A Comparison of the Extraction Methods used in the UK Nitrate Residues Monitoring Program

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Summary

It has been reported that significantly different nitrate levels may be obtained from the same crop at the same harvest when analysed by different laboratories. These differences may be due to the methods of analysis used by the laboratories, and in particular the extraction procedures that may be applied to extract nitrates from the crops. In this project a suitable homogenisation procedure for the preparation of samples was developed for preparation of samples to reduce this variability. Several nitrate extraction methods, including that described in BS EN 12014-2:1997, were evaluated. The BS method was subjected to a robustness test and then a collaborative trial involving 9 laboratories. Of the extraction methods compared, the hot water extraction method (BS EN 12014-2:1997) was found to produce the most reliable results. Cold water extraction of nitrate from lettuce and spinach samples was found to give significantly low, and variable, recoveries of nitrate when the sample had not been previously frozen. Laboratories should therefore adopt a hot water extraction method.

Introduction

Commission Regulation No. 466/2001 [1], as amended by Regulation 563/2002 [2], requires that Member States monitor nitrate levels in lettuce and spinach and report results to the EC. The UK Code of Good Agricultural Practice for the production of lettuce and spinach in minimising nitrate residues specifically makes provision for enforcement of Article 2.2 of these Regulations [3].

Nitrate concentrations in both UK-produced and imported lettuce and spinach occasionally exceed the maximum levels specified by the Regulation, as shown in recent surveys undertaken by the UK Food Standards Agency [4-6]. It is important that data obtained in the course of monitoring residues in the harvested crop are accurate and reliable in order to inform the EU and to protect the consumer against excessive dietary exposure.

The EC has produced guidelines for laboratories carrying out determination of nitrate in lettuce and spinach[7]. These do not specify particular analytical methods but set criteria for analytical performance (including recovery and precision) that should be met by all methods used. It has been reported that significantly different results may be obtained from the same crop at the same harvest when analysed by different laboratories. One cause of this may be that different extraction procedures have been used. Burns has reported [8] that extraction with cold water can lead to variable recoveries of nitrate. Inconsistencies may therefore have been due to the extraction procedure applied.

Colorimetric analysis of nitrate has been applied generally to foods, e.g. the BS method for the analysis of nitrate in meat [9] which uses hot borax as the extractant. A number of researchers have applied colorimetry specifically to the analysis of vegetables [10, 11]. The extraction procedures involved hot water followed by de-proteinisation using Carrez solutions. An automated colorimetric method using continuous flow[12] recommended that samples should be stored at a temperature of at least -18^{0}C and that homogenisation be carried out on the frozen sample. Storage at –18^{0}C before homogenisation is important to assure cell-breakage and complete extraction. This method was subsequently adopted as a BS/CEN method [13].

Colorimetry has been subject to criticism when compared to detection based on HPLC. A comparison of methods for quantitative estimation of nitrate and nitrite in vegetables [14] found significant differences between these methods. The classical colorimetric Cd-Griess method detected an average of 63% less nitrate, was less precise, and had significantly lower recoveries of added nitrate than the HPLC method. These differences were probably the result of incomplete colour development attributable to poor control of the pH after addition

of the acidic colour development reagent. The US National Academy of Sciences has also declared that cadmium reduction followed by colorimetry was generally unreliable since the success depended on the degree to which nitrate is reduced to nitrite, a reaction that is difficult to control and reproduce.

Methods based on chromatographic separation of the nitrate prior to detection have employed either UV or conductivity detection. Several researchers have employed a cold water extraction procedure, in some cases with a preliminary drying stage [15-17], and in others on the fresh sample [18]. A comparison of three extraction procedures [19] involving boiling water, blending with water, and an alkaline extraction found that nitrate was recovered without loss from vegetables that were heated or blended with water at room temperature and from vegetables that were extracted under alkaline conditions. In contrast to methods based on room temperature water extraction of nitrate, Burns has reported [8] that extraction of nitrate from lettuces with hot water gave results nearly double those obtained by using cold water. The current BS/CEN method [20] uses hot water to extract homogenised fresh samples. Extracts are either subjected to treatment with Carrez I and II solutions, or eluted through a solid phase extraction column prior to filtration through a membrane filter and analysis by either HPLC or Ion chromatography.

