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2,6-Difluorosubstituted benzoylurea compounds – Determination of 2,6-difluorobenzoic acid in urine by GC-MS

Biomonitoring Method – Translation of the German version from 2022

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method.

The analytical method described herein allows for the sensitive and specific determination of 2,6-difluorobenzoic acid (2,6-DFBA) in the urine of individuals occupationally exposed to 2,6-difluorosubstituted benzoylurea compounds. 2,6-DFBA is determined by gas chromatography with mass-spectrometric detection (GC-MS) after derivatisation. To this end, urine samples are mixed with an internal standard (ISTD), acidified, and purified using solid-phase extraction (SPE). The samples are derivatised with *N*-methyl-*N*-*tert*-butyldimethylsilyltrifluoroacetamide (MTBSTFA), and the analyte concentration is quantified by GC-MS. The ISTD used is 5-bromo-2-fluorobenzoic acid. Calibration is performed using standard solutions prepared in pooled urine.

1 Characteristics of the method

Matrix	Urine
Analytical principle	Gas chromatography with mass-spectrometric detection (GC-MS)

Parameter and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
Bistrifluron (*N*-[[2-Chloro-3,5-bis(trifluoromethyl)phenyl]carbamoyl]-2,6-difluorobenzamide)	201593-84-2		
Chlorfluazuron (*N*-[[3,5-Dichloro-4-[3-chloro-5-(trifluoromethyl)pyridine-2-yl]oxyphenyl]-carbamoyl]-2,6-difluorobenzamide)	71422-67-8		
Diflubenzuron (*N*-[(4-Chlorophenyl)carbamoyl]-2,6-difluorobenzamide)	35367-38-5		
Fluazuron (*N*-[[4-Chloro-3-[3-chloro-5-(trifluoromethyl)pyridine-2-yl]oxyphenyl]-carbamoyl]-2,6-difluorobenzamide)	86811-58-7		
Flucycloxuron (*N*-[[4-[[(*E*)-[(4-Chlorophenyl)cyclopropylmethylidene]amino]oxymethyl]-phenyl]carbamoyl]-2,6-difluorobenzamide)	113036-88-7; 94050-52-9		
Flufenoxuron (*N*-[[4-[2-Chloro-4-(trifluoromethyl)phenoxy]-2-fluorophenyl]carbamoyl]-2,6-difluorobenzamide)	101463-69-8	2,6-DFBA	385-00-2
Hexaflumuron (*N*-[[3,5-Dichloro-4-(1,1,2,2-tetrafluoroethoxy)phenyl]carbamoyl]-2,6-difluorobenzamide)	86479-06-3		
Lufenuron (*N*-[[2,5-Dichloro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]carbamoyl]-2,6-difluorobenzamide)	103055-07-8		
Novaluron (*N*-[[3-Chloro-4-[1,1,2-trifluoro-2-(trifluoromethoxy)ethoxy]phenyl]-carbamoyl]-2,6-difluorobenzamide)	116714-46-6		
Noviflumuron (*N*-[[3,5-Dichloro-2-fluoro-4-(1,1,2,3,3,3-hexafluoropropoxy)phenyl]-carbamoyl]-2,6-difluorobenzamide)	121451-02-3		
Penfluron (2,6-Difluoro-*N*-[[4-(trifluoromethyl)phenyl]carbamoyl]benzamide)	35367-31-8		
Teflubenzuron (*N*-[(3,5-Dichloro-2,4-difluorophenyl)carbamoyl]-2,6-difluorobenzamide)	83121-18-0		

Reliability data

2,6-DFBA

Within-day precision:	Standard deviation (rel.)	s_w = 7.74%, 4.15%, or 2.93%
	Prognostic range	u = 21.5%, 11.5%, or 8.13%
	at a spiked concentration of 2.5 µg, 20 µg, or 100 µg 2,6-DFBA per litre of urine and n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 8.92%, 2.06%, or 3.75%
	Prognostic range	u = 24.8%, 5.73%, or 4.40%
	at a spiked concentration of 2.5 µg, 20 µg, or 100 µg 2,6-DFBA per litre of urine and n = 5 determinations	
Accuracy:	Recovery rate (rel.)	r = 99.2%, 101%, or 101%
	at a spiked concentration of 2.5 µg, 20 µg, or 100 µg 2,6-DFBA per litre of urine and n = 5 determinations	
Detection limit:	0.416 µg 2,6-DFBA per litre of urine	
Quantitation limit:	1.32 µg 2,6-DFBA per litre of urine	

2 General information on the hazardous substances

Benzoylphenylurea derivatives are chitin-synthesis inhibitors used worldwide as pesticides in plant and livestock production as well as for wood protection due to their low mammalian toxicity. The compounds inhibit the transport of UDP-*N*-acetyl glucosamine across the biomembranes of cells and, consequently, chitin synthesis. In this way, moulting is prevented, resulting in the death of the larvae or pupae or in non-viable adults. The effect of the benzoylphenylurea derivatives is therefore not immediate, but rather occurs only during the transition to the next stage of development (Cohen 2001; Sun et al. 2015).

