibration curve is poor.

Once the data has been entered, make sure each analysis result is satisfactory by checking that the message 'within range' is printed alongside it in the worksheet. If the comment 'out of range' appears instead, the result is unreliable because the sample extract gave a mean colour reading above that for the top standard. Should this occur, use the graduated pipettes to dilute 10ml of the extract with 10 ml (or, if necessary, 20 ml) of pure water and mix thoroughly to bring its reading within range. Enter the data for any diluted extract in the bottom table of the *Template* worksheet in the ***Plant Nitrate Calculator*** to get a more reliable result.

The analysis is most accurate where the colour readings fall between about 100 and 400 on the meter. If the method regularly gives colour readings which are closer to that for the top standard, or produce the 'out of range' comment in the ***Plant Nitrate Calculator***, try using a smaller weight of sample for the extraction to improve accuracy.

Using the Nitrachek 404 meter

Switch on the meter by flipping open the hatch to expose the slot where the test strips are inserted. When the display shows *CAL,* insert an unused dry strip vertically, with the colour-sensitive zones facing the body of the meter, and close the hatch. (The meter then monitors any existing background colouration on the strip).

When the display shows *GO,* open the hatch, remove the strip and quickly transfer it to the standard or sample solution, fully immersing both colour-sensitive zones for 1

to 2 seconds. Shake off any excess moisture and wait while the timer counts down from 60 seconds.

Re-insert the strip in the same position in the meter when the latter starts to bleep (with about 3 seconds left) and wait until the meter displays a colour reading. Any delay in inserting the strip will result in the meter display showing *Shut*. Do not delay inserting the strip for too long if this occurs, as this will bias the result.

Record the colour reading displayed on the meter (ignoring any units given with it), and repeat the whole process for the next standard or sample solution. Note that, because of variability between individual test strips, duplicate measurements on the same solution may not give exactly the same colour reading. Colour readings above the maximum of the meter will be displayed as *HI*; these should be diluted and reanalysed. Test strips showing very pale colours may be displayed by the meter as *LO*; these can be considered as equal to 0 (zero).

If *both* colour sensitive zones on the test strips turn pink or violet, then the measured nitrate concentration will be unreliable and *should not be used*. This may occur if a sample is extracted but not analysed on the same day, or if the extracted samples have a strong red colour.

For further information on the Nitrachek meter, consult its Operating Manual.

Using the Plant Nitrate Calculator Excel file

Before using the Excel file for the first time, take care to make a secure copy of the original in case you need to reinstall it. The file may be copied to any destination folder you choose, but will only run successfully if you have Excel installed on your computer.

See below for details on how to use the ***Plant Nitrate Calculator*** Excel file for calculating the results. The file consists of three separate worksheets:

- an *Instructions* worksheet with additional guidelines on completing the template
- a blank *Template* for entering new data
- an *Example* worksheet to show how the template should appear after completion

To enter new data, select the *Template* worksheet of the ***Plant Nitrate Calculator***. You may enter data in any of the pale yellow areas of the tables on this worksheet; do not attempt to enter values in the other areas (these are protected). Note that you should type the data values into the tables directly from the keyboard. If you make a mistake during data entry, delete the erroneous value and re-enter it correctly. Do not copy and paste values into the tables from elsewhere in this or other worksheets, because you may inadvertently alter some of the equations and create errors which may not be immediately obvious.

For reference, record the date of the analysis, the name of the analyst, and the Batch No of the test strips and their Expiry Date (given on their box or container) at the top of the template.

Enter the concentrations and colour readings for the nitrate standards in ascending order of concentration in the table entitled *Calibration data for different nitrate standards*. Use a new line for each standard up to a maximum of ten. There is space for up to five separate colour readings for each standard if required. If you have less than five colour readings for any standard, leave the other cells empty (do not enter zeros).

Check the curve of the calibration graph on the right of the worksheet to make sure it fits closely to the data points. The curve will normally reduce in slope slightly at higher concentrations. If it is a good fit, enter the parameters for x^2 and x (from the equation printed on the curve) into the small table immediately to the left of the graph.

