

Appendix U Methods of 24-hour urine analysis and quality control

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This appendix has been updated in 2017 (since first publication in December 2017) to include details of the derivation of the correction factor applied to the 24-hour urinary sodium excretion data to adjust for analytical bias and allow comparison with results from recent and previous urinary sodium surveys of adults which have also been corrected.^{1,2,3,4,5}

U.1 Introduction

This appendix describes the methods used to analyse urinary analytes and provides details of the quality control procedures for these assays in Years 2 to 5 of the National Diet and Nutrition Survey Rolling Programme (NDNS RP). The quality of the laboratory analyses was assured by rigorous instrument maintenance, staff training, adherence to standard operating procedures and good laboratory practice. The quality control and assessment practices used at HNR are all standard procedures for the type of assay used and HNR is ISO certified (BS EN ISO 9001:2008).

U.2 Analysis of urine samples

U.2.1 Sodium and potassium

Urinary sodium and potassium were measured using ion-specific electrodes (ISEs) during the NDNS RP. The sodium and potassium methods on the Siemens Dimension® Xpand clinical chemistry system with the QuikLYTE® module are *in vitro* diagnostic tests intended for the quantitative measurement of sodium and potassium in urine, which use indirect sample sensing with the QuikLYTE® Integrated Multi-sensor Technology (IMT) to develop an electrical potential proportional to the activity of each specific ion in the sample. Each urine sample is diluted automatically and then transferred automatically to the sensor, where Na⁺ and K⁺ ions establish equilibrium with the electrode surface. A potential is generated proportional to the logarithm of the analyte activity in the sample. The electrical

potential generated by a sample is compared with the electrical potential generated by a standard solution, and the concentration of the desired ions is calculated. Sampling, dilution, reagent delivery, mixing, processing, calculation and printing of results are automatically performed by the Dimension® system. Samples are identified with bar codes; the instrument automatically uploads barcode and concentration information to a results spreadsheet, thus eliminating transcription errors. The assay range for sodium is 5–300mmol/L and for potassium is 1–300mmol/L.

The performance of the Siemens Dimension Xpand was closely monitored during analysis, it showed good precision but gave quality assurance results which were lower than those given by other analytical methods. Crossover studies were performed in the HNR laboratories, comparing sodium concentrations in NDNS RP urines as measured using the Siemens Dimension with those obtained using a Roche Cobas C111 which gives results consistent with the consensus All Laboratory Trimmed Mean (ALTM) established by the UK National External Quality Assessment Scheme (UK NEQAS).⁶ The ALTM is regarded as the best indication available of the accurate concentration.

The crossover studies showed that at the time the urines for this survey were assayed urinary sodium measured on the Siemens Dimension Xpand was approximately 5.2% lower than when measured on the Roche Cobas C111, which was believed from its NEQAS performance to give accurate results. Therefore in order to improve accuracy and to facilitate comparison with other surveys, urinary sodium concentrations have been multiplied by 1.052 to correct for this analytical bias. Application of this factor resolved the negative bias shown in unchanged Siemens Dimension urine results relative to the ALTM for external quality assessment samples (see table U.9).

Data will be made available in the UK Data Archive for urinary sodium concentration (mmol/L) and excretion (mmol/24-hour) with and without application of the factor.

U.2.1.1 Quality controls for Sodium and potassium

U.2.1.1.1 Internal quality controls

Internal commercially-prepared quality control (QC) samples (Biorad Liquichek, Level 1 and Level 2) were run on the analyser to check for correct calibration and function before the samples were analysed, and included in every batch to determine between-assay precision. Once a bottle was opened, the remaining volume was aliquoted into smaller tubes and frozen at -20°C and then brought to room temperature and mixed thoroughly before use. The batch was accepted provided that the QC result obtained was within the manufacturer's specified range and also within the more stringent range determined within the laboratory; if the QC results were out of range the analysis was repeated.

The QC results demonstrate good precision throughout Years 2 to 5 of the NDNS RP.