In 1975, diflubenzuron became the first member of this group of active substances to be marketed, using the trade name Dimilin™. Since then, more than 10 000 benzoylphenylurea derivatives have been synthesised, although only fifteen compounds have achieved commercial importance. Twelve of the commercially approved benzoylphenylurea derivatives have a 2,6-difluorosubstitution on the benzoyl ring, as this substitution pattern exhibits the highest larvicidal effect (Figure 1) (Sun et al. 2015).

There is no data available concerning the metabolism of 2,6-difluorosubstituted benzoylphenylurea derivatives in humans. Depending on their respective areas of application, 2,6-difluorosubstituted benzoylurea compounds have been investigated with regard to absorption, metabolism, and elimination in experimental animals, livestock, and house pets. The active substances, administered either dermally or orally, were, depending on the specific substance, administered dosage, application type, and the species in question, absorbed, metabolised, and excreted in varying degrees. Elimination took place either unmetabolised with the faeces or metabolised with the urine.

2,6-DFBA has been confirmed as a urinary metabolite of fluazuron in rats (FAO 1998; WHO 1998); of diflubenzuron in rats, sheep, cattle, and pigs (FAO 2002 a, b; Willems et al. 1980); of teflubenzuron in rats (Koerts et al. 1997); of novaluron in rats (FSCJ 2003); of hexaflumuron in rats (EMA 2015); of flufenoxuron in rats (FSCJ 2007); and of lufenuron in rats and goats (FAO 2018). For bistrifluron, chlorfluazuron, flucycloxuron, noviflumuron, and penfluron, there are currently no data available for metabolism in mammals. It can, however, be assumed that these substances are also partially metabolised to 2,6-DFBA and excreted with the urine.

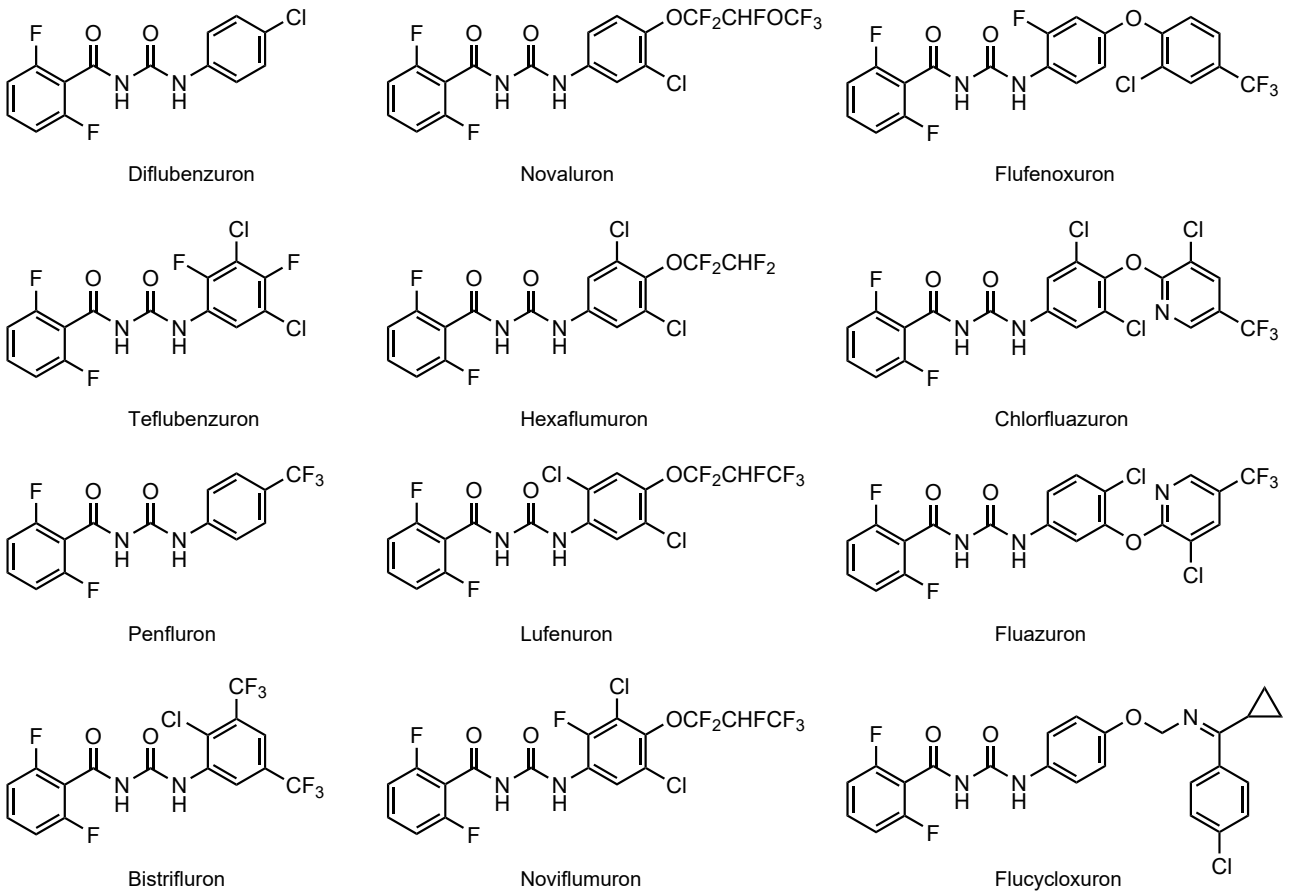

Fig. 1 Chemical structures of the 2,6-difluorosubstituted benzoylurea compounds that have achieved commercial importance (according to Sun et al. 2015)