Then enter the required values in the table of *Sample Data for different plant extracts,* using a new line for each solution (up to a maximum of twenty). This includes the sample type (selected from a drop-down menu), your sample number, the weight of plant material used, and the associated colour readings. Again, there is space for up to five separate colour readings for each solution. If you have less than five colour readings for any solution, leave the other cells empty.

The mean nitrate concentration in each extract solution and in the original plant sample (on a fresh weight basis) is then calculated automatically. Check the comment alongside each analysis result to make sure it reads ‘within range’. If it reads ‘out of range’, the result is likely to be unreliable, and the extract solution should be diluted and reanalysed (see above).

Enter details for any sample that is reanalysed after dilution in the bottom table entitled *Repeat analyses on 'out of range' samples after further dilution of the extract*. This includes its original sample number and plant weight, together with the volumes of extract and water used for the dilution, and the new colour readings. The table recalculates the nitrate concentration in the diluted extract and in the original plant sample, automatically adjusting the latter for the dilution factor. If you have carried out the dilution carefully, then this value is likely to be more accurate than the original one.

Once complete, use the 'File/Save As' commands to rename the workbook and transfer it to your chosen folder.

5. Sourcing the equipment, apparatus and reagents

Most of the equipment, laboratory apparatus, disposables and reagents can be purchased from standard Laboratory Suppliers (or their Distributers) such as:

VWR International Ltd
Hunter Boulevard
Magna Park
Lutterworth
Leics LE17 4XN
(Tel: 0800 22 33 44)

or

Fisher Scientific UK
Bishop Meadow Road
Loughborough
Leics LE11 5RG
(Tel: 01509 231166)

The nitrate test strips can also be purchased through VWR International.

The Nitrachek 404 Meter can be purchased directly from:

KPG Products Ltd
34 St Keyna Avenue
Hove
East Sussex BN3 4PP
(Tel: 01273 708796)

6. Need more help?

For further help or advice, contact Julian Davies at STC (Tel: 01757 268275).

Prepared by Ian Burns
Warwick HRI

Appendix 3

Plant Nitrate Calculator

(printout of electronic Excel file provided on floppy disc)

This Excel workbook file consists of three separate worksheets:

1. Instructions
2. Template (currently empty, but used for entering new data)
3. Example

These are reproduced on the following three pages.

Instructions for the use of the Plant Nitrate Concentration Worksheet Template (to be used in conjunction with the Plant Nitrate Protocol)

Before attempting to use this template, save it somewhere securely in case you need to re-install it subsequently.

Select the ***Template*** worksheet by clicking on the tab below and enter your data directly from the keyboard.
Note - Areas of the worksheet where you can enter data are highlighted in pale yellow.
Do not attempt to cut/copy and paste data from other parts of this or other worksheets because this may change the embedded equations.

If necessary, check the ***Example*** worksheet to find out how the blank template should appear after entering new data.

1 Enter the date of analysis, the name of the analyst and the details of the test strips (given on their container) in cells C3 to C6, for reference purposes.

2 Enter the concentration of the lowest nitrate standard used in cell A13 and the corresponding colour readings (up to a maximum of five) in cells B13 to F13, with the first reading in B13. *Leave any unused cells empty.* The mean colour reading is then displayed automatically in cell G13.

3 Repeat step 2 for each of the standards in turn (in increasing order of concentration) on the following lines of the table. Avoid leaving any blank lines. Use as many lines as necessary, up to the maximum of ten available. The curve on the calibration graph will update automatically as the data are entered.

4 Check that the curve on the calibration graph fits closely to the experimental data points. If it does not, you may need to make additional colour readings on any errant standards. Expect the R^2 value given on the curve to be between 0.99 and 1.0.

5 When you are satisfied that the calibration curve is a good fit
- accurately transfer the parameter value immediately in front of **x^2** in the equation on the calibration curve into **cell K13** (including the decimal point, all of its numbers and any minus sign)
- accurately transfer the parameter value immediately in front of **x** in the equation on the calibration curve into **cell K14** (including the decimal point, all of its numbers and any minus sign)

6 Enter the data for the first plant sample in line 30 as follows:
- sample type in cell A30, selecting from the drop-down menu
- your sample number in cell B30
- the weight of plant tissue used for the extraction in cell C30
- each of the colour readings (up to a maximum of five) for this extract in cells D30 to H30, starting in D30

Leave any unused cells empty. The mean colour reading is then displayed automatically in cell I30.