The evidence from the literature indicates that the analyst potentially has a choice of suitable extraction and detection methods for the analysis of nitrate in lettuce and spinach. With the exception of Burns' observations, where the recovery and precision data are given these comply with EC guidelines. However, where claims can be substantiated by collaborative trial evidence there is considerably more confidence in the robustness of the procedure. In view of the conflicting evidence from the literature, particularly regarding the effectiveness of cold water extraction, and the concerns raised regarding the reliability of colorimetry, scientific evidence is required to reinforce the confidence associated with the UK nitrate monitoring programme.

Extraction Method Evaluation

Selection of a Suitable Method

Most procedures for the extraction and analysis of nitrate in vegetables have one or more of the following characteristics :-

- Sample pre-treatment; samples are either pre-dried (oven or freeze-dried); analysed on a "fresh material" basis, or the frozen sample is extracted.
- Extraction technique; this can involve shaking, ultrasonic extraction or maceration/blending.
- Extraction solvent; hot or cold water with or without the addition of borax or pH adjustment, 50/50 water/methanol.
- Clean up; techniques include activated charcoal, deproteinisation using Carrez solution, or a solid phase extraction column.
- Filtration; including glass fibre, nitrate free paper, membrane filtration.

Seven extraction methods were selected for evaluation which reflected different reported extraction conditions:-

Method 1 [20]:

10 g sample + 400 ml hot water. Stand in a boiling waterbath for 15 min, cool. Dilute and filter through a membrane filter for aqueous solutions with a pore size of 0.45 µm.

Method 2:

As method 1 with clean up using Carrez solution. (Addition of Carrez solution No. 1 to the sample solution, followed by Carrez solution No. 2, dilution, mixing and filtering through filter paper, followed by membrane filtration).

Method 3:

As method 1 with clean up using solid phase extraction column. (Elution of the sample extract through a solid phase extraction column with reverse phased RP C18 cartridge, followed by membrane filtration).

Method 4 [18]**:**

As method 1, but substituting fresh sample for the oven dried sample. 1 g plus 0.5 g active charcoal plus 50 ml water; shake for 30 min, filter through a 0.45 µm membrane suitable for aqueous solutions.

Method 5 [14]**:**

5 g sample plus 50 ml water, mix in high speed blender for 3-4 min, clarify with Carrez I and II solutions, filter through a 0.45 µm membrane suitable for aqueous solutions.

Method 6 [16]**:**

10 g sample plus 70 ml water/ 12 ml 2% NaOH adjusted to pH 8; blend for 5 min. Heat in a waterbath at 50-60^{O}C, add $ZnSO_4$. Cool dilute and filter.

Method 7 [21]**:**

10 g sample plus 5 g borax (50g/l), plus 100 ml water. Heat to 70^{O}C in a waterbath for 15 min. Cool, deproteinise with Carrez solution, followed by solid phase extraction column clean up.

Homogenised bulk lettuce samples were prepared and frozen prior to use. In order to eliminate variability due to the detection technique all samples were analysed using the same HPLC method. The chromatographic procedure used throughout was HPLC (Partisil 10 SAX column) with UV detection. For each analytical batch of lettuce the 7 methods were employed 4 times under repeatability conditions. This procedure was repeated on the following two days giving a maximum of 12 data points for each of the 7 methods.

Table 1

Comparison of results Obtained from the Analysis of Homogenised Lettuce Samples Using 7 Methods

	Nitrate Concentration (mg/kg)						
	Method						
	1	**2**	**3**	**4**	**5**	**6**	**7**
N	12	10	12	12	12	10	10
Mean mg/kg	3336	3325	3331	3266	3164	3031	3083
RSD %	3.3	4.2	2.5	4.3	4.7	7.2	7.2
U mg/kg	222	280	165	279	297	437	444
r mg/kg	48	74	68	334	161	603	903
RSD_r%	1.4	2.2	2.0	10.2	5.1	19.9	29.3
%BSI	-	-	-	98.0	95.0	91.0	92.6

