European Commission

JRC TECHNICAL REPORTS

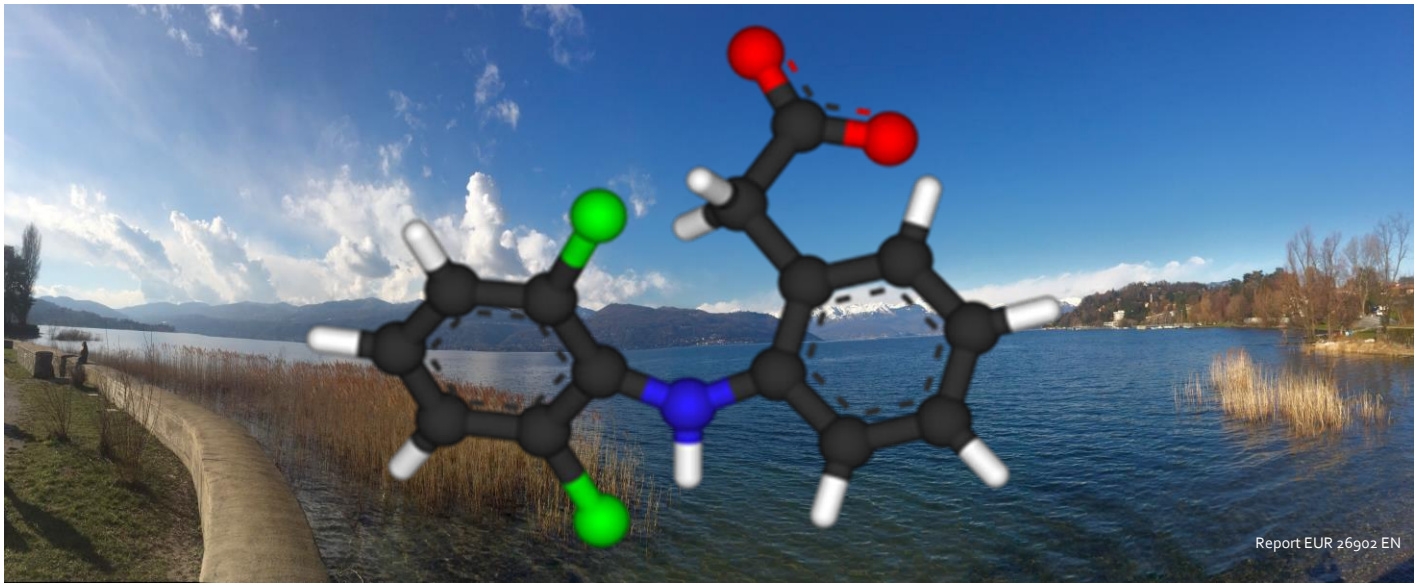
Water Framework Directive Watch List Method

Analysis of diclofenac in water

Validation report, according to ISO 17025 requirements

S. Tavazzi, B. Paracchini, G. Suurkuusk,
G. Mariani, R. Loos, M. Ricci
and B. M. Gawlik

2014



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Contact information
Simona Tavazzi
Address: Joint Research Centre, Via Enrico Fermi 2749, TP 120, 21027 Ispra (VA), Italy
E-mail: simona.tavazzi@jrc.ec.europa.eu
Tel.: +39 0332 78 3683
Fax: +39 0332 78 9328

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This publication is a Technical Report by the Joint Research Centre of the European Commission.

JRC91354

EUR 26902 EN

ISBN 978-92-79-43859-2 (PDF)

ISSN 1831-9424 (online)

doi:10.2788/19818

Luxembourg: Publications Office of the European Union, 2014

Printed in Luxemburg

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List of Abbreviations and Symbols

Throughout this report, the following abbreviations and symbols are used:

ANOVA	Analysis of variance
CAD	Collision Gas
CUR	Curtain Gas
CRM	Certified reference material
CXP	Collision Cell Exit Potential
DG	Directorate-General
EC	European Commission
EP	Entrance Potential
EU	European Union
GS1	Ion Source gas 1
GS2	Ion Source gas 2
HLB	Hydrophilic-lipophilic balance
IES	Institute for Environment and Sustainability
IS	Internal standard/Ion Transfer voltage
ISO	International Organization for Standardization
JRC	Joint Research Centre
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NSAID	Non-steroidal anti-inflammatory drug
PPG	Polypropylene glycol
PS	Priority substances
QC	Quality controlled sample
R^2	Coefficient of determination
RSD	Relative standard deviation
SD	Standard deviation
SPE	Solid-phase extraction
TEM	Temperature
UHPLC	Ultra-high-pressure liquid chromatography
WFD	Water Framework Directive

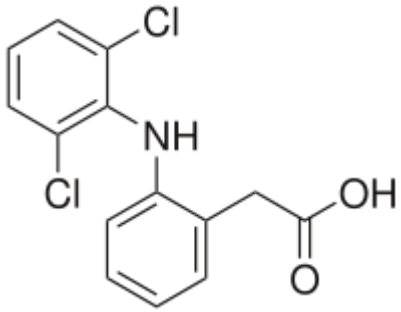
Chemical elements are identified by their respective symbols as defined by the International Union of Pure and Applied Chemistry (IUPAC)

Acknowledgements

The authors gratefully acknowledge the support provided by Grainne Mulhern (EC-JRC) for the revision of English language and improving the readability of the text. Input and constructive comments received from France on the comparability of LoQ are acknowledged, too.