7 The values for the nitrate concentration in both the extract solutions and the plant tissue are calculated automatically in cells J30 and K30 respectively. Check cell L30 to make sure each analysis result is 'within range'.

8 Repeat steps 6 and 7 for each of the subsequent plant extracts on the following lines of the table. Use as many lines as necessary, up to the maximum of the twenty available.

9 If any of the results are 'out of range', consider diluting and re-measuring its extract as described in the Protocol to obtain a more accurate result. Once the measurements have been made, enter the original sample type, sample number and plant weight, together with the volumes of original extract and water used in the dilution into cells A57 to E57. Then enter the new colour readings on the diluted solutions (up to a maximum of five) in cells F57 to J57. The mean colour reading and the nitrate concentration in the diluted extract are given in cells K57 and L57. The recalculated nitrate concentration in the original plant tissue is then displayed automatically in cell M57 after adjustment for the dilution factor.

10 Enter data for any other 'out of range' extracts on the following lines of the table. There are enough lines for the re-analysis of ten diluted samples. Check column N to make sure each new result is now 'within range'. If not, you will either need to further dilute the *original* extract with a larger volume of water.

11 Use the '**File/Save As'** commands to rename the workbook and transfer it to your chosen folder.

Units:
for nitrate concentration in solution: 1000 ppm = 1000 mg/l = 1000µg/ml

Template for Calculating Nitrate Concentration in Plant Tissue

Date of analysis:	
Name of analyst:	
Test strip batch no:	
Expiry date:	

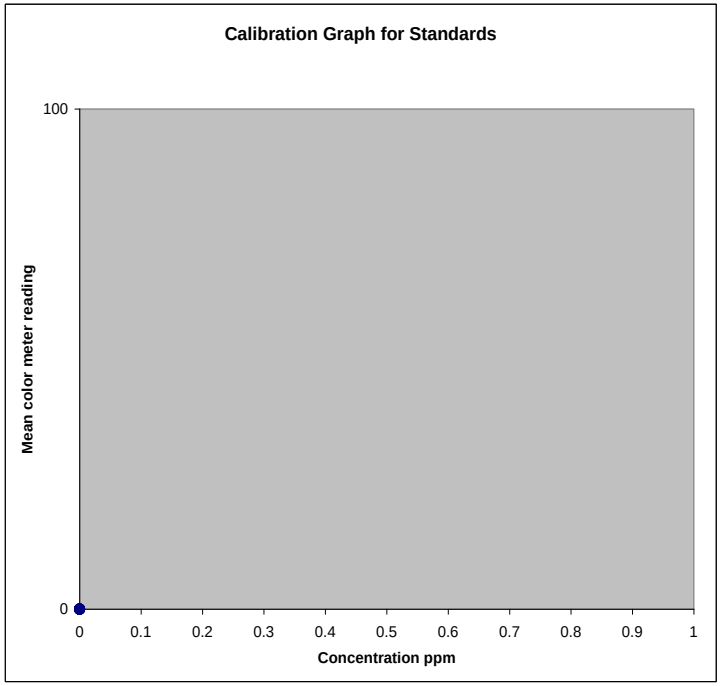
Calibration data for different nitrate standards:

Nitrate concn (ppm)	Colour reading 1	Colour reading 2	Colour reading 3	Colour reading 4	Colour reading 5	Mean colour

Parameters from calibration equation:

for x^2:	
for x:	

(include any 'minus' signs in the above values)



Sample data for different plant extracts:

Sample type	Sample number	Plant weight (g)	Colour reading 1	Colour reading 2	Colour reading 3	Colour reading 4	Colour reading 5	Mean colour	Nitrate concn in extract (ppm)	Nitrate concn in plant (ppm FW)	Comment on analysis result

Dilute and repeat any samples which are 'out of range'.