Table U.1 Year 2 QC data for sodium assay

	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	28	8	28	8
Mean	79.0	77.2	161	163
sd	1.36	0.46	2.07	1.04
%cv	1.72	0.60	1.29	0.64

Table U.2 Year 3 QC data for sodium assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	35	35
Mean	76.4	162
sd	1.09	3.14
%cv	1.43	1.93

Table U.3 Year 4 QC data for sodium assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	20	20
Mean	76.3	161
sd	1.20	2.43
%cv	1.57	1.51

Table U.4 Year 5 QC data for sodium assay

	Lvl 1 lot 63291	Lvl 2 lot 63292
N	10	10
Mean	77.8	162
sd	1.20	1.89
%cv	1.54	1.17

Table U.5 Year 2 QC data for potassium assay

	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	28	8	28	8
Mean	28.2	29.2	62.3	64.9
sd	0.52	0.51	1.11	1.33
%cv	1.84	1.76	1.78	2.04

Table U.6 Year 3 QC data for potassium assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	35	35
Mean	29.7	67.5
sd	0.73	3.28
%cv	2.45	4.86

Table U.7 Year 4 QC data for potassium assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	20	20
Mean	31.2	69.9
sd	2.22	5.51
%cv	7.11	7.89

Table U.8 Year 5 QC data for potassium assay

	Lvl 1 lot 63291	Lvl 2 lot 63292
N	10	10
Mean	32.4	68.2
sd	0.34	0.91
%cv	1.06	1.34

The HNR Nutritional Biomarker Analysis Laboratory is a member of NEQAS (National External Quality Assessment Scheme) for urinary sodium and potassium - this scheme sends samples to all hospitals and many other analytical laboratories in the UK for analysis and subsequent comparison of results, to improve harmonization of data between laboratories. NEQAS control samples are urine or artificial matrices spiked to simulate the range of concentrations found in human urine.

During NDNS RP Years 2 to 5, NEQAS control samples were run concurrently with NDNS urines.

Tables U.9 and U.10 summarise the results for the NEQAS control samples obtained using the HNR Siemens Dimension assay relative to those obtained by other laboratories using the same method, and relative to the All Laboratories Trimmed Mean (ALTM). The ALTM is the target concentration derived from the results from all participating laboratories, regardless of method used, and is considered to be the best indication available of the accurate concentration.

Tables U.9 and U.10 demonstrate that there was consistency of assay performance for urinary sodium and potassium across the four years of the NDNS RP. The first four rows of table U.9 show that urinary sodium as measured on the DimensionTM analyser was in general within 1 SD of the mean results obtained by all labs using the same method, but lower than the ALTM. The bottom two rows demonstrate that when each individual sodium result was multiplied by the method-specific factor (1.052) as defined from the cross-calibration experiments carried out at HNR, the bias was resolved and the overall accuracy improved.

Table U.9 NEQAS results for urinary sodium analyses (Years 2 to 5)

NDNS year (NEQAS distributions)	Year 2 (52 to 55)	Year 3 (61 to 66)	Year 4 (80 to 87)	Year 5 (92 to 96)
% bias from method mean	-1.5	-4.0	-0.7	-1.7
SD of bias	1.8	3.4	1.9	2.3
% bias from ALTM	-2.9	-6.1	-5.5	-6.1
SD of bias	2.1	1.8	3.1	1.8

National Diet and Nutrition Survey. Results from Years 2-5 (combined) of the Rolling Programme (2009/2010 – 2012/13): Wales. REVISED JUNE 2017

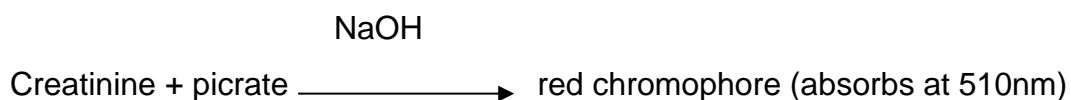
% bias from ALTM after application of factor	2.1	-1.2	-0.6	-1.2
SD of bias after application of factor	2.2	1.9	3.2	1.8