As such, 2,6-DFBA constitutes a sum parameter for the detection of exposures to 2,6-difluorosubstituted benzoylurea compounds. As long as only one hazardous substance is applied at a time, the detection of 2,6-DFBA indicates specific exposure.

The metabolism of diflubenzuron has been investigated most thoroughly. Figure 2 shows the metabolism scheme of diflubenzuron. Besides 2,6-DFBA, the figure shows additional metabolic products of diflubenzuron which have been detected in various mammalian species (FAO 2002 a, b; Willems et al. 1980).

With regard to workplace exposure, no data have been published in the scientific literature to date and no data are currently available on background exposure in the general population. Since 2,6-difluorosubstituted benzoylurea compounds are used for pest control in plant cultivation, animal husbandry, and building preservation, exposure can occur both in the workplace and in private settings, such as via residues on food products. During the application of diflubenzuron against the oak processionary moth, both inhalation and dermal exposure was determined in the workers examined (Roitzsch et al. 2019).

According to the German *Datenbank der gemeldeten Biozidprodukte* (Database of Authorised Biocidal Products), diflubenzuron, flufenoxuron, and hexaflumuron are available on the market until the end of 2024 (BAuA 2022) and can be applied as products to control pests (Product Type 18, diflubenzuron and hexaflumuron), as products in veterinary hygiene (Product Type 3, diflubenzuron), and as wood preservatives (Product Type 8, flufenoxuron). In Germany, lufenuron is applied as a veterinary drug against fleas and worms in dogs and cats (BfArM 2022).

Furthermore, in Europe, diflubenzuron and teflubenzuron are used against parasites in fish farming (NVI 2016), and, in Australia, bistrifluron, chlorfluazuron, hexaflumuron, and novaluron are used against termites (APVMA 2022).