Repeat analyses on 'out of range' samples after further dilution of the extract

Sample type	Sample number	Plant weight (g)	Volume of extract (ml)	Volume of water (ml)	Colour reading 1	Colour reading 2	Colour reading 3	Colour reading 4	Colour reading 5	Mean colour	Nitrate concn in dil. extract (ppm)	Corrected plant nitrate concn (ppm FW)	Comment about analysis result

Signed: _____

Date: _____

Template for Calculating Nitrate Concentration in Plant Tissue

Date of analysis:	13-Aug-04
Name of analyst:	Ian Burns
Test strip batch no:	OC332590
Expiry date:	Jul-06

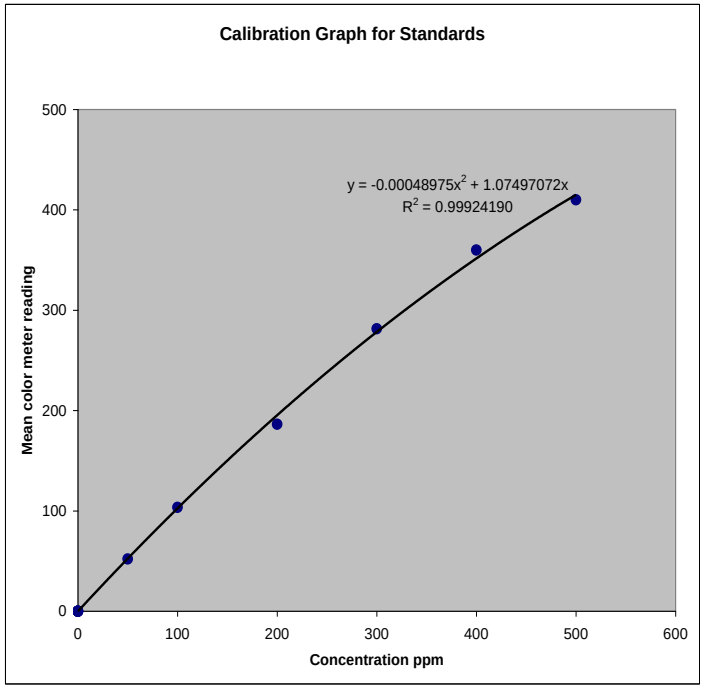
Calibration data for different nitrate standards:

Nitrate concn (ppm)	Colour reading 1	Colour reading 2	Colour reading 3	Colour reading 4	Colour reading 5	Mean colour
50	50	54				52.0
100	98	109				103.5
200	158	212	189			186.3
300	289	274				281.5
400	365	355				360.0
500	400	420				410.0

Parameters from calibration equation:

for x^2:	-0.00048975
for x:	1.0749707

(include any 'minus' signs in the above values)



Sample data for different plant extracts:

Sample type	Sample number	Plant weight (g)	Colour reading 1	Colour reading 2	Colour reading 3	Colour reading 4	Colour reading 5	Mean colour	Nitrate concn in extract (ppm)	Nitrate concn in plant (ppm FW)	Comment on analysis result
green lettuce	1005	79.4	302	300				301.0	377.3	4751	within range
green lettuce	1006	58.1	231	196	168			198.3	232.8	4008	within range
green lettuce	1007	79.5	280	288				284.0	351.8	4425	within range
lollo rossa (pale pink)	1008	78.9	271	278				274.5	279.1	3537	within range
lollo rossa (pale pink)	1009	79.8	290	280				285.0	291.8	3657	within range
lollo rossa (pale pink)	1010	82.2	288	282				285.0	291.8	3550	within range
green spinach	1011	85.7	297	284	298			293.0	273.6	3192	within range
green spinach	1012	78.8	284	273	292			283.0	262.4	3330	within range
green spinach	1013	88.1	244	261	284			263.0	240.6	2732	within range
green spinach	1014	110.9	325	271	286			294.0	274.7	2477	within range
green lettuce	1015	125.4	406	420				413.0	568.6	4534	***out of range***
green lettuce	1016	93.7	382	336	350			356.0	465.4	4967	within range
green lettuce	1017	184.9	451	442				446.5	637.1	3446	***out of range***
lollo rossa (pale pink)	1018	187.5	488	463				475.5	581.1	3099	***out of range***
green lettuce	1019	173.4	356	320	371	345		348.0	452.0	2607	within range

Dilute and repeat any samples which are 'out of range'.