1 Introduction

Diclofenac chemical structure:



Chemical property data	
Formula	$C_{14}H_{11}Cl_2NO_2$
Molecular mass	296.148 g/mol
CAS number	15307-86-5
Systematic IUPAC name	2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid

Since the pharmaceutical market has been growing in recent decades, the issue of pharmaceuticals in the environment gives rise to increasing public concern because various types of medicinal products (hormones, anti-cancer drugs, antidepressants, antibiotics, etc.) have been detected in various environmental compartments, such as surface water, groundwater, soil, air, and biota. Although it is unlikely that the concentrations of pharmaceutical residues in the environment could provoke direct toxicological effects in human beings, the long-term (chronic) exposure has been reported to have effects on wildlife. The two most prominent and well-documented cases of direct ecotoxicological effects of pharmaceutical residues in wildlife address the presence of ethinylestradiol in water bodies, which causes the feminisation of male fish (Caldwell et al., 2008; Kidd et al., 2007), and the use of diclofenac.

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) taken or applied to reduce inflammation and used as an analgesic to reduce certain types of pain. It is supplied as or contained in medication under a variety of trade names, and is also administered to livestock as an anti-inflammatory medication.

Diclofenac has been found to directly affect wildlife as a consequence of its widespread use as a veterinary drug for cattle. Tens of millions of vultures in India died as a result of feeding on the corpses of cows treated with diclofenac (Oaks et al., 2004; Risebrough, 2004).

In the first review of the list of priority substances (PS) under the European Water Framework Directive (WFD), the Commission legislative proposal introduced an environmental quality standard (EQS) for diclofenac in the aquatic environment. The annual average EQS value (AA-EQS) for diclofenac was set at 0.1 µg/l in inland surface waters (including rivers, lakes and related artificial or heavily modified water bodies), and at 0.01 µg/l in other (coastal) surface waters. Diclofenac has been put on the newly introduced "watch list" mechanism to collect European Union-wide monitoring data (EU, 2013).

The analytical method reported in this report is based on solid-phase extraction (SPE) using a universal reversed-phase sorbent (HLB: hydrophilic-lipophilic balance) followed by ultra-high-pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) analysis. The procedure has been fully characterised in terms of linearity, working range, selectivity, precision, repeatability, trueness and uncertainty.

The objective of this method validation exercise is to contribute to the chemical analysis of diclofenac in the aquatic environment in compliance with the first watch list exercise for the implementation of the Water Framework Directive (WFD).

2 Materials and methods

2.1 Chemicals

2.1.1 Standards

Diclofenac sodium salt, CAS 15307-79-6, batch 30266, purity 99.5%, expiry date March 11, 2017, Sigma Aldrich, MO (USA).

Diclofenac-(Acetophenyl ring-$^{13}C_6$) sodium salt 4.5-hydrate, batch SZBB264XV, purity 99.9%, expiry date September 21, 2014, Sigma Aldrich, MO (USA).

2.1.2 Materials and reagents

Ethyl acetate for trace analysis (Carlo Erba Reactifs-SDS).

Methanol, code 701091.1612, (LC-MS) PAI, Panreac Quimica, Barcelona (Spain).

MilliQ water obtained from a MilliQ water system, Millipore, Bedford, MA (USA).

Ammonium acetate 99.99+%, code 431311, Sigma Aldrich, MO (USA).

Acetonitrile, code 701881.1612, (LC-MS) PAI, Panreac Quimica, Barcelona (Spain).

Acetic acid, code 07692, TraceSelectUltra for trace analysis, Sigma Aldrich, MO (USA).

Ammonium hydroxide, 28% in water, 99.99 metals basis, code 338818, Sigma Aldrich, Germany.

OASIS HLB cartridges 6CC (0.2 g), code WAT106202, Waters, Milford, MA, USA.

2.1.3 Reagent solutions

Mobile phase A: 5 mM ammonium acetate

a. Weigh 0.385 g of ammonium acetate in a 1-l volumetric flask.
b. Dissolve and dilute to volume with water.

Mobile phase B: methanol;acetonitrile 50;50, % v/v

a. Transfer 500 ml of methanol and 500 ml of acetonitrile into a 1-l bottle.
b. Mix and degas in ultrasonic bath for 20 seconds.

UHPLC Autosampler strong washing solution

a. Transfer 900 ml of water, 47.5 ml of methanol, 47.5 ml of acetonitrile, and 5 ml of glacial acetic acid into a 1-l bottle.
b. Mix and degas in ultrasonic bath for 20 seconds.

UHPLC Autosampler weak washing solution

a. Transfer 45 ml of water, 475 ml of methanol, 475 ml of acetonitrile, and 5 ml of glacial acetic acid into a 1-l bottle.
b. Mix and degas in ultrasonic bath for 20 seconds.

UHPLC Seal washing solution

a. Transfer 100 ml methanol and 900 ml of water into a 1-l bottle.
b. Mix and degas in ultrasonic bath for 20 seconds.

UHPLC-MS/MS Reconstituting solution for LC-MS/MS analysis

a. Transfer 900 ml of mobile phase A into a 1-l bottle.
b. Add 100 ml of mobile phase B and mix.

2.1.4 Standard solutions

Diclofenac stock standard solution (970 µg/ml)

a. Accurately weigh approximately 9.7 mg of diclofenac in a 10-ml volumetric flask.
b. Dissolve and dilute to volume with methanol and mix.

Intermediate Standard Solution 1 (970 ng/ml)

a. Transfer 10 µl of diclofenac stock standard solutions (970 µg/ml) into a 10-ml volumetric flask.
b. Dilute to volume with methanol and mix.