RSD% internal relative reproducibility standard deviation

U expanded measurement uncertainty with a coverage factor of k=2

r repeatability

RSD_r repeatability relative standard deviation

%BSI the mean value for methods 4 to 7 expressed as a percentage of the overall mean value obtained using the 3 variants of the BS method [20]

The results from extraction of the lettuce sample (Table 1) show better performance for all 3 variants of the BS method [20] in terms of repeatability, and improved method uncertainty. The overall mean results obtained using the BS extraction method are also higher than those from other methods indicating better recovery. Higher blank values were observed for samples subjected to clean-up using Carrez solutions. The application of the BS method [20] to spinach, either without clean up or applying Carrez solution or a solid phase extraction, also gave satisfactory results, although the precision obtained for Method 1 was slightly worse.

These results indicate that the hot water extraction technique employed in the BS method [20] gives better precision and higher recovery of nitrate than is obtained using a variety of other extraction methods tested under repeatability conditions.

Effect of Freezing Samples Prior to Extraction

In order to investigate the performance characteristics of nitrate analytical methodology it is necessary to have a supply of stable and homogenous samples. Freezing homogenised samples ensures this, however freezing alters sample structure. Beljaars et al. [12] recommended that samples should be stored at a temperature of at least –18^{0}C before homogenisation to assure cell-breakage and complete extraction of nitrate if extraction was to be undertaken at room temperature (i.e. "cold water" extraction). Burns [8] has reported that extraction of lettuces with hot water gave results nearly double those obtained using cold water. Conversely Lyons et al. [19] reported that extraction of nitrate from vegetables is quantitative at room temperature and have compared room temperature and hot water extraction. They do not mention having frozen samples for storage prior to analysis and presumably did not. Schuster and Lee [14] undertook extraction at room temperature but mainly reported work on carrots, it is not clear whether other vegetables analysed had been stored frozen prior to analysis. Hertz and Baltensperger [18] also employed a room temperature extraction procedure but did not make any comparison with hot water.

Table 2

Comparison of Hot and Cold Water Extraction Procedures on the Nitrate Content of Fresh and Frozen lettuce and Spinach

Sample	Nitrate Concentration (mg/kg)		
	Fresh / Cold Water Extraction	Fresh / Hot Water Extraction	Frozen / Hot Water Extraction
Lettuce Mean (n = 4)	1892	1991	2002
As % frozen	94.5%	99.5%	-
Spinach Mean (n = 4)	103.3	1246	1375
As % frozen	75.1%	90.6%	-
Spinach mean (n = 4)	1914	1990	1982
As % frozen	96.6%	100.4%	-

Two samples of spinach and one round lettuce were prepared and analysed "fresh", i.e. prior to freezing for subsequent storage. Samples were subjected to hot water and cold water extraction. The same samples were analysed after they had been frozen, using hot water extraction. Table 2 details the data obtained. Although the results from cold water extraction of fresh samples are closer to the hot water extraction results in 2 cases it is concluded from this work that extraction using cold water gives inconsistent recoveries and should be avoided.

Effect of Temperature on Sample Storage

Sub-samples of a pureed lettuce sample were stored under different temperature conditions. A sample of pureed lettuce was split into 3 batches. One batch was stored in a freezer at -25^{0}C, one in a refrigerator at 1^{0}C, and one at ambient. Samples were analysed in duplicate over a period of 9 days. Samples are stable over a period of at least 9 days when stored in the freezer (Table 3).

In the case of lettuce samples stored at ambient temperature the low results obtained on day 1 may be anomalous, but in view of the potential for onset of mould, and the generally lower results obtained when storing at ambient, it is not advisable to take this risk.

Mould also occurred in spinach samples when examined after 8 days at ambient (Table 4). There was some trend towards lower results when the spinach samples were stored in the refrigerator but this is unlikely to be significant in the case of overnight storage.

No significant trend in nitrite formation was noted on storage at ambient temperature, levels were approximately 100 mg/kg throughout. However the practice of storing homogenised samples in the refrigerator overnight should not give rise to a nitrate losses. Longer-term storage in a freezer is acceptable.