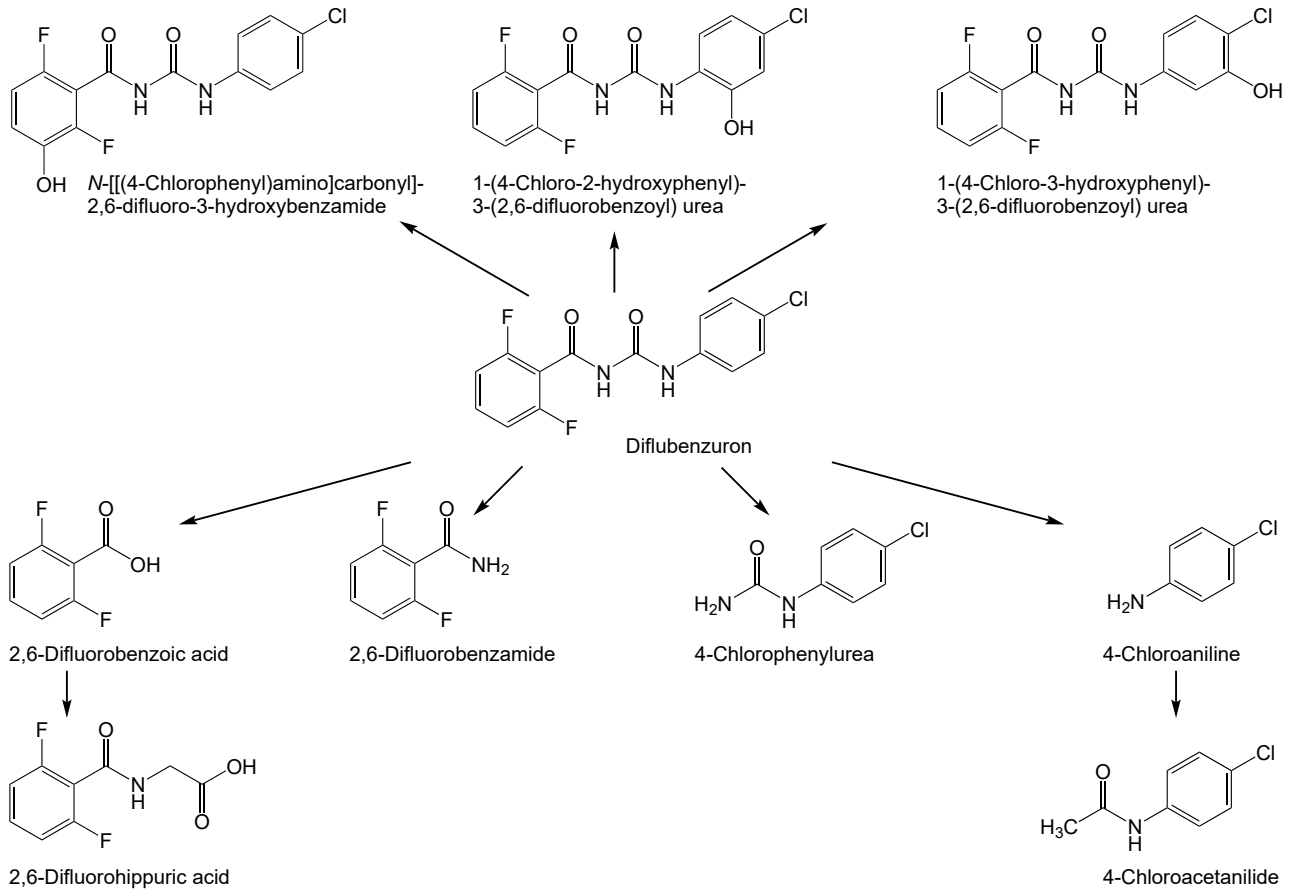

Fig. 2 The metabolism of diflubenzuron in mammals (according to FAO 2002 b)

Initial studies using the method described herein show that 2,6-DFBA is an appropriate parameter to detect occupational exposure to 2,6-difluorosubstituted benzoylphenylurea derivatives. The results presented in Table 1 show 2,6-DFBA concentrations in 34 urine samples from three workers after handling and spraying diflubenzuron against the oak processionary moth.

Tab. 1 2,6-DFBA in the urine of three workers after occupational exposure to diflubenzuron (n = 34)

Analyte	Mean ± standard deviation [µg/l]	Range [µg/l]	Median [µg/l]
2,6-DFBA	42.1 ± 47.2	0.786–151	12.8

3 General principles

The analytical method described herein allows for the sensitive and specific determination of 2,6-DFBA in the urine of individuals occupationally exposed to 2,6-difluorosubstituted benzoylurea compounds. 2,6-DFBA is determined by GC-MS after derivatisation. To this end, urine samples are mixed with an ISTD, acidified, and purified using solid-phase extraction (SPE). The samples are derivatised with MTBSTFA and the analyte concentration is quantified by

GC-MS. The ISTD used is 5-bromo-2-fluorobenzoic acid. Calibration is performed using standard solutions prepared in pooled urine.

4 Equipment, chemicals, and solutions

4.1 Equipment

- Gas chromatograph Agilent 8890 GC with autosampler (7693A) and mass-spectrometric detector (5977B MSD); evaluation software (Enhanced ChemStation) (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Capillary gas-chromatographic column: stationary phase: 5%-phenyl-methylpolysiloxane; length: 60 m; inner diameter: 0.25 mm; film thickness: 0.25 µm (e.g. HP-5ms, No. 19091S-436, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Laboratory centrifuge (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- Drying cabinet (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- Analytical balance (e.g. BP 211D, Sartorius AG, Göttingen, Germany)
- Microlitre pipettes, 10–100 µl and 100–1000 µl with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Multipette® Plus (e.g. Eppendorf AG, Hamburg, Germany)
- pH meter (e.g. SCHOTT AG, Mainz, Germany)
- Vortex mixer (e.g. VWR International GmbH, Darmstadt, Germany)
- SPE vacuum manifold (e.g. IST VacMaster, Biotage Sweden AB, Uppsala, Sweden)
- SPE cartridges (e.g. ISOLUTE ENV+, 100 mg, 3 ml, Biotage Sweden AB, Uppsala, Sweden)
- 10-ml, 50-ml, and 100-ml volumetric flasks (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 5-ml sample vials (e.g. VWR International GmbH, Darmstadt, Germany)
- 1.8-ml GC vials with screw caps (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- Various beakers and measuring cylinders (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- Polypropylene urine cups (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- 10-ml urine tubes (e.g. Urine Monovette®, Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade.

- 2,6-Difluorobenzoic acid (2,6-DFBA) 98% (e.g. No. D57450, Merck KGaA, Darmstadt, Germany)
- 5-Bromo-2-fluorobenzoic acid 97% (e.g. No. 636452, Merck KGaA, Darmstadt, Germany)
- *N*-*tert*-Butyldimethylsilyl-*N*-methyltrifluoroacetamide with 1% *tert*-butyldimethylsilyl chloride (MTBSTFA with 1% TBDMSCl) ≥ 95% (e.g. No. 375934, Merck KGaA, Darmstadt, Germany)
- Hydrochloric acid 25% (e.g. No. 100316, Merck KGaA, Darmstadt, Germany)
- Methanol MS SupraSolv® (e.g. No. 100837, Merck KGaA, Darmstadt, Germany)