Table U.10 NEQAS results for urinary potassium analyses (Years 2 to 5)

NEQAS	Year 2	Year 3	Year 4	Year 5
% bias from method mean	2.9	1.4	5.8	4.3
SD of bias	3.0	1.8	3.6	1.8
% bias from ALTM	-3.8	-4.0	1.0	-1.9
SD of bias	3.0	2.0	1.6	2.7

U.2.2 Creatinine

The creatinine method employs a modification of the kinetic Jaffe reaction (Larsen). In the presence of a strong base such as sodium hydroxide, picrate reacts with creatinine to form a red chromophore. The rate at which absorbance at 510nm increases due to the formation of the chromophore is directly proportional to the creatinine concentration in the sample and is measured using a bichromatic (510nm, 600nm) rate technique. Bilirubin is oxidised by ferric ferricyanide to prevent interference.



Creatinine in boric acid treated urine is stable for two to three days at room temperature and therefore posting of the urine aliquots to the laboratory was acceptable.

The assay range is 0 – 1768 μ mol/L. The limit of detection of the creatinine method is 4 μ mol/L; this represents the lowest concentration of creatinine that can be statistically distinguished from zero (Siemens data).

Creatinine excretion is affected by muscle mass and therefore varies considerably from person to person.

U.2.2.1 Quality controls for Creatinine

U.2.2.1.1 Internal quality controls

The creatinine assay on the Siemens Dimension® Xpand was controlled with Lyphochek QC 1 and 2 produced by Bio-Rad Laboratories, included in every batch to determine between-batch precision. Once a bottle was opened, the remaining volume was aliquoted out into smaller tubes and frozen at -20°C, and then brought to room temperature and mixed properly before use.

The batch was accepted provided that the QC result obtained was within the manufacturer's specified range and also within the more stringent range determined within the laboratory; if the QC results were out of range the analysis was repeated.

Table U.11 Year 2 QC data for creatinine assay

	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	30	8	30	8
Mean	5.33	5.87	11.9	12.7
sd	0.11	0.12	0.27	0.19
%cv	2.05	2.05	2.31	1.48

Table U.12 Year 3 QC data for creatinine assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	45	45
Mean	5.68	12.0
sd	0.18	0.40
%cv	3.26	3.29

Table U.13 Year 4 QC data for creatinine assay

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	30	30
Mean	5.27	11.2
sd	0.12	0.26
%cv	2.20	2.27

Table U.14 Year 5 QC data for creatinine assay

	Lvl 1 lot 63291	Lvl 2 lot 63292
N	10	10
Mean	4.89	10.2
sd	0.07	0.13
%cv	1.46	1.25

U.2.2.1.2 External quality assessment

HNR subscribes to NEQAS for urinary creatinine (as described in section U.2.1.1.2).

NEQAS control samples for creatinine were run concurrently with NDNS urines.

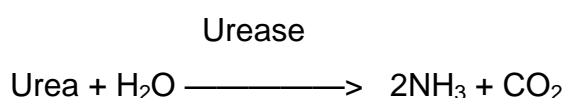
Table U.15 summarises the results obtained using the HNR Siemens Dimension assay relative to those obtained by other laboratories using the Siemens Dimension kinetic Jaffe method, and relative to the ALTM.

Table U.15 NEQAS results for urinary creatinine analyses (Years 2 to 5)

	Year 2	Year 3	Year 4	Year 5
% bias from method mean	-5.5	-3.5	-6.4	-0.6
SD of bias	2.3	2.8	2.8	4.6
% bias from ALTM	-2.6	-3.3	-2.5	1.2
SD of bias	3.3	3.8	1.7	4.2

U.2.3 Urea

The urea method employs a urease/glutamate dehydrogenase coupled enzymatic technique. Urease specifically hydrolyzes urea to form ammonia and carbon dioxide. The ammonia is used by the enzyme glutamate dehydrogenase (GLDH) to reductively aminate α -ketoglutarate (α -KG), with simultaneous oxidation of reduced nicotinamide-adenine dinucleotide (NADH). The change in absorbance at 340nm due to the disappearance of NADH is directly proportional to the urea concentration in the sample and is measured using a bichromatic (340nm, 383nm) rate technique.