Table 3

Effects of Storage Conditions on Nitrate Content of Pureed Lettuce

Day	Nitrate Concentration (mg/kg)					
	Ambient A	Ambient B	Fridge A	Fridge B	Freezer A	Freezer B
0	3084	3245	3163	3325	3145	3056
1	2614	2473	3256	3275	2964	3155
7	2940	2926	3414	3435	3216	3142
8	2937	*	3304	3359	3247	3266
9	3011	*	3338	3372	3290	3289

* mouldy sample

Table 4

Effects of Storage Conditions on Nitrate Content of Pureed Spinach

Day	Nitrate Concentration (mg/kg)					
	Ambient A	Ambient B	Fridge A	Fridge B	Freezer A	Freezer B
0	1350	1308	1475	1258	1176	1312
1	1281	1271	1359	1295	1360	1403
7	1278	1280	1283	1265	1364	1320
8	*	*	1268	1264	1309	1221
9	*	*	1149	806	1301	1282

* mouldy sample

Homogenisation Method Evaluation

Nitrate is not distributed uniformly within a crop of lettuce, or within an individual lettuce head. Burns [8], reported a 10 to 15% variability between individual heads due to inherent (mainly genetic) differences within a crop; 8 to 14% between individual heads of soil-grown crops in winter; 17 to 44% between individual heads of soil-grown crops in summer; and 8 to 19% between bulked 10-head samples (as recommended in the UK nitrate monitoring guidelines) of soil-grown protected lettuce in year round production. Dejonckheere et al.[21] also reported great variations in nitrate contents of individual lettuce heads even when they originated from the same greenhouse or were taken from adjacent positions in the same planting area. They concluded that taking 6 heads of lettuce might not guarantee the representativeness of a complete batch. The nitrate residue-monitoring programme provides a minimum of 10 individual lettuce heads to the laboratory. These should be combined to form a single sample, after removal of non-edible or damaged outer leaves or adhering soil. It is essential to ensure adequate mixing of shredded samples. Samples processed through a Hobart homogeniser had to be processed a second time to ensure sufficiently thorough mixing of samples. Double shredding of the sample decreased the overall standard deviation of results to 4.0% compared to 9.1% (Iceberg) and 8.2% (Round) initially obtained using single shredding.

Experimental Methodology

A variation on the standard BS method [20] was used throughout. The standard method was amended to include, where necessary, a double maceration step during initial sample comminution. No Carrez or solid-phase clean-up were used. Hot-water extraction was used throughout and all sample extract analyses were undertaken by HPLC as standard.

Method Validation

Ruggedness

The Youden ruggedness test applies a "fractional factorial" design to evaluating a number of variables in a relatively small number of analytical experiments. A seven-factor plan ruggedness test was applied to the adapted standard method. The variables studied comprised; the time of residence of the extract in the water bath, the ratio of sample to water, blending versus shaking, delaying extraction, introducing a Sep-Pak solid phase clean-up stage, hot water versus hot borax solution, variation in the filtration procedure. None of these factors were found to be significant.

Table 5 shows the results obtained. The effect of introducing the factors can be seen in context by comparing with the expected repeatability of the method. Applying a %RSDr of 1.4% (See Table 1) to both lettuce and spinach gives approximate repeatability values of 60 and 45 mg/kg respectively. Factor C exceeds this, giving lower results when the sample is periodically taken out of the water bath and placed in a blender, as opposed to occasionally manually shaking the sample as it stands in the water bath. It is highly unlikely that a laboratory would introduce this factor. Substituting borax as the extractant resulted in lower overall results but this is unlikely to be significant. Other factors do not appear to be significant. The exercise confirms that the procedure is robust to relatively minor in-house variations.

Table 5

Robustness Testing - Effects of Individual Factors on Results

Condition Altered	Nitrate Concentration (mg/kg)					
	Lettuce			Spinach		
	With	Without	Difference	With	Without	Difference
Time in water bath	1490	1506	-16	1094	1079	+15
Sample:water ratio	1509	1487	+22	1108	1065	+43
Extraction procedure	1460	1536	-76	1049	1124	-75
Store extract in fridge	1486	1510	-24	1070	1102	-32
Clean-up using Sep-Pak	1512	1484	+28	1092	1081	+11
Borax extractant	1472	1524	-52	1058	1114	-56
Filter after make to vol.	1507	1489	+18	1085	1087	-2

Additionally, Hunt and Seymore [16] have noted that conventional filter papers contain variable amounts of nitrate which makes it difficult to use a blank. In order to overcome this problem it is routine in-house practice to discard the first 20 ml of the filtrate before final filtration through a membrane.