- Acetonitrile, anhydrous (e.g. No. 83676.269, VWR International GmbH, Darmstadt, Germany)
- Toluene MS SupraSolv® (e.g. No. 100849, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Milli-Q plus VE System (> 18 MΩ), Merck KGaA, Darmstadt, Germany)
- Pooled urine from individuals with no known exposure to 2,6-difluorosubstituted benzoylurea compounds, proteins frozen out and filtered off
- Helium 5.0 (e.g. Linde GmbH, Pullach, Germany)

4.3 Solutions

- Diluted hydrochloric acid (1 : 20 (v/v), 0.4 mol/l)
  80 ml of ultra-pure water are placed in a 100-ml volumetric flask and 5 ml of 25% hydrochloric acid are added using a pipette. The flask is then made up to the mark with ultra-pure water.
- Diluted hydrochloric acid (pH 1.5)
  200 ml of ultra-pure water are placed in a beaker. The pH value of the solution is adjusted to pH 1.5 by adding 25% hydrochloric acid.
- Diluted hydrochloric acid (pH 1.5) with 5% (v/v) methanol
  5 ml of methanol are placed in a 100-ml volumetric flask, which is then made up to the mark with diluted hydrochloric acid (pH 1.5).

4.4 Internal standard (ISTD)

- ISTD stock solution (500 mg/l)
  Exactly 25 mg of 5-bromo-2-fluorobenzoic acid are weighed out into a 50-ml volumetric flask, which is then made up to the mark with acetonitrile and thoroughly mixed.
- ISTD spiking solution (10 mg/l)
  200 µl of the ISTD stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile.

The stock and spiking solutions of the ISTD can be stored at –20 °C for at least six months without substance loss.

4.5 Calibration standards

- Stock solution (500 mg/l)
  Exactly 25 mg of 2,6-DFBA are weighed out into a 50-ml volumetric flask, which is then made up to the mark with acetonitrile and thoroughly mixed.
- Spiking solution I (1000 µg/l)
  20 µl of the stock solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile and thoroughly mixed.
- Spiking solution II (100 µg/l)
  1000 µl of spiking solution I are pipetted into a 10-ml volumetric flask, which is then made up to the mark with acetonitrile and thoroughly mixed.

The stock solution and the spiking solutions of 2,6-DFBA can be stored at –20 °C for at least six months without analyte loss.

The calibration standards are prepared using pooled urine from individuals with no known exposure to 2,6-difluoro-substituted benzoylurea compounds after the proteins have been frozen out and filtered off. To this end, the spiking solutions are pipetted into 5-ml screw-cap vials according to the pipetting scheme given in Table 2 and pooled urine is added. The calibration standards are processed analogously to the urine samples as described in Section 5.

Tab. 2 Pipetting scheme for the preparation of calibration standards for the determination of 2,6-DFBA in urine

Calibration standard	Spiking solution	Volume of spiking solution [µl]	Volume of pooled urine [µl]	Analyte concentration [µg/l]
0	–	–	2000	0
1	II	50	1950	2.5
2	II	100	1900	5
3	I	40	1960	20
4	I	120	1880	60
5	I	200	1800	100

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine samples are collected in sealable urine cups and–if necessary–drawn into Urine Monovettes®. Urine samples from occupationally exposed workers are collected at the end of the workday or–in cases of prolonged exposure–at the end of the workweek.

The urine samples can be stored in the refrigerator at 4 °C for up to three days. For long-term storage, it is recommended to store the urine samples at –20 °C.

5.2 Sample preparation

The urine samples are brought to room temperature and thoroughly mixed. 2 ml of the urine sample are placed in a 5-ml screw-cap vial, adding 10 µl of the ISTD spiking solution and 1 ml of diluted hydrochloric acid (0.4 mol/l) to each vial. The vials are sealed and the samples are mixed on a vortex mixer.

The SPE cartridges are conditioned with 1.3 ml of acetonitrile and 3 ml of diluted hydrochloric acid (pH 1.5). The acidified urine samples, to which ISTD has been added, are loaded onto the SPE cartridges. The cartridges are washed first with 1 ml of the diluted hydrochloric acid with 5% methanol and then with 1 ml of the diluted hydrochloric acid (pH 1.5). After applying a gentle vacuum, the cartridges are centrifuged at 2500 × *g* for ten minutes and dried under vacuum for one hour. Afterwards, the analyte is eluted by washing the cartridge twice with 900 µl of acetonitrile in 1.8-ml sample vials, and the eluates are evaporated to dryness under a stream of nitrogen. The residues are mixed with 10 µl of MTBSTFA each and the samples are derivatised at 80 °C for one hour. After adding 1 ml of toluene, the samples are mixed on a vortex mixer and the analyte content is determined by GC-MS.

In the samples prepared for analysis, a precipitate may remain which does not dissolve in toluene. This precipitate is separated by centrifugation at 3000 × *g* and the clear supernatant is used for analysis by GC-MS.

