91/414/EEC, Annex II, Part A, 4.2.3
as amended in commission directive 96/46/EC

REPORT

VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF DODINE RESIDUES IN WATER

OCSPP Guideline

850.6100

10. PURPOSE

The objective of this study was to develop and to validate a method for the quantitative analysis of dodine residues in water. The target limit of quantification (=limit of determination) for tap water and ground water was 0.1 µg/l. The target limit of quantification of surface water was 0.008 µg/l.

Validation of the analytical method includes optimisation of the response on a suitable chromatographic system and determination of the stability of the response, stability of dodine in relevant solvent(s) and linearity of the response.

Validation of the extraction procedure from water includes determination of specificity, precision, recovery (=accuracy) and limit of quantification (=limit of determination).

11. TEST CHEMICAL

11.1 Dodine

NOTOX analytical standard	AS480/A
Identification	DODINE TECH. (1-dodecylguamidimium acetate)
Description	White crystalline powder
CAS number	2439-10-3
Chemical Formula	$C_{15}H_{33}N_3O_2$
Molecular weight	287.4
Batch number	005/044/01.01
Purity	98.6%
Test substance storage	At room temperature in the dark
Expiry date	01 January 2006
Supplier	Chimac-Agriphar s.a.

12. TEST SYSTEM

Drinking water: Notox tap water was used as test system for drinking water.

Surface water: water from the river Waal (Loenen, The Netherlands) was used as test system for surface water. The water was sampled on October 14, 2001. The parameters pH, hardness, DOC and suspended solids were determined.

pH	7.97
Total hardness	14 German degrees, medium-hard
DOC	7.0 mg/l
Suspended solids	26.4 mg/l

Ground water: water from a groundwater well (Loosbroek, The Netherlands) was used as test system for ground water. The water was sampled on August 13, 2001.

13. MATERIALS AND METHODS

13.1 Chemicals

Water (MQ)	Purified by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).
Methanol (MeOH)	HPLC-grade (Labscan, Dublin, Ireland)
Acetonitrile (ACN)	HPLC-grade (Labscan, Dublin, Ireland)
Potassiumdihydrogenphosphate	p.a. (Merck, Darmstadt, Germany)
Sodium hydroxide	1N (Merck, Darmstadt, Germany)
Ammonium hydroxide	p.a. 25% (Merck, Darmstadt, Germany)
Acetic acid	p.a. 100% (Merck, Darmstadt, Germany
Glycerol	purity about 87% (Merck, Darmstadt, Germany)
Heptafluorbutyric acid (HFBA)	purity 99% (Aldrich, Steinheim, Germany)
End solution	45/55 (v/v %) ACN/water + 0.1% HFBA 450 volumes of acetonitrile was mixed with 550 volumes of water and 1 volume of HFBA
45/55 ACN/MQ	45 volumes of acetonitrile mixed with 55 volumes of water
Mobile phase	45/55 (v/v %) ACN/water + 0.1% HFBA 450 ml acetonitrile mixed with 550 ml water and 1 ml HFBA
Pump 2 eluent	45/55 (v/v %) ACN/water + 0.1% HFBA
2% Acetic acid	20 ml acetic acid was mixed with 980 ml water
2% ammonium hydroxide	20 ml ammonium hydroxide was mixed with 980 ml water
0.05M phosphate buffer	3.40 g kaliumdihydrogenphosphate was dissolved in 250 ml (pH=7) water, sodium hydroxide was added till pH=7 (about 16 ml) and water is added up to 500 ml
Wash solution 1	21 ml ACN + 9 ml MeOH was mixed with 70 ml 2% ammonium hydroxide
Wash solution 2	21 ml ACN + 9 ml MeOH was mixed with 70 ml 2% acetic acid
Elution solution	49 ml ACN + 21 ml MeOH are mixed with 30 ml 2% acetic acid
Keeper solution	100 µl glycerol will be mixed with 99.9 ml methanol
Washing solvent autosampler	80/20 (v/v%) methanol/water 8 volumes of methanol was mixed with 2 volumes of water.
Purge solvent autosampler	1/1 (v/v%) methanol/water 1 volume of methanol was mixed with 1 volume of water.

13.2 Chromatographic and tandem mass spectrometric conditions

HPLC	Agilent 1100 Series (Agilent Technologies, Portland, USA)
HPLC (pump 2)	510 HPLC pump (Waters Corporation, Milford MA, USA)
Auto sampler	Gerstel MPS-3C (CTC Analytics, Switzerland)
Detection	API 3000 mass spectrometer (Applied biosystems, Sciex, Toronto, Canada)
Interface	Turbo ion spray, Positive mode (No split) Temp.: 500°C Flow: 6000 ml/min.
Hardware/Software	Microsoft Windows NT Operation System 4.00 + PE Sciex Analyst Version 1.1
Mobile phase	45/55 (v/v %) ACN/water + 0.1% HFBA
HPLC Column	Xterra MS C_8, 50 x 2.1 (ID) mm; d_p=3.5 µm (Waters Corporation, Milford, MA, USA)
Flow	0.3 ml/min
Injection volume	10 µL
Column temperature	20°C
Auto sampler temperature	4°C (samples stored in the dark during analyses)
Monitored m/z values	MRM m/z 228.2 → 186.2 Dwelltime: 1200 ms
Retention time	3.5 minutes
Total run time	5.0 minutes

A switching valve was positioned after the HPLC column and was used to prevent the entrance of matrix in the interface during the first 2.0 minutes of the injection.