U.2.3.1 Quality controls for Urea

U.2.3.1.1 Internal quality controls

The urea assay on the Siemens® Dimension Xpand was controlled with Lyphochek QC 1 and 2, produced by Bio-Rad Laboratories, included in every assay run to determine between-batch precision. Once a bottle was opened, the remaining volume was aliquoted out into smaller tubes and frozen at -20°C, and then brought to ambient temperature and mixed before use.

The batch was accepted provided that the QC result obtained was within the manufacturer's specified range and also within the more stringent range determined within the laboratory; if the QC results were out of range the analysis was repeated.

Table U.16 Year 2 inter-assay variation for urinary urea

	Lvl 1 lot 62871	Lvl 1 lot 63221	Lvl 2 lot 62872	Lvl 2 lot 63222
N	21	8	21	8
Mean	170	164	283	282
sd	4.01	3.77	6.58	10.8
%cv	2.36	2.29	2.33	3.84

Table U.17 Year 3 inter-assay variation for urinary urea

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	28	28
Mean	167.5	285
sd	3.82	5.97
%cv	2.28	2.10

Table U.18 Year 4 inter-assay variation for urinary urea

	Lvl 1 lot 63221	Lvl 2 lot 63222
N	14	14
Mean	160	270
sd	7.12	13.9
%cv	4.44	5.16

Table U.19 Year 5 inter-assay variation for urinary urea

	Lvl 1 lot 63291	Lvl 2 lot 63292
N	10	10
Mean	160	270

sd	2.76	3.03
%cv	1.73	1.12

U.2.3.1.2 External quality assessment

HNR subscribes to NEQAS for urinary urea (as described in section U.2.1.1.2). NEQAS controls were run concurrently with NDNS RP urines. Table U.20 summarises the results obtained using the HNR Siemens Dimension assay relative to those obtained by other laboratories using the Siemens Dimension kinetic Jaffe method, and relative to the ALTM.

Table U.20 NEQAS results for urinary urea analyses (Years 2 to 5)

NEQAS	Year 2*	Year 3	Year 4	Year 5
% bias from method mean	-4.5	-4.7	-7.8	0.0
SD of bias	2.9	3.3	4.1	2.5
% bias from ALTM	-4.5	1.5	-0.9	4.3
SD of bias	3.7	2.4	5.9	3.6

* 1 gross outlier omitted

U.2.4 Nitrogen

Twenty-four hour nitrogen excretion can be used to validate protein intake estimated by various dietary record methods.

The urinary nitrogen assay was subcontracted to the Institute of Grassland and Environmental Research (IGER), now the Institute for Biological, Environmental and Rural Sciences (IBERS), at the University of Aberystwyth, UK. The laboratory is accredited to ISO 17025.

Urinary nitrogen was determined using the FP-428 LECO Nitrogen Determinator. In this method, encapsulated samples of approximately 200mg weight are combusted in high purity oxygen. The combustion products, mainly CO₂, H₂O, NO_x and N₂, are passed through a thermoelectric cooler to remove most of the water.

A 10 cm³ aliquot of the gas is swept through a hot copper catalyst to change NO_x to N₂, then through magnesium perchlorate and sodium hydroxide to remove CO₂ and H₂O respectively.

The remaining product, N₂, is measured by a thermal conductivity cell.

U.2.4.1 Quality controls for Nitrogen

U.2.4.1.1 Internal quality controls

Two (Year 2 to Year 3) or three (Year 4 to Year 5) controls were used to determine between-batch precision.