Aliquots of filtrates from lettuce extracts were analysed to investigate if the first 20 ml of filtrate differed from the remainder. The first 20 ml of the extract contains a slightly higher nitrate content than the remainder. This is not statistically significant ($P = 0.05$) and could be due to leaching of nitrate from the filter paper. The effect is small but it is advisable to discard the first portion of the filtrate as a precaution.

Internal Reproducibility Assessment

A sample of lettuce was analysed, in duplicate, over a period of 10 days using the amended methodology. The following performance characteristics were determined:

- mean = 3624 mg/kg
- internal reproducibility standard deviation = 91.5 mg/kg
- internal relative reproducibility standard deviation = 2.5%

Method Recovery

A sample of lettuce was fortified at a range of nitrate levels (20-3000 mg/kg) and the recovery assessed using the amended methodology. The overall mean recovery was 98.3 ± 4.8 %. (Mean ± 2 x standard deviation).

Limit of Detection and Quantitation.

A standard solution was prepared containing 0.04 mg/l of nitrate, equivalent to 2 mg/kg in a weight of 10 g. This was analysed 20 times using the amended methodology and the mean and relative standard deviation measured. The relative standard deviation was <10%, acceptable to indicate that this level can be detected reliably. From this the limit of detection is estimated as 2 mg/kg, with an associated limit of quantitation of 20 mg/kg.

Comparison with Reference Materials

There are no available Certified Reference Materials for nitrate in lettuce and spinach. A FAPAS® sample T1516 was analysed where the acceptable values are 878 ≤ 991 ≤ 1103 mg/kg. This sample was analysed daily, in duplicate, over a period of 3 days. The mean result ±2 standard deviations = 1038 ± 77 mg/kg. All results lie within the indicative value range and the mean is 105% of the FAPAS® mean.

Linearity

Seven incremental concentrations of sodium nitrate solution, ranging from 0.5 µg/ml to 200 µg/ml in solution, equivalent to 18 - 7300 mg/kg nitrate, showed satisfactory linearity when injected into the HPLC system, ($R^2 = 0.9991$, $y = 0.0035$ x).

Collaborative Trial

Nine participating laboratories were identified and invited to participate in the trial by the UK Food Standards Agency. Participants were provided with "familiarisation" samples before taking part in the collaborative trial and reported satisfactory results overall. Samples of lettuce, spinach and rocket (all previously tested for homogeneity), as blind duplicates, were sent to participants using an overnight courier in insulated boxes with ice packs. Participants were requested to analyse the samples using the amended methodology supplied. Raw data obtained is given in Table 6 along with the calculated statistical parameters in Table 7. All statistical analyses were undertaken according to the IUPAC Harmonised Guidelines [24]. These data compare favourably with those reported in the literature previously [12 & 20].

The ratios between the observed RSD_R and the values of RSD_R predicted by the Horwitz equation ($2^{(1 - 0.5 \log C)}$); and the corresponding ratios for RSD_r are designated as the HORRAT (Ho_r, Ho_R) values. Codex[23], adopt the HORRAT as an indication of the acceptability of a method with respect to its precision. In an interlaboratory performance study a series of HORRAT ratios close to 1.0 or consistently smaller indicates acceptable precision of a method. IUPAC (1990) consider that an RSD_R value no higher than twice the predicted value (i.e. HORRAT values <2) indicates an acceptable method.

Taking into consideration the criteria set by Codex, and in the context of previous collaborative trials, the results from the present trial indicate acceptable precision. With the exception of the Rocket sample HORRAT values for both repeatability and reproducibility were consistently smaller than 1.0. The amended BS methodology described enables nitrate monitoring laboratories to achieve consistent results on homogenised samples.