Intermediate Standard Solution 2 (9.7 ng/ml)

a. Transfer 100 µl of diclofenac intermediate standard solution 1 (970 ng/ml) into a 10-ml volumetric flask.
b. Dilute to volume with methanol and mix.

Standard Solution A (0.194 ng/ml)

a. Transfer 20 µl of intermediate standard solution 2 into a 1-ml dark vial.
b. Dilute to 1 ml with acetone:hexane 50:50, (%, v/v) and mix.

Standard Solution B (0.97 ng/ml)

a. Transfer 100 µl of intermediate standard solution 2 into a 1-ml dark vial.
b. Dilute to 1 ml with acetone:hexane 50:50, (%, v/v) and mix.

Standard Solution C (1.94 ng/ml)

a. Transfer 200 µl of intermediate standard solution 2 into a 1-ml dark vial.
b. Dilute to 1 ml with acetone:hexane 50:50, (%, v/v) and mix.

Standard Solution D (38.8 ng/ml)

a. Transfer 40 µl of intermediate standard solution 1 into a 1-ml dark vial.
b. Dilute to 1 ml with acetone:hexane 50:50 (%, v/v), and mix.

Standard Solution E (97 ng/ml)

a. Transfer 100 µl of intermediate standard solution 1 into a 1-ml dark vial.
b. Dilute to 1 ml with acetone:hexane 50:50 (%, v/v), and mix.

Standard Solution Low QC (2.91 ng/ml)

a. Transfer 0.3 ml of intermediate standard solution 2 into a 1-ml dark vial.
b. Dilute to 1 ml with acetone:hexane 50:50 (%, v/v), and mix.

Standard Solution High QC (87.3 ng/ml)

a. Transfer 90 µl of intermediate standard solution 1 into a 1-ml dark vial.
b. Dilute to 1 ml with acetone:hexane 50:50 (%, v/v), and mix.

2.1.5 Internal standard solutions

Internal standard stock solution (diclofenac $^{13}C_6$, 10 mg/ml)

a. Accurately weigh approximately 10 mg of diclofenac $^{13}C_6$ and dissolve them in 1 ml of methanol.

Internal standard working solution 1 (diclofenac $^{13}C_6$,10 µg/ml)

a. Transfer 10 µl of diclofenac $^{13}C_6$ 10 mg/ml into a 10-ml volumetric flask.
b. Dilute to 10 ml with methanol and mix.

Internal standard working solution 2 (diclofenac $^{13}C_6$,1 µg/ml)

a. Transfer 1 ml of Internal Standard working solution 1 (10 µg/ml) into a 10-ml volumetric flask.
b. Dilute to 10 ml with methanol and mix.

2.2 Apparatus

2.2.1 Laboratory equipment

Analytical balance:	Model AX204, Mettler-Toledo SpA
Automatic pipettes:	Eppendorf research (Milan, Italy)
Microsyringes:	Microliter Syringes, Hamilton (Reno, CA, USA)
Autosampler vials for LC-MS:	Micro-V vials target Dp clear, 1.5 ml, 12x22 mm National Scientific (Germany)
Volumetric flasks:	Grade A various sizes, Duran®
Volumetric pipettes:	Grade A various sizes, Duran®

Dionex Autotrace AT280 automated SPE system (Thermo Scientific, Waltham, MA, USA)

TurboVap II (Caliper Life Science, Mountain View, CA, USA)

Vortex Genius, Ika, Staufen, Germany

2.2.2 Instrumental equipment and conditions

2.2.2.1 UHPLC equipment and conditions

Pumps:	Binary Solvent Manager, Model UPB, Waters (Milford, MA, USA)
Autosampler:	Sample Manager, Model UPA, Waters (Milford, MA, USA)
Detector:	QTRAP 5500, Applied Biosystems MDS SCIEX, (Foster City, CA, USA) equipped with Turbo V™ ion source
Flow rate:	400 µl/min
Injection volume:	5 µl
Analytical column:	Triart C18, 1.9 µm, 50 x 2.1 mm, YMC (Dinslaken, Germany) equipped with UHPLC column saver (Fortis, Technologies)
Mobile phase A:	5 mM ammonium acetate
Mobile phase B:	Acetonitrile-methanol (50:50, % v/v)

The chromatography was performed in gradient mode according to the following scheme:

Time	A	B	Flow (ml/min)
0	90	10	0.4
1	90	10	0.4
9	5	95	0.4
9.1	5	95	0.4
9.2	90	10	0.4
12	90	10	0.4

Under these conditions, the retention time of diclofenac was about 6.2 minutes. The run time was 12.5 minutes.

2.2.2.2 QTRAP 5500 operative condition

An AB Sciex QTRAP5500 mass spectrometer equipped with Turbo V™ ion source was used. The instrument was previously tuned and calibrated in electrospray mode using

polypropylene glycol (PPG). Prior to analysis, all the specific parameters were optimised infusing a 1 µg/ml standard solution of analyte and internal standard (IS).

The eluent from the column was introduced directly into the ion source. The rapid desolvation and vaporisation of the droplets minimises thermal decomposition and preserves their molecular identity. The data were collected using the software programme Analyst 1.6.