Table U.21 Internal quality controls for urinary nitrogen

	Year 2		Year 3		Year 4			Year 5		
	1	2	1	2	1	2	3	1	2	3
n	82	75	92	89	185	204	57	145	114	49
Mean (%N)	9.58	1.70	9.64	1.72	9.61	1.70	1.00	9.60	1.66	0.98
SD	0.033	0.023	0.033	0.028	0.031	0.035	0.045	0.036	0.030	0.060
% CV	0.34	1.35	0.34	1.63	0.33	2.05	4.51	0.38	1.80	6.12

U.2.4.1.2 External quality assessment

There is no external quality assessment scheme available for urinary nitrogen analysis.

U.2.5 PABA (*para* aminobenzoic acid)

In Year 2, all samples were initially analysed using the conventional colorimetric procedure (see section U.2.5.1). This assay is subject to interference from substances containing a primary aromatic amine, such as paracetamol. In such urines the apparent PABA concentration in 24 hour urine collections may be greater than 100% of the oral dose ingested during the test day. Therefore, following the protocol of Bingham and Cummings,⁷ where the colorimetric assay indicated that >119% of the dose had been recovered in the urine the sample was re-analysed by a more specific HPLC method (see section U.2.5.2), which is unaffected by

interfering substances. For Years 3 to 5 all samples were analysed by the specific HPLC method only, in order to better standardise interpretation.

U.2.5.1 PABA Analysis by colorimetry

The basis of the assay for analysis of PABA in 24-hour urine is the Bratton and Marshall⁸ diazocoupling reaction. The method was adopted by Bingham and Cummings⁷ and HNR use this version of the method, adapted to a microplate format. The amine group of the PABA reacts with nitrous acid to form a diazonium salt. Excess nitrite is removed with sulphamate to prevent nitrosamine formation. The diazonium salt is then coupled with a colour reagent and the stable dye thus formed can be measured colorimetrically and the absorbance at 530nm interpolated from a calibration curve.

In order to prepare urines for the above procedure an alkaline hydrolysis step is performed. This is required because PABA metabolites, formed as PABA passes through the liver, may be acetylated on the amine group and are therefore unavailable for diazonium salt formation. A two-hour hydrolysis with sodium hydroxide converts the metabolites back to the free molecule. For NDNS RP Year 2, the assay was performed in duplicate and if duplicates differed by more than 10% the sample was reanalysed.

The reference range for PABA excretion as assessed by the colorimetric assay indicating a complete 24-hour urine collection was confirmed at HNR as 85-119% of the 240 mg dose, using 50 adult volunteers (unpublished data).

Comparison at HNR of the PABA excretion kinetics in adults and children after taking a single tablet indicated that there is no reason to expect the range indicating a complete collection to be different in children aged 4 to 10 years (unpublished data).

U.2.5.2 PABA Analysis by HPLC

The traditional colorimetric method may overestimate PABA in some urine collections as it cannot discriminate between PABA and primary aromatic amines

that may originate from drugs, most commonly paracetamol.⁷ HPLC is not subject to this interference.

PABA metabolites in urine are hydrolysed under alkaline conditions, the solution is then neutralised and the resultant PABA determined by HPLC. The HPLC method is a reverse-phase method using an internal standard to compensate for volume losses during hydrolysis. The PABA HPLC method used at HNR is based upon that previously used at the MRC Dunn Nutrition Unit which in turn was based on the method described by Jakobsen *et al.*, (1997).⁹ The PABA HPLC method was then modified at HNR to replace the acetonitrile in the mobile phase with methanol because of the unavailability of acetonitrile.

The reference range for PABA excretion as assessed by the HPLC assay indicating a complete 24 hour urine collection was confirmed at HNR as 70-104% of the 240 mg dose, using 50 adult volunteers (mean \pm 2 sd range).

Comparison of the PABA excretion kinetics in adults and children after taking a single tablet indicated that there is no reason to expect the range indicating a complete collection to be different in children aged 4 to 10 years (unpublished data).

U.2.5.3 Quality controls for PABA by colorimetry

U.2.5.3.1 Internal quality control

A 24-hour urine collection from a participant who had taken 3 x 80 mg PABA tablets was stored as single-use aliquots and frozen at -80°C. It was assayed with every sample set (i.e. on each microplate) during the period when urines from Year 2 were being assayed in order to determine between-batch precision.