Table 6

Collaborative Trial Results

Lab	Nitrate Concentration (mg/kg)									
	Spinach 1		Lettuce 1		Rocket		Lettuce 2		Spinach 2	
	A	G	C	F	B	D	H	J	E	I
1	1253	1234	978	976	486	491	1875	1914	1991	1928
2	1366	1400	1004	1008	412	457	1954	1943	1924	1838
3	1220	1190	937	911	392	398	1820	1780	1790	1720
4	1192	1271	976	956	344	345	1887	1878	1754	1785
5	1296	1264	1017	989	477	472	1896	1939	1913	1899
6	1213	1241	964	964	423	419	1875	1912	1878	1913
7	1158	1244	986	1026	445	425	1875	1921	1830	1850
8	1325	1273	1024	1017	451	403	1903	1866	1969	2005
9	1278	1258	992	1000	437	440	1855	1850	1944	1981

Table 7

Mean and Precision Parameters for all Valid Results

Statistics	Rocket	Lettuce 1	Spinach 1	Spinach 2	Lettuce 2
Mean (mg/kg)	428.72	984.72	1259.78	1844.00	1885.72
s_r	16.40	14.13	34.13	34.73	23.63
RSD_r	3.83	1.44	2.71	1.84	1.25
r	45.92	39.56	95.56	97.25	66.17
Ho_r	0.90	0.38	0.75	0.54	0.37
s_R	43.60	31.57	61.70	86.32	44.63
RSD_R	10.17	3.21	4.90	4.58	2.37
R	122.08	88.39	172.75	241.70	124.97
Ho_R	1.58	0.57	0.90	0.89	0.46

s_r Repeatability standard deviation

RSD_r Repeatability relative standard deviation

r Repeatability limit

Ho_r (Observed RSDr) / (Horowitz RSDr)

s_R Reproducibility standard deviation

RSD_R Reproducibility relative standard deviation

R Reproducibility limit (2.8 x sr)

Ho_R (Observed RSDR) / (Horwitz RSDR)

Discussion and Conclusions

There are a number of extraction and detection methods available to analysts involved in determining nitrate levels in lettuce and spinach, not all of these are equally reliable in terms of precision and recovery.

Preliminary evaluation of seven extraction methods selected from consideration of the literature, and information on commonly adopted extraction procedures employed in the UK, indicated that the method published by the British Standards Institute [20] gave better precision and recoveries than others tested when applied to pre-frozen homogenised spinach and lettuce. This method is based on a hot water extraction of the sample. Cold water extraction gave slightly lower recoveries, in this case, however significant differences would not be expected as freezing of the sample disrupts cells and makes it easier to extract the nitrate. It is therefore recommended that a hot water extraction method is utilised.

The BS method recommends two clean up methods to protect the analytical column and remove matrix interference. These are based on solid phase extraction and Carrez precipitation of proteins. Clean-up using a membrane filter alone has given consistently reliable results. However if a clean-up stage is employed for the analysis of lettuce or spinach from the evidence available solid phase extraction is preferable to Carrez solution as there is more likelihood of losses with the latter. The efficiency of cold water extraction was tested using samples which had not been previously frozen, this work indicated that using cold water extraction gives inconsistent results and should be avoided. The choice of chromatographic detection method (HPLC or ion chromatography) makes no difference to the results but using colorimetry as the detection method gave a significantly high bias in results. Chromatography is therefore recommended as the detection method.

Nitrate is not distributed uniformly throughout individual plants or between different plants even if they are taken from close proximity and at the same harvest. Thorough homogenisation of the samples is essential and a suitable protocol was developed which laboratories should adopt.

A slightly amended BS method (minus Carrez or solid phase clean-up) was investigated for ruggedness using a fractional factorial design with 7 variables and shown to be robust to relatively minor in-house variations. In-house validation of the amended BS method confirmed that recoveries and precision were acceptable and within the requirements specified by the EC for nitrate monitoring.

The amended BS method was subjected to a full collaborative trial according to internationally recognised harmonised guidelines. Repeatability and reproducibility were comparable to those obtained in previous trials and acceptable when assessed against Codex and IUPAC criteria.

In conclusion, it is recommended that hot water extraction is employed for the determination of nitrate in lettuce and spinach, that the analytical procedure described in BS EN 12014-2 (which has been found to be robust) should be the detection method of choice. Thorough homogenisation using the developed protocol should be employed. These recommendations should be incorporated into the industry code of practice to minimise nitrate content of lettuce grown under protected cropping culture in UK.

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