Repeat analyses on 'out of range' samples after further dilution of the extract

Sample type	Sample number	Plant weight (g)	Volume of extract (ml)	Volume of water (ml)	Colour reading 1	Colour reading 2	Colour reading 3	Colour reading 4	Colour reading 5	Mean colour	Nitrate concn in dil. extract (ppm)	Corrected plant nitrate concn (ppm FW)	Comment about analysis result
green lettuce	1015	125.4	10	10	248	240	258			248.7	300.9	4799	within range
green lettuce	1017	184.9	10	10	280	273				276.5	340.7	3686	within range
lollo rossa (pale pink)	1018	187.5	10	10	300	272	313			295.0	304.2	3245	within range

Signed: _____

Date: _____

Appendix 4

Introduction and Background to the Workshops

European Commission (EC) Regulation No. 194/97 specifies the maximum levels of nitrate in lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea)*. The Regulation came into force in EU on 15 February 1997, and was amended in April 1999 by EC Regulation No. 864/99. The two regulations set maximum nitrate levels in protected lettuce of 4,500ppm fresh weight in winter (harvested between October and March inclusive) and 3,500ppm in summer (harvested between April and September). Corresponding limits for nitrate in spinach are 3,000ppm when harvested in November to March inclusive, or 2,500ppm from April to October. UK growers currently have a derogation provided they can demonstrate that they follow an agreed Code of Practice, but the derogation for protected (and outdoor) lettuce crops will be withdrawn on 1 January 2005, when they must comply with the EU limits. The derogation for spinach remains in place, at least in the short term.

In order to help ensure compliance with the EU Regulations on nitrate concentrations, it is necessary for producers to have regular analyses of nitrate concentrations carried out on lettuce and spinach crops. At present, these tests need to be undertaken at a FAPAS accredited laboratory. The costs of these analyses can be expensive, and it can often take between 7 and 10 days before the results are available.

The ability to assess the nitrate content of lettuce and spinach quickly and in a reliable way on grower holdings or produce distribution centres would clearly be very useful to the industry. Procedures for nitrate analysis that could be used in a rapid test were initially evaluated by Nottingham Trent University as part of the LINK project '*Development of a decision support system for nitrogen fertiliser application in soil-grown lettuce crops'* (HDC project PC 88a). This work concluded that the portable Nitrachek® meter has considerable potential for use on industry premises provided a suitable extraction procedure could be developed. Based on both the fixed costs of the equipment needed and the additional recurrent costs of doing the analyses, it would take only 14 analyses at £40 per sample from a commercial laboratory to recover the outlay for the new method.

Further work has now been completed at Warwick HRI to develop a complete protocol for the extraction and measurement of nitrate using the Nitrachek® meter for lettuce and spinach samples (HDC project PC 218). This rapid test method is suitable for samples of green lettuce and spinach and for lettuce with pale pink leaves. However, it must <u>not</u> be used for any plants with a strong red colour, as the latter can interfere with the analysis and bias the results. This document provides protocols on the sampling, extraction and the measurement of nitrate levels in spinach and lettuce using the Nitrachek® meter, together with a Plant Nitrate Calculator (provided as an Excel file) for converting the meter measurements into tissue nitrate concentrations.

This rapid and cost-effective nitrate testing procedure will provide growers with further support to the measures outlined in the Industry Code of Good Practice, and the Crop Specific Protocol for Protected Lettuce of the Assured Produce Scheme with respect to monitoring nitrate concentrations in salad crops. Although the rapid Nitrachek® meter test will not completely remove all need for fully certified analyses,

its adoption should assist growers and suppliers in demonstrating due diligence regarding EU nitrate limits. Regular records of nitrate analysis may also enable growers to link nitrate levels with management practices such as fertiliser application and adjust these accordingly.

Appendix 5

Summary of Procedure for the Extraction and Analysis of Lettuce and Spinach Samples

(a laminated two-sided reference sheet for reference for use during analysis)

***Important*:** This method must not be used for any plants with a strong red colour, as the latter can interfere with the analysis and bias the results.

Remove Nitrate test strips and standard nitrate solutions from fridge to allow them to get to room temperature. Switch on the water bath to minimise time delays latter on.