All calculations were based on chromatographic peak area ratios for the multiple reaction monitoring (MRM) precursor-product ion transitions for analyte to the precursor-product ion transition of the internla standard. The general operating conditions were as follows:

Scan Type:	Scheduled MRM
Polarity:	Negative
Ion Source:	Turbo Spray
Resolution Q1:	Unit
Resolution Q3:	Unit
MR Pause:	5.0000 msec

Analyte MRM	Time(min)	Declustering Potential (DP)	Collision Energy (CE)
Diclofenac (294>250)	6.2	-42	-16
Diclofenac (294>214)	6.2	-42	-29

Curtain gas (CUR):	25
Collision gas (CAD):	Medium
Temperature (TEM):	550
Ion Transfer Voltage (IS):	-4 500.
Entrance Potential (EP) -	10.00
Collision cell Exit Potential (CXP)	-11.00
Ion Source gas 1 (GS1)	55
Ion Source gas 2 (GS2)	45

3 Experimental set up of method validation

Different experiments were carried out for the characterisation of the developed procedure in terms of limit of detection and quantitation, linearity and working range, recovery, trueness, repeatability, intermediate precision and sample and extract stability. In our approach, a calibration curve and quality controlled samples (QCs) were freshly prepared in triplicate for five different days. Some of the experiments were used in the evaluation of different parameters.

The analyte / internal standard peak area ratios will be used as target parameters for quantitation. A weighted (1/c) least-square regression analysis of data was performed to determine the calibration curve parameters and the coefficient of determination (R^2).

The equation obtained with the linear regression method is as follows:

$$X = \frac{Y - B}{A}$$

where:

X = analyte concentration

Y = peak area ratio = $\frac{\text{analyte peak area}}{\text{I.S. peak area}}$

A = slope

B = intercept

Analyst 1.6 software was used for data acquisition and data processing.

Statistical calculations will be performed using Excel software.

3.1 Calibration curve

Calibration standards in MillliQ water (six different spiking levels, including a blank sample) covering the studied calibration range (0.2-97 ng/l) were freshly prepared and processed on each day of validation.

The relationship (goodness of fit) between peak area ratios of analyte / I.S. and concentrations in the investigated concentration range was assessed by the coefficient of determination (R^2) and by the shape of the distribution of residuals around the horizontal axis.

The acceptance criteria set for calibration curves were:

- $R^2 \geq 0.9900$ calculated over five calibration curves and
- random dispersion of residuals around the horizontal axis, proving the pertinence of the linear regression model to interpret the data.

3.2 Repeatability and intermediate precision

Quality controlled samples (QCs) were freshly prepared in MilliQ water and analysed in triplicate during validation days at two spiking levels (2.91 and 87.3 ng/l) for a total of 15 independent sample preparations.

The acceptance criterion set for the relative standard deviation (RSD) of the repeatability and intermediate precision was 15% at both spiking levels.

3.3 Limits of detection and quantification

The limits of detection and quantification were estimated by analysing 15 different blank samples.

The mean value of blank samples (b) and the RSD served for LOD and LOQ estimation, in accordance with the following equations:

LOD = b + 3SD

LOQ = b + 10SD.

3.4 Trueness

Trueness was assessed by analysing 15 different QCs at each spiking level and by the application of the significance test (t-test) at the 95% confident level. The difference between the mean value of spiked MilliQ water samples and their nominal concentration was evaluated according to the following formula:

$$t = \frac{(x - \mu)\sqrt{n}}{SD}$$

where (x) is the mean value of (n) samples with standard deviation (SD), and (μ) is the nominal concentration.

3.5 Recovery

Recovery was evaluated by extracting and analysing in triplicate 1-litre MilliQ water samples spiked, before extraction, with native analytes only. The internal standard was then added to

the extracts at the end of the sample preparation with the aim of allowing the estimation of analyte loss during processing.

The recovery was evaluated by comparing the ratios analyte/IS in spiked samples to the same ratios obtained by analysing a standard solution containing native compounds and the labelled solution at the same concentration levels, not subject to any handling.

The spiking level was 9.7 ng/l.

3.6 Stability

3.6.1 Stability of extract

Three extracts of QC at each spiking level were re-injected after storage at 4°C for one week in the dark. In order to be considered stable, the concentration of re-injected samples had to fall within about twice the standard deviations of the concentration of QCs used for the repeatability study.

3.6.2 Stability of sample

The stability of the analyte in water samples during storage was studied by analysing low and high QCs, prepared on 3 August 2012 and stored under identical temperature and lighting conditions to those of the real water samples (i.e. 5°C, in darkness).

Stability samples for diclofenac were extracted and analysed on days 0, 38, 83 and 96 after spiking.

Concentrations in samples had to fall within twice the standard deviation of the concentrations of QCs used for repeatability evaluation.

4 Preparation of calibration standards and water samples

4.1 Calibration standards and Quality Control samples (QCs)

a. Fill 1-l glass bottle with 1 l MilliQ water.
b. Add 1 ml of working standard solution according to the following:

Standard solution	Diclofenac concentration ng/ml	Diclofenac concentration in water ng/l		IS concentration in water (ng/l)
A	0.19	0.19	Calibration samples	10
B	0.97	0.97	„	10
C	1.94	1.94	„	10
D	38.8	38.8	„	10
E	97	97	„	10
Low QC	2.91	2.91	QC samples	10
High QC	87.3	87.3	„	10

4.2 Water sample extraction

a. Add 10 µl of diclofenac $^{13}C_6$ working solution 2 to 1 l water standard and QC samples
b. Shake the water sample
c. Condition SPE OASIS HLB cartridge with 10 ml of ethyl acetate
d. Condition SPE cartridge with 10 ml of methanol

e. Condition SPE cartridge with 10 ml of water
f. Load water samples at 10 ml/minute
g. Dry the sorbent under nitrogen for 30 minutes
h. Elute the sample with 10 ml ethyl acetate at 5 ml/minute.

Half of the extract (i.e. about 5 ml) evaporated due to dryness and was reconstituted in 0.2 ml of reconstituting solution for LC-MS/MS analysis.