6 Operational parameters

6.1 Gas chromatography

Capillary column:	Material:	Fused silica
	Stationary phase:	5%-phenyl-methylpolysiloxane
	Length:	60 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
Detector:	Mass-selective detector (MSD)	
Temperatures:	Column:	Initial temperature of 90 °C, 1 min isothermal; increase at a rate of 25 °C/min to 110 °C, 5 min isothermal; increase at a rate of 5 °C/min to 150 °C, 10 min isothermal; increase at a rate of 10 °C/min to 250 °C, 0.1 min isothermal; increase at a rate of 30 °C/min to 315 °C, 7 min at final temperature
	GC transferline:	280 °C
	Injector:	280 °C
	Interface:	280 °C
Carrier gas:	Helium 5.0	
Flow rate:	1.4 ml/min	
Injection:	1.5 µl, splitless, split opened after 1 min	

6.2 Mass spectrometry

Ionisation:	Electron-impact ionisation
Ionisation energy:	70 eV
Source temperature:	230 °C
Quadrupole temperature:	150 °C
Dwell time:	100 ms
Detection mode:	Selected Ion Monitoring (SIM)

All parameters are instrument-specific and must be individually optimised by the user. The given parameters can only serve as cursory guidance.

7 Analytical determination

For the analytical determination of the urine samples prepared as described in Section 5, 1.5 µl of each processed sample is injected into the GC-MS system. The time courses of the ion traces listed in Table 3 are recorded in SIM mode.

Tab. 3 Retention times and detected ion traces for the determination of 2,6-DFBA in urine

Analyte/ISTD	Retention time [min]	Ion trace [*m/z*]	
		Quantifier	Qualifier
2,6-DFBA	22.29	215	113; 141
5-Bromo-2-fluorobenzoic acid	31.81	277	175; 196

The retention times given in Table 3 are intended only as a point of reference. Users must ensure proper separation performance of the capillary column used and the resulting retention behaviour of the analyte. Figures 3 and 4 show representative GC-MS chromatograms of ultra-pure water and a pooled urine sample, respectively, spiked with 20 µg/l. These figures also depict the reliable separation of 2,6-DFBA from the isomeric DFBAs (2,3-DFBA; 2,4-DFBA; 2,5-DFBA; 3,4-DFBA; 3,5-DFBA). Figure 5 shows the chromatogram of a native urine sample from a worker exposed to diflubenzuron (measured 2,6-DFBA concentration: 27.6 µg/l urine). A different temperature program was used to measure the native urine sample, leading to shorter retention times for both the analyte and the ISTD.