10) Take a representative sample of plant material (between 50 and 200 g) either from a trimmed individual plant or from several plants. If the sample is not to be analysed immediately, transfer it to a polythene bag and store it either in a fridge for up to 24 hours, or in a freezer for longer periods until required. (Note to conform to EC regulations, a bulk sample from ten plants should be used for each analysis). Record weight of plant material to the nearest 0.1 g.

11) Weigh the plant tissue and then homogenise it with between 200 and 350 ml of **pure** water in a blender. Add the sample and water in stages as the lumps of tissue are broken down. **Do not use too much water.** Be especially careful not to spill any sample when removing the lid, which can accumulate both liquid and bits of plant tissue. ***Make sure the blender lid is always tightly fitted before switching it on to avoid losing any sample.***

12) Once all of the large lumps in the whole sample have been broken up, leave the blender on for a full minute, rocking it gently during operation. Remove the lid carefully and rinse down the lid and sides of the blender with *a minimum amount* of pure water from the plastic wash bottle. Replace the lid and run the blender continuously for another minute, before visually checking that the pulp is fully homogenized. Should any sample or solution be accidentally lost during any part of the extraction procedure, **abandon** the process and start the extraction again with a new sample.

13) Transfer the homogenised pulp plus **all residues** to a 1 litre conical flask. The final volume should be no more than about 800ml. Stand it in a boiling water bath for 20 to 30 minutes until its temperature is greater than 70^{o}C.

14) Cool the homogenised pulp to room temperature under a tap (avoid any water entering into the flask) or in a sink of cold water. Transfer it to a 1 litre graduated flask using a funnel - slowly tilting the flask if necessary to minimise frothing. Use additional **pure** rinse water as needed to help transfer all of the solution and make it up to the mark with **pure** water.

15) Shake the flask thoroughly. Pour some out into a 100ml beaker and put in a folded filter paper to obtain a pulp free solution inside. Note that the final solution may be lightly coloured, but should not be unduly cloudy.

16) Dip the test strips in a series of standard solutions of known nitrate concentration **(see over page for making standard solutions)** and measure the colour developed using the Nitrachek meter. Make sure all test strips used each day have the same Batch No. and / or Expiry Date. **See over page for instructions to use Nitrachek meter**.

17) Dip the test strips into the filtered sample extracts and measure the colour developed using the Nitrachek meter; measure each solution at least twice. Record the readings.

18) Repeat Step 8 for testing subsequent lettuce samples.

19) After testing the final lettuce sample repeat Step 7 as a final calibration.

20) Using the *Plant Nitrate Calculator* Excel file, enter the concentrations and colour readings for the nitrate standards in ascending order of concentration in the table on the blank *Template* worksheet entitled *Calibration data for different nitrate standards*. Use a new line for each standard up to a maximum of ten.

21) Check the curve of the calibration graph on the right of the worksheet to make sure it fits closely to the data points. If it is a good fit, enter the parameters for x^2 and x from the equation printed on the curve to the small table immediately to the left of the graph.

22) Enter the data into the *Sample data for different plant extracts* table in the *Template* worksheet of the ***Plant Nitrate Calculator*** to calculate the nitrate concentration in the original plant tissue. The Calculator adjusts the result for differences in the type of plant material analysed.

Preparation of the standard nitrate solutions

- Prepare a series of *at least five* standards of different nitrate concentration fresh each week using graduated pipettes to dilute the stock 1000ppm nitrate standard solution with **pure** water.
- Make sure the sample of stock solution is at room temperature before use. Use one pipette to measure out all volumes of stock solution, and a separate one to measure out the water. Alternatively carefully weigh out the required weight of stock 1000ppm nitrate solution and **pure** water – use a 0.01g balance. See table below.
- Shake the diluted standards thoroughly and make sure they are at room temperature before use. Keep each diluted solution in a srew top bottle and avoid contamination by keeping out of direct sunlight, store in fridge when not in use and with the lid always on. It is recommended to replace the diluted standards every 7-10 days.