5 Validation procedure and results

5.1 Selectivity

For the identification of diclofenac, two multiple reaction monitoring (MRM) transitions between the precursor ion and the two most abundant fragment ions were monitored. The first was used for quantification purposes, whereas the second ("qualifier") was used to confirm the presence of the target compound in the sample. The quantified analyte was identified by comparing the retention time of the corresponding standard and the isotopic ratio between two ions recorded (±30%), in the standard and water samples.

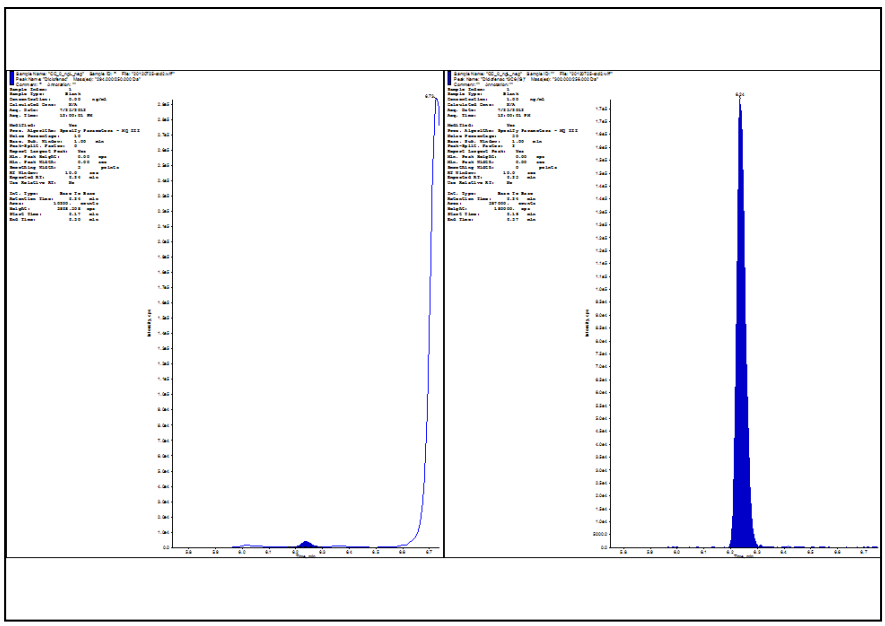
The selected mass transitions used for quantification were 294 > 250 for diclofenac, and 300 > 256 for diclofenac $^{13}C_6$.

5.2 Limit of detection (LOD) and limit of quantification (LOQ)

Limits of detection and quantification were estimated by analysing blank samples.

A typical blank chromatogram is depicted in Figure 1.

Figure 1 – Chromatogram of a blank sample



The mean values of the blank samples (b) and standard deviation (SD) were calculated using the data resulting from these experiments. LOD and LOQ were estimated according to the formula reported in 3.3.

The results of the LOD and LOQ estimation are shown in Table 1.

Table 1 - LOD and LOQ values

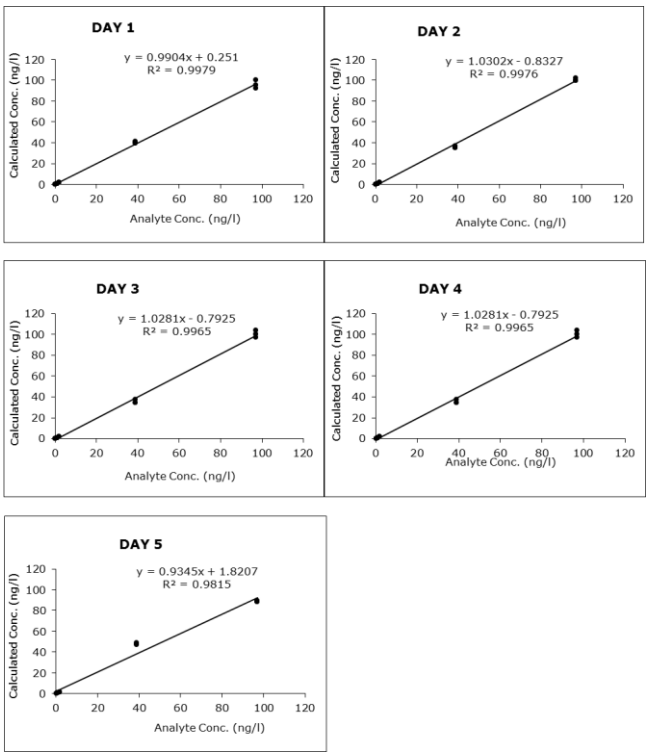
Analyte	Nr of blanks analysed	LOD (ng/l)	LOQ (ng/l)
Diclofenac	15	0.1	0.2

5.3 Linearity study

The linearity of the whole SPE-LC-MS-MS procedure was studied in the concentration range 0.19-97 ng/l.

In order to verify the linearity of the calibration curve, a blank sample spiked only with labelled IS and five spiked MilliQ water samples (i.e.: 0, 0.19, 0.97, 1.94, 38.8, 97 ng/l) were extracted and analysed in three replicates on five different days. The calibration curves are illustrated in Figure 2.