Table U.22 PABA controls during Year 2 (by colorimetry)

In-house QA	PABA mg/litre
N	226
Mean	95.3
sd	15.3
%cv	16.1

No colorimetric PABA assays were performed during Years 3 to 5.

U.2.5.4 Quality controls for PABA by HPLC

U.2.5.4.1 Internal quality control

A sample of urine containing PABA was analysed with each batch of samples in order to determine inter-assay variation. Assay results for each run are accepted if the QC results fall within limits defined within the laboratory, otherwise the batch is re-assayed. Completeness of hydrolysis was monitored by including a sample containing PAHA (para amino hippuric acid) with each batch. This is hydrolysed to PABA which is then quantitated by HPLC. 2mM PAHA theoretically yields PABA 2mM (i.e. 275 mg/L).

Table U.23 PABA controls during Year 2

	PABA mg/litre	PAHA hydrolysis control
N	44	44
Mean	73.1	258
sd	5.88	10.0
%cv	8.05	3.88

Table U.24 PABA controls during Year 3

	PABA mg/litre	PAHA hydrolysis control
N	57	58
Mean	76.6	259
sd	6.98	8.72
%cv	9.11	3.37

Table U.25 PABA controls during Year 4

	PABA mg/litre	PAHA hydrolysis control
N	52	51
Mean	72.5	265
sd	7.15	11.0
%cv	9.86	4.15

Table U.26 PABA controls during Year 5

	PABA mg/litre	PAHA hydrolysis control
N	58	59
Mean	77.6	268
sd	5.59	12.8
%cv	7.20	4.77

U.2.5.4.2 External quality assessment – colorimetric and HPLC assays

There is no external quality assessment scheme for PABA.

¹ National Diet and Nutrition Survey - Assessment of dietary sodium in adults (aged 19 to 64 years) in England, 2011 report.

https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/127916/Sodium-Survey-England-2011_Text_to-DH_FINAL1.pdf.pdf (accessed 27/06/16)

² A survey of 24 hour urinary sodium excretion in a representative sample of the Scottish population as a measure of salt intake. Published April 2011.

<http://www.foodstandards.gov.scot/survey-24-hour-urinary-sodium-excretion-representative-sample-scottish-population-measure-salt> (accessed 27/06/16).

³ National Diet and Nutrition Survey (NDNS): Assessment of dietary sodium for adults (19 to 64 years) in England, 2014 report.

https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/509399/Sodium_study_2014_England_Text_final.pdf Published 2016 (accessed 27/06/16).

⁴ National Diet and Nutrition Survey (NDNS): Assessment of dietary sodium for adults (19 to 64 years) in Scotland, 2014 report.

<http://www.foodstandards.gov.scot/national-diet-and-nutrition-survey-assessment-dietary-sodium> Published 2016 (accessed 27/06/16).

⁵ National Diet and Nutrition Survey: Assessment of dietary sodium - Adults (19 to 64 years) in Northern Ireland 2015.

<https://www.food.gov.uk/northern-ireland/nutritionni/national-diet-and-nutrition-survey-assessment-of-dietary-sodium> Published 2016 (accessed 06/10/16).

⁶ The consensus is the All Laboratory Trimmed Mean (ALTM).

⁷ Bingham S and Cummings J H. The use of 4-aminobenzoic acid as a marker to validate the completeness of 24h urine collections in man. *Clin Sci (Lond)* 1983; 64(6):629-35.

⁸ Bratton AC, Marshall GK. A new coupling component for sulfanilaxnide determination. *J Biol Chem* 1939;128:537-50.

⁹ Jakobsen J, Ovesen L, Fagt S, et al. Para-aminobenzoic acid used as a marker for completeness of 24-hour urine: Assessment of control limits for a specific HPLC method. *Eur J Clin Nutr* 1997; 5: 514.