Standard concentration (ppm of nitrate)	Volume/weight of stock solution required (ml or g)	Volume/weight of pure water required (ml or g)
50	1.0	19.0
100	2.0	18.0
200	4.0	16.0
300	6.0	14.0
400	8.0	12.0
500	10.0	10.0

Using the Nitrachek 404 meter

- Switch on the meter by flipping open the hatch to expose the slot where the test strips are inserted. When the display shows *CAL,* insert an unused dry strip vertically, with the colour-sensitive zones facing the body of the meter, and close the hatch.
- When the display shows *GO,* open the hatch, remove the strip and quickly transfer it to the standard (lowest concentration first) or sample solution, fully immersing both colour-sensitive zones for 1 to 2 seconds. Shake off any excess moisture and wait while the timer counts down from 60 seconds.
- Re-insert the strip in the same position in the meter when the latter starts to bleep (with about 3 seconds left) and wait until the meter displays a colour reading.
- Record the colour reading displayed on the meter and repeat the whole process for the next standard or sample solution.

For further help or advice, contact Julian Davies at STC (Tel: 01757 268275).

Prepared by:

Julian Davies
STC Ltd

Ian Burns
Warwick HRI

Appendix 6

Report on the Lettuce Nitrate Workshops

A series of workshops were run by Julian Davies (STC Ltd.) in different locations around the country to demonstrate the new test developed by Ian Burns and colleagues at Warwick HRI.

Planning and organisation

Workshops were held at the following venues:

1. Stockbridge Technology Centre, Yorkshire – mainly attended by Lancashire and Lincolnshire growers
2. Snaith Salad Growers, East Yorkshire – Yorkshire glasshouse and outdoor growers
3. Pershore College, Worcestershire – Evesham growers
4. Harlow, Essex – Lea Valley, Cambridgeshire and Kent growers
5. Chichester, West Sussex – South coast and Kent growers

Attendance was good with a total of people attending including growers, technologists, independent consultants, QA staff, FSA staff and seed company technical representatives. The events were registered with NRoSO so that growers could obtain CPD points.

Venue	Date	Time of day	Number of people
STC	30 November	Morning	13
Snaith	30 November	Afternoon	8
Pershore	1 December	Morning	6
Harlow	8 December	Morning	9
Chichester	9 December	Morning	13
Chichester	9 December	Afternoon	9

At each demonstration the background of the project was explained and then the technique gone through in detail. Each attendee was given a handout, a laminated summary sheet plus a sheet with some web sites for getting second hand equipment.

Three samples were prepared with growers invited to volunteer to get ‘hands on experience’ after the preparation of the first sample. This went down very well and showed that although the technique might appear quite daunting it was simple to follow.

There was good interaction and also discussions on when the test should be carried out. A common approach would be to sample 10 days before expected harvest date and also a few days closer to harvest. The first sample would identify if the crops were at risk of exceeding the limit and would give growers a quick result (1.5 hours) which could be used to decide harvesting options. It was considered that if the result was below the limit at this early sample date then it was likely to remain low unless late fertiliser application was made. If high then a second sample closer to harvest

should indicate the extent of any reduction with harvesting staff informed on how to trim the heads.

The calibration of the Nitrachek meter was discussed in detail ending with the inputting of the data into the Excell spreadsheet.

Feedback from the participants

Overall they were positive as they could see that the test could help them gain a better understanding of how crops take up the nitrate. The result would be available within 1-2 hours at a fraction of the cost of sending to a commercial laboratory. They were put off by having to buy the equipment – they were advised that it would be about £800 to set up for doing 12 samples.

There were no obvious problems with the technique but they were reminded to pour the liquid at an angle to avoid getting a 'frothy head'. Could there be potential to use a 1 litre measuring cylinder? Water volumes were always well below the 1 litre during sample preparation and washing out.

All of the butterhead samples were 'outside range' and dilution was required. It was recommended that they dilute based on weight and the accuracy of this compared to using a pipette needs to be checked as the liquid might have a different specific gravity to pure water.

The potential shelf life of the 5 stock solutions when stored in a fridge needs to be checked as this would save time if they only had to make these up every month.

Overall comments

- Growers felt it was easy to use
- They liked the laminated summary sheet
- They appreciated being shown the technique at a venue close to them - except Lancashire growers who would have preferred one in the North West
- They appreciated the telephone contact details of STC to help them if any problems
- They would write additional comments on the print out to provide information to use in following seasons

I did suggest that an email user group might be set up at STC so that if there are any problems then all the companies attending the workshops would be kept informed.

Julian Davies
STC Ltd
10 December 2004