Figure 2 – Calibration curves



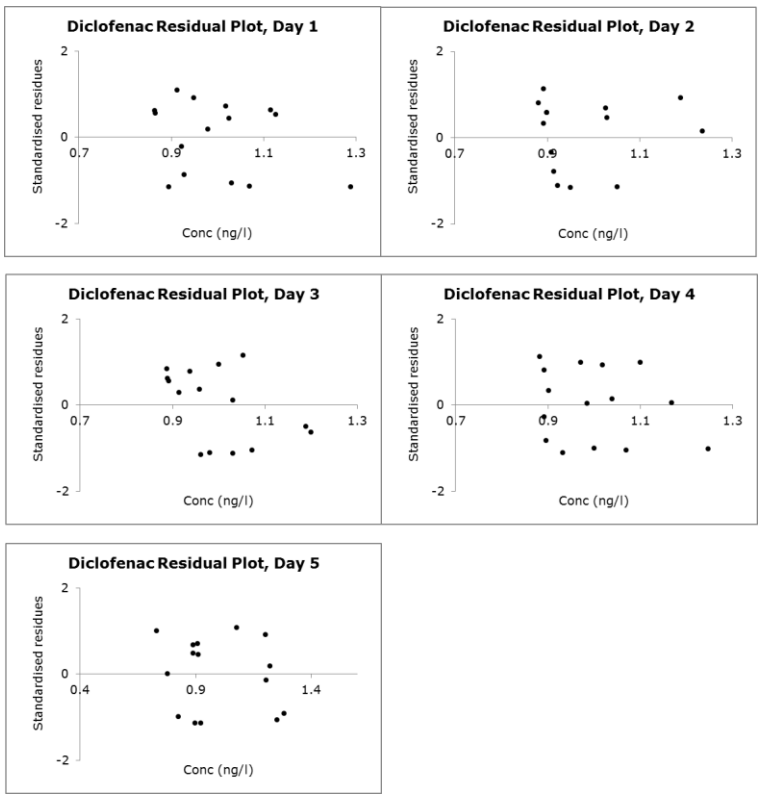
As reported in Table 2, the mean coefficient of determination (R^2) values, calculated over five calibration curves, was ≥0.994, with an RSD of 0.7 %.

Table 2 - Coefficient of determination (R^2) values for calibration curves on different days

Validation Day	R^2
1	0.9979
2	0.9976
3	0.9965
4	0.9965
5	0.9815
Average	**0.994**
RSD (%)	**0.7**

The study of the distribution of residuals revealed shapes randomly dispersed around the horizontal axis, proving the pertinence of the linear regression model for interpreting the data. The residual plots are shown in Figure 3.

Figure 3 – Residual plots



5.4 Working Range

The working range, defined as the range of concentrations for which the chosen calibration curve is valid, was defined by the limits of quantification and highest point in the respective calibration curve. Table 3 summarises the working range established for the procedure.

Table 3 - Working range of the analytical method

Analyte	Working range (ng/l)
Diclofenac	0.2-97.3

5.5 Trueness

Fifteen QCs at low and high concentration levels (i.e. about 3 and 90 ng/l) were extracted and analysed, and the back-calculated concentrations were evaluated to demonstrate the truthfulness of the null hypothesis (H_0: the analytical method is not subject to systematic error).

As reported in Table 4, the t-values were found to be lower than the critical values for the target analyte at the studied concentration levels, demonstrating the absence of evidence of systematic errors in analyte quantification.

Table 4 - Results of the trueness study at the different concentration levels

Analyte	Mean value (x) ng/l	Nr of samples (n)	Nr of degrees of freedom	Theoretical value (μ) ng/l	Standard Equation of samples (SD) ng/l	Calculated t-value	Critical t_4 P=0.05	Decision
Diclofenac	2.94	15	10	2.91	0.41	0.29	2.23	OK
	88.4	15	10	87.3	7.3	0.59	2.23	OK

5.6 Recovery

The results of the recovery experiments, carried out according to section 3.5, are reported in Table 5.

Table 5 - Recovery

Analyte	Spike Level (ng/l)	Mean Recovery (%) (n=3)	SD (ng/l)	RSD (%)
Diclofenac	9.7	71.2	3.61	5.1

5.7 Repeatability and intermediate precision

For repeatability and intermediate precision, QCs at two concentration levels were tested on five different days. For each sample, three replicate injections were made. Using one-way ANOVA, the results were obtained as shown in Table 6.

Table 6 – Relative standard deviations of repeatability and intermediate precision

Concentration level	Relative standard deviation (RSD_{Rep}) of Repeatability measurements	Relative standard deviation (RSD_{Ip}) of Intermediate precision measurements

Low	2.6	14.6
High	3.1	8.2

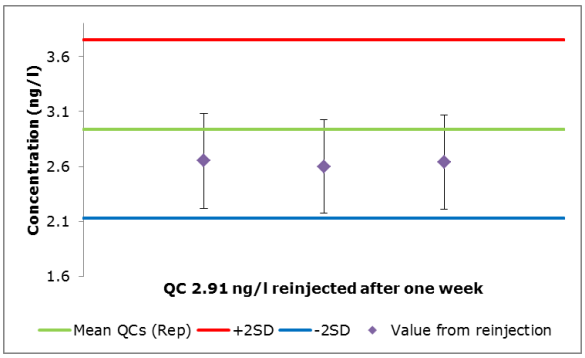
5.8 Stability

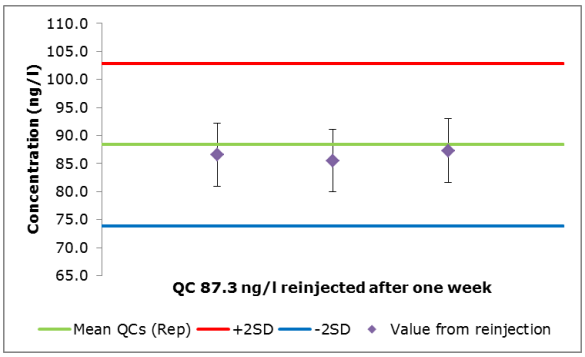
5.8.1 Stability of extract

A graphical representation of the stability of extracted data is reported in Figure 4.