Switching valve program	0-2.0 minutes position B (mobile phase is switched to waste and a second pump is delivering a flow to the MS-MS) 2.0-5 minutes position A (mobile phase is switched to the MS-MS)

After measuring an analytical batch, the column was washed with 100% acetonitrile for at least 1 hour (flow 0.3ml/min) before stopping the flow. This was necessary due to retention time shift if the column was left standing in the mobile phase. The column was conditioned for at least 1 hour, if a new batch of mobile phase was made, otherwise the retention time was not stable.

13.3 Materials

Solid Phase Extraction column	Waters Oasis HLB 6cc (200 mg) (W1261B2; Waters Corporation, Milford, MA, USA)
Vacuum manifold	24 positions (Alltech)
Vacuum pump	Divac 2.4L (Leybold vacuum products inc., USA)
Vortex mixer	Reax 2000 (Heidolph-Instruments GmbH&Co KG, Kelheim, Germany)
Sample concentrator	Techne DRI block DB30 (Labo Scientific BV, Ede, The Netherlands)

13.4 Preparation of solutions

13.4.1 Stock solutions

Two stock solutions of dodine were prepared by dissolving dodine in methanol. The concentration of the stock solutions are given in Table 1. The stock solutions were stored in the refrigerator (0-10°C).

Table 1 Preparation of stock solutions containing dodine

Stock solution code	Dodine weight (mg)	Dissolved in methanol (ml)	Concentration dodine[1] (mg/ml)
ST_D	13.53	10.552	1.00
ST_E	13.40	10.451	1.00

[1] Corrected for purity and acetate

Aliquots of both stock solutions were diluted with mobile phase to a concentration of 1.00 µg/ml. The difference between stock solution D and E was determined by measuring (in triplicate) the dilutions with LC-MS-MS. The difference was 1.1% (were stock solution D was set as 100%) indicating that both stock solutions had the same concentration. Therefore only stock solution D was used for the validation.

Stock solution ST_D was freshly d luted in acetonitrile/MQ (45/55 v/v%) according Table 2.

Table 2 Preparation of diluted stock solutions containing dodine

Dilution code	Solution no.	Aliquot (µl)	Final volume (ml)	Nominal conc. (µg/l)
ST_D_1000	ST_D	50.0	50	1000
SP 0.8	ST_D_1000	40.0	50	0.8
SP 8.0	ST_D_1000	80.0	10	8.0

13.4.2 Preparation of calibration standards

Six calibration standards were prepared in end solution (see Table 3). The concentrations of the calibration standards were corrected for the concentration factor (400x, see 13.6).

Table 3 Preparation scheme for standards in end solution

Calibration Standard Code	Diluted stock-solution No.	Aliquot dodine (µl)	Final volume End solution (ml)[1]	Concentration (µg/l)	Final concentration in extracts (µg/l)[2]
W 2	ST_D_1000	50.0	25	2	0.00500
W 5	ST_D_1000	50.0	10	5	0.0125
W 10	ST_D_1000	50.0	5	10	0.0250
W 20	ST_D_1000	100.0	5	20	0.0500
W 30	ST_D_1000	150.0	5	30	0.0750
W 40	ST_D_1000	200.0	5	40	0.100
W 3.2[3]	ST_D_1000	80.0	25	3.2	0.00800

1 The end solution contained ACN/water (45:55; v/v) + 0.1% HFBA
2 A concentration factor of 400 was applied
3 W 3.2 was not used in the calibration curve but was used for the matrix effect

13.4.3 Preparation standard solution for assessment of stability

A standard solution was prepared by adding 100.0 µl of ST_D_1000 in 5 ml of end solution, leading to concentration of 0.050 µg/l.

13.5 Preparation quality control samples

Spiked samples were prepared by transferring an appropriate amount of spiking solution to 100 ml of test matrix. Blank samples consisted of test matrix that was not spiked with dodine.

A test concentration of 0.008 µg/l was prepared by adding 1000 µl diluted stock solution SP 0.8 (0.8 µg/l) in 99 ml of test matrix.
A test concentration of 0.08 µg/l was prepared by adding 1000 µl diluted stock solution SP 8.0 (8.0 µg/l) in 99 of ml test matrix.