Since the concentrations evaluated by reinjections after storage for one week at 4°C in the dark fall within about twice the standard deviations of the concentration of QCs used for repeatability evaluation, the extracts could be considered as being stable in the studied conditions.

Figure 4 – Stability of extract after one week storage at 4°C in the dark



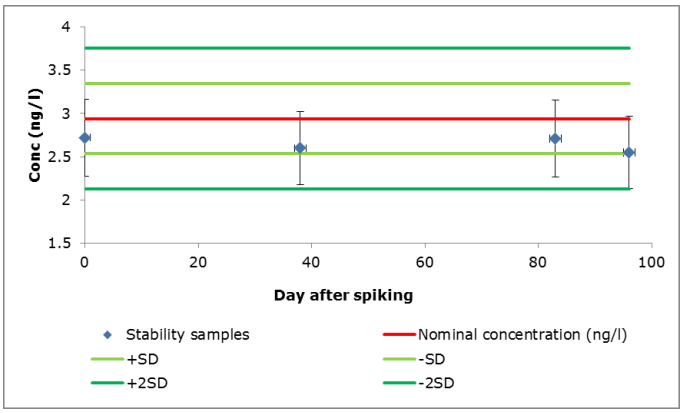


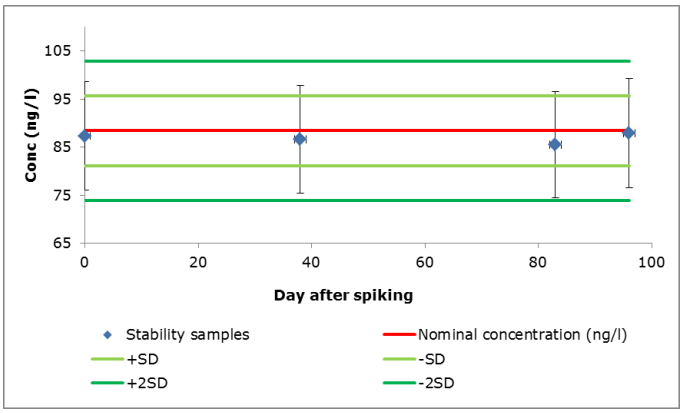
5.8.2 Stability of water samples

A graphical representation of stability data of diclofenac in water samples is reported in Figure 5.

Since the concentrations in stability samples fall within about twice the standard deviation of the concentrations of quality control samples used for repeatability evaluation, the samples could be considered stable.

Figure 5 – Stability of water samples





5.9 Uncertainty estimation

The estimation of measurement uncertainty was carried out following a top-down approach based on in-house validation data. The data derived from the validation of the method includes the sample preparation, standard dilution, and chromatographic and MS detection variability. This approach takes account of the relative standard deviation of repeatability, intermediate precision and trueness measurements. The uncertainty of prepared standard stock solution is also considered, as another source of uncertainty.

The expanded uncertainty was calculated using the following formula:

$$U=k\sqrt{(u_{Tness})^2+(u_{Rep})^2+(u_{ip})^2+(u_{Std})^2}$$, where:

U is the expanded relative uncertainty,

k is the coverage factor (k=2),

u_{Tness} is the relative standard uncertainty of trueness estimation,

u_{Rep} is the relative standard uncertainty of repeatability,

u_{Ip} is the relative standard uncertainty of intermediate precision and

u_{Std} is the relative standard uncertainty related to calibration standards including weighing, purity and dilution contributions.

5.9.1 Uncertainty of trueness

u_{Tness} is the standard relative uncertainty associated with the trueness, and has been calculated from the standard deviation (SD) of the mean of QCs used for trueness as follows:

$$u_{Tness}=\sqrt{\frac{(SD)^2}{c\sqrt{n}}}$$, where:

C is the average result of the QCs analyses,

n is the number of QCs that have been analysed.

5.9.2 Uncertainty of repeatability and intermediate precision

u_{Rep} and u_{Ip} are the standard relative uncertainties related to repeatability and intermediate precision measurements, respectively. Individual contributions are calculated according to the following equations:

$$u_{Rep}=\sqrt{\frac{(RSD_{Rep})^2}{n_{Rep}}}$$

and

$$u_{Ip}=\sqrt{\frac{(RSD_{Ip})^2}{n_{days}}}$$, where:

RSD_{Rep} standard deviation of repeatability measurements

RSD_{Ip} standard deviation of intermediate precision measurements

n_{Rep} number of total replicates for repeatability measurements

n_{days} number of days for intermediate precision measurements.

5.9.3 Uncertainty of standard

u_{Std} is the standard relative uncertainty associated with diclofenac and diclofenac $^{13}C_6$ standards used, and is calculated as follows:

$$u_{Std}=\sqrt{(u_{Diclofenac})^2+(u_{IS})^2+(u_{Flask})^2+2(u_{Syringe})^2+(u_{Balance})^2}$$, where:

$u_{Diclofenac}$ is the uncertainty relative to the diclofenac standard used in the calibration solution preparation. The Manufacturer's Certificate of Analysis reports the $U_{Diclofenac}$ as having an expanded combined uncertainty at 95% confidence level equal to 0.5%. Consequently:

$$u_{diclofenac}=U_{diclofenac}/2 = 0.25.$$

u_{IS} is the uncertainty relative to the diclofenac $^{13}C_6$ standard used as internal standard.

The Manufacturer's Certificate of Analysis reports the $U_{IS,}$ as expanded combined uncertainty at 95% confidence level, with n=6, equal to ±0.5%. Consequently, the relative uncertainty is 0.2, according to the following formula:

$$u_{diclofenac\ ^{13}C6}=U_{diclofenac\ ^{13}C6}/2.45 = 0.2$$

u_{Flask} is the uncertainty related to the volumetric flask. The tolerance of the class A 10-ml volumetric flask (given by the manufacturer) is set to 0.04 ml. As this value is not correlated with a confidence level or distribution information, a rectangular distribution is assumed. For the uncertainty estimation, the relative tolerance value (i.e. 0.4%) must by divided by $\sqrt{3}$, giving a value of 0.231 for u_{Flask}.

$u_{Syringe}$ is the uncertainty related to the withdrawal of the standard solution using a 1 000-µL Hamilton syringe. As these syringes are manufactured to be accurate within ± 1% of the nominal value and this value is not correlated with a confidence level or distribution information, a rectangular distribution is assumed. For the uncertainty estimation the relative uncertainty (i.e. 1 ml/1 000 ml*100=0.1%) must by divided by $\sqrt{3}$, giving a value for $u_{Syringe}$ equal to 0.058.

$u_{Balance}$ is the contribution from the weight of standards, and it is due to the linearity uncertainty of the balance from Calibration Certificate. From balance linearity (± 0.03 mg), a rectangular distribution is assumed to obtain a standard uncertainty; this contribution is considered twice, once for the tare and once for the gross weight. According to this approach, the $u_{Balance}$ as RSD % is:

$$u_{Balance}=\sqrt{2 \times \left(\frac{0.03}{\sqrt{3}}\right)^2}=0.02$$

$$u_{Balance}=\frac{0.02mg}{10\ mg}\% = 0.2\%.$$

As the repeatability and trueness of the measurement were estimated for two different concentration levels, the uncertainty can also be estimated separately for low and high concentration levels.

5.10 Final uncertainty budget

The dominating uncertainty contribution came from the trueness assessment, which was 6.5 and 3.7% at low and high concentration levels respectively. The other uncertainty contributions from repeatability, trueness and standard preparation were around 1%.

The expanded relative uncertainty was estimated as 13 and 8% at low and high concentration levels respectively, based on 15 replicate measurements on 5 days, and as 30 and 18% for a single measurement performed on a single day.

The detailed uncertainty budget and results of the uncertainty estimations are reported in Table 7.

Table 7 - Uncertainty budget and estimated uncertainty of measurements

Estimated uncertainty	Values	
	Low concentration level (2.91 ng/l)	**High concentration level (87.3 ng/l)**
u_{Tness} (%)	0.5	1.5
u_{Rep} (%)	0.7	0.8
u_{Ip} (%)	6.5	3.7
u_{Std} (%)	0.5	0.5
Expanded Relative Uncertainty (U, %)) (k=2), (n_1=15, n_2=5)	**13**	**8**
Expanded Relative Uncertainty (U, %) (k=2), (n_1=1, n_2=1)	**30**	**18**

5.11 Remark on LoQ

As regards the afore-described method it has to be annotated that in 2013, the French standardisation body AFNOR, has published a standard for the analysis of medical waste products, i.e. *XP T90-223: Water quality - Determination of Some drug residues in the water-Dissolved fraction - Method using solid-phase extraction (SPE) and liquid chromatography tandem mass spectrometric analysis with investigation (LC-MS / MS)*. In addition to that the French Reference Laboraratory for Water (AQUAREF) has test in two intercomparison exercises in 2009 and 2012, respectively, a method available on the website AQUAREF (http://www.aquaref.fr/system/files/Fiche%20MA14-230309%20.pdf).

While both methods feature similar results in terms of extraction recovery, differences can be observed with regard to the limits of quantification.

This may be ascribed to the different water matrices used (artificial mixtures in case of the method described above vs. processed real water samples). In addition, the approach used to evaluate the limit of quantification is different form the procedures applied in France.

However, in both scenarios, the LOQs of the methods meet the requirements for the implementation of a watch list monitoring, i.e.10 ng/l vs. 0.2 ng/l.

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European Commission
EUR 26902 – Joint Research Centre – Institute for Environment and Sustainability

Title: Water Framework Directive. Watch list method. Analysis of diclofenac in water

Author(s): S. Tavazzi, B. Paracchini, G. Suurkuusk, G. Mariani, R. Loos, M. Ricci and B. M. Gawlik

Luxembourg: Publications Office of the European Union

2014 – 24 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series –ISSN 1831-9424 (online)

ISBN 978-92-79-43859-2 (PDF)

doi:10.2788/19818

Abstract

Validation of an analytical method is a necessary step in controlling the quality of quantitative analysis. Method validation is an established process which provides documentary evidence that a system fulfils its pre-defined specification, or shows that an analytical method is acceptable for its intended purpose. The purpose of the present study was to validate an SPE-LC-MS/MS method for the determination of diclofenac in surface water samples according to the requirements laid down in ISO 17025. The calibration curves, working ranges, recoveries, detection and quantification limits, trueness as well as repeatability were determined. The uncertainty budget was estimated following a top-down approach based on in-house validation data. The expanded relative uncertainty was estimated as 13 and 8% at low and high concentration levels respectively, based on 15 replicate measurements on 5 days, and as 30 and 18% for a single measurement performed on a single day.

LB-NA-26902-EN-N

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DOI:10.2788/19818

ISBN 978-92-79-43859-2