13.6 Sample extraction procedure

100 ml of water sample was transferred in to a glass (e.g. an erlenmeyer flask) and 10 ml of phosphate buffer (pH=7) was added. The sample was mixed.
SPE columns (Oasis HLB 6cc 200mg (Waters, part no. WAT106202), was conditioned with 1 ml methanol and 1 ml water. The sample was transferred on the SPE column. The glass was rinsed with 3 ml water (MQ), this was also transferred on the SPE column. The column was washed with 2 ml wash solution 1 and 2 ml wash solution 2. Dodine was eluted with 2.5 ml elution solution and was collected in a 5 ml glass vial (Emergo, AR glas). To the eluent 1 ml of Keeper solution was added. The sample was dried at 60°C under nitrogen stream and after about 20 minutes 1 ml methanol was added, this was repeated about each 20 minutes until the solvent was evaporated. The residue was dissolved in 250 µl end solution and analysed with 10 µl single injection on LC/MS-MS.

During sample processing, the sample is concentrated 400 times (corresponding to a decrease of sample volume from 100 ml to 0.250 ml of end solution).

It is advised to use disposable glass or use the glass only once. Due to the low LOQ it is possible to have carry over of Dodine if glasswork is used more than once.

14. VALIDATION PROCEDURE

14.1 Specificity

The specificity of the analytical method was demonstrated by the retention time of the analyte on the chromatographic system. The interferences in processed blank samples should not exceed 30% of the LOQ.

14.2 Linearity

Chromatography was performed on each independently prepared calibration standard (single injection). The area response of dodine was recorded. The results of the peak area were used to calculate a regression equation. The coefficient of determination (r^2) should be ≥ 0.9801 and the accuracy of the back calculated concentration of each individual calibration points must be within 90 –110% of the nominal concentration. If the accuracy exceeded this range, the calibration point was removed, and the calculation was repeated. The number of calibration standards should be at least 75% of the used calibration standards and not less than 5.

14.3 Precision

For each concentration level the within batch, between batch and total precision were statistically determined (see Chapter 15) and should be ≤ 20%.

14.4 Recovery

The recovery (=accuracy) refers to the extraction efficiency of the sample processing procedure for dodine. For each sample, the recovery was calculated by comparing of the nominal spiked concentration with the actual measured concentration. The recovery of dodine was expressed as the overall mean recovery per concentration level of the samples ≥ LOQ, and should be in the range 70-110%.

The contribution of the matrix effect on the recovery was determined. The effect of the matrix on the quantification of dodine was tested by extracting blank surface water. After evaporation, the eluate was dissolved in 250 µl end solution that contained dodine (solution W 3.2 see Table 3). The matrix effect on the analysis of dodine was calculated by normalising the actual measured concentration with the nominal spiked concentration.

14.5 Limit of Quantification (LOQ)

The limit of quantification (=limit of determination) was set at the lowest test concentration, 0.008 µg/l.

14.6 Stability

14.6.1 Stability of the stock solution

The stability of the stock solution was determined by LC/MS-MS, according to the NOTOX standard operating procedure SOP CHE/K/011 over a period of 18 days. Maximum allowed deviation in response between stored and freshly prepared stock solutions was 5%.

14.6.2 Stability in sample extract end solution and end solution

The stability in sample extract end solution and end solution was determined by comparing the back-calculated concentration of freshly prepared sample extract with the back-calculated concentration of the same sample under storage condition of the auto sampler.

15. DATA HANDLING

Response	Peak area test substance [units]
Response factor	Response/concentration [units*(l/µg)]
Mean	$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$ x_i = measured value n = number of measurements
Standard deviation	$s_{n-1} = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{(n-1)}}$ where x_i = measured value n = number of measurements
Coefficient of Variation (C.V.) (Precision)	[standard deviation/mean value] x 100%

Accuracy [E/T] x 100%

E = experimental concentration
T = theoretical concentration

Within-batch precision [%][1] Calculated as:

$$\frac{S_{pwr}}{mean} \times 100\,\%$$

S_{pwr}: standard deviation (pooled) within run component of precision.

Between-batch precision [%][1] Calculated as:

$$\frac{S_{Br}}{mean} \times 100\,\%$$

S_{Br} : standard deviation between run component of imprecision

Total precision [%][1] Calculated as:

$$\frac{\sqrt{S_{Br}^2 + S_{pwr}^2}}{mean} \times 100\%$$

16. RESULTS

Concentration level 1 (=0.008 µg/l) was successfully analysed in surface water. The response for all blank types of water was < 30%. Therefore, the validation of dodine in water was done only with surface water. A total of 2 analytical batches were performed.

16.1 Analytical batch history

The analytical method for surface water was validated according to the scheme shown in Table 4. The same operator and using identical methods, test material and equipment, processed two batches in short time intervals.

Table 4 Analytical batch history

Analytical Batch	Extraction	Analysis	Validation subjects
1	31/10/2001	31/10/2001	Blanks[1], LOQ, 10x LOQ, matrix effect
2	01/11/2001	01/11/2001	Blanks[1], LOQ, 10xLOQ

[1] Blanks: surface, tap and ground water

16.2 Specificity

Specificity of the analytical method for dodine was demonstrated by the retention time of the analyte on the HPLC-column and by the specific response (MRM) in the MS–MS detector. The specificity is based on selection of the precursor ion (parent) in the detector, followed by fragmentation and detection of a product (daughter) ion. The retention time of dodine was 3.5 min, in calibration standards and processed spiked samples.