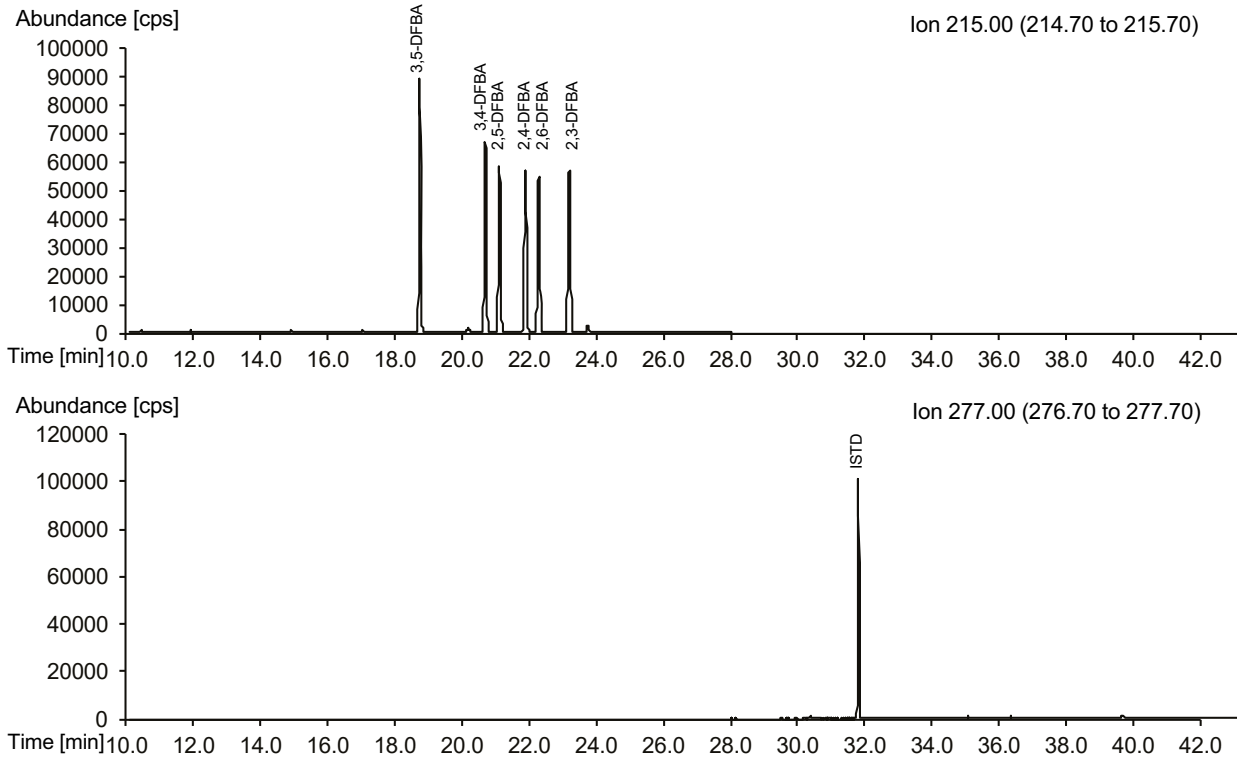

Fig. 3 GC-MS chromatogram of ultra-pure water spiked with 20 µg DFBA/l

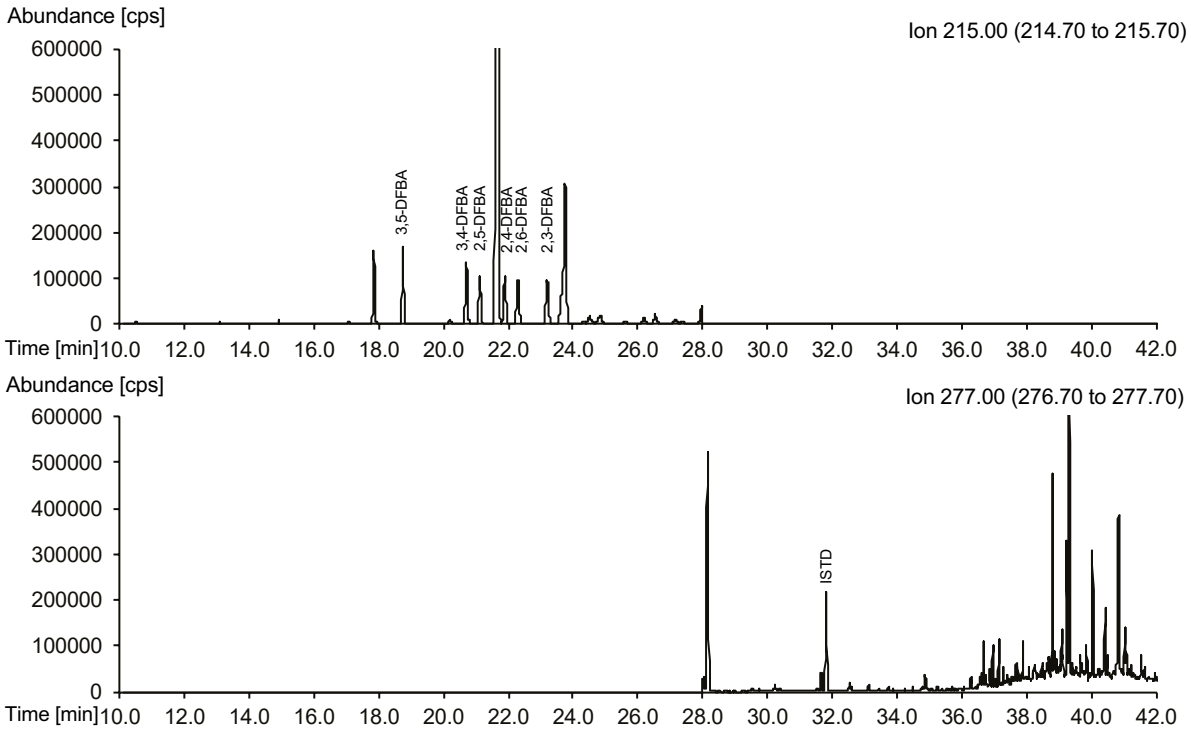

Fig. 4 GC-MS chromatogram of pooled urine spiked with 20 µg DFBA/l

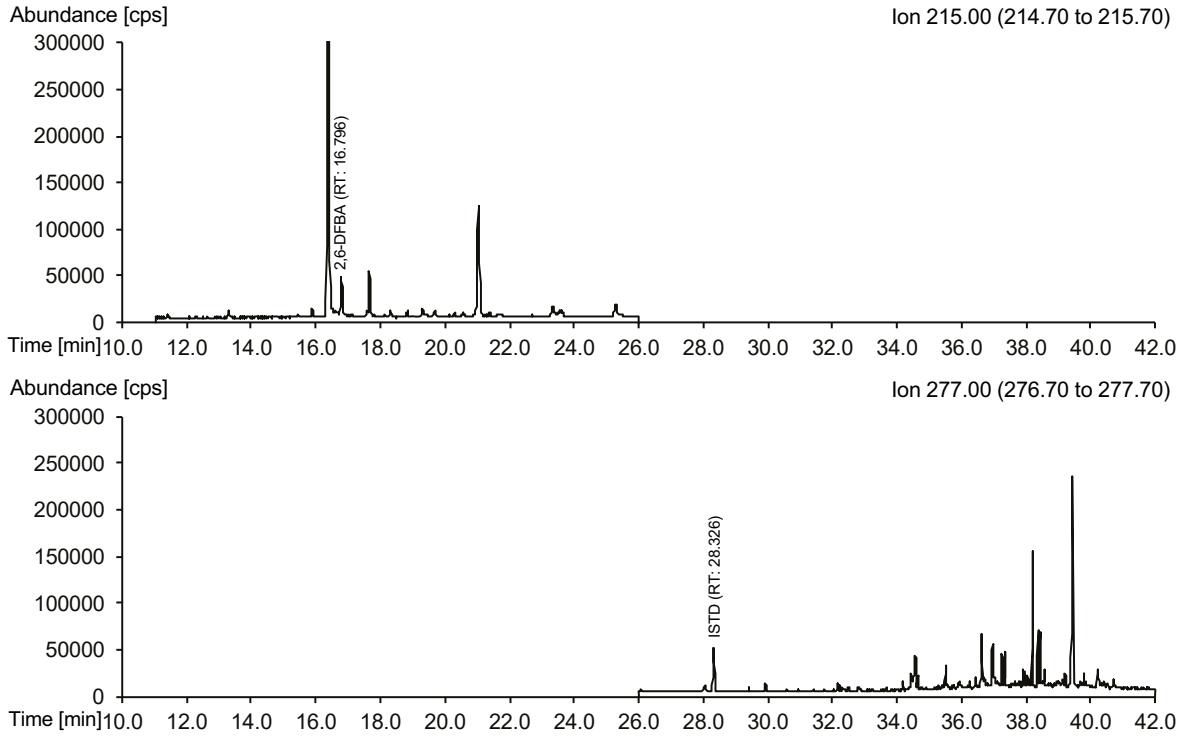

Fig. 5 GC-MS chromatogram of a native urine sample following occupational exposure to diflubenzuron (measured 2,6-DFBA concentration: 27.6 µg/l urine)

8 Calibration

For the calibration of the method, the calibration standards prepared in pooled urine as described in Section 4.5 are processed according to Section 5.2 and analysed by GC-MS. The calibration curve is obtained by plotting the peak area ratio of the analyte and the ISTD against the respective analyte concentration. The calibration curve is linear in the relevant concentration range under the analytical conditions described. Figure 6 shows a representative calibration curve for the determination of 2,6-DFBA in urine.

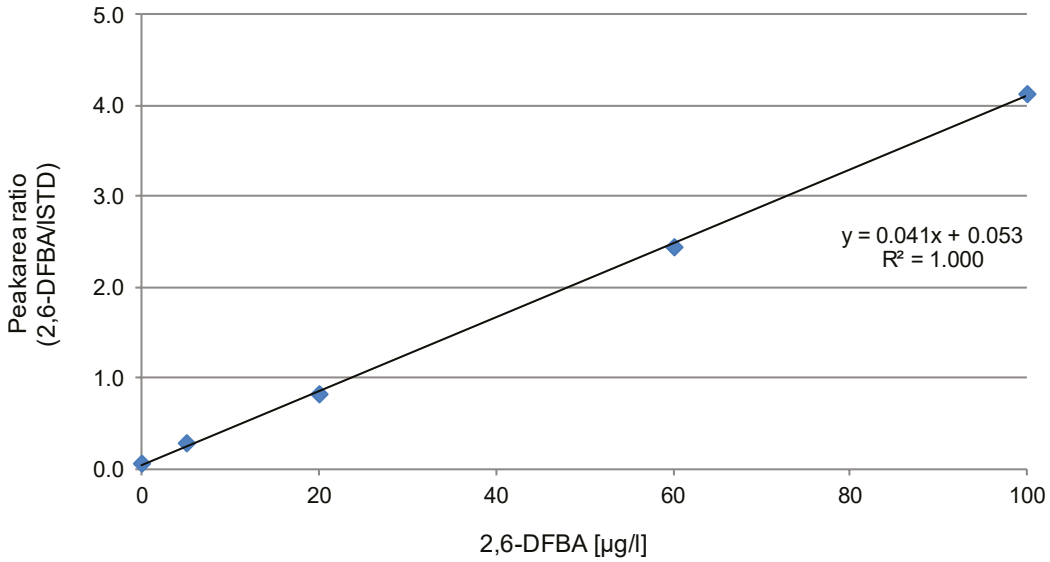

Fig. 6 Calibration curve for the determination of 2,6-DFBA in a concentration range of 2.5 µg/l to 100 µg/l

9 Calculation of the analytical results

The analyte concentration in the urine samples is calculated using the calibration function corresponding to the analytical run in question (Section 8). The peak area ratio of 2,6-DFBA and the ISTD is entered into the calibration function to yield the analyte concentration in µg/l. Any reagent blank values observed are deducted from the analytical results of the real samples.

If the measured result lies above the calibration range, the corresponding sample is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

To check precision, two quality-control samples with low or high analyte concentrations are included in each analytical run. As such reference material is not commercially available, it must be prepared in the in-house laboratory by spiking pooled urine from individuals with no known exposure to 2,6-difluorosubstituted benzoylurea compounds with 2,6-DFBA. The spiked concentration of the materials must lie within the relevant concentration range. The quality-control materials thus prepared are aliquoted (2 ml) into 5-ml screw-cap vials and frozen at –20 °C until analysis. The nominal value and tolerance range (mean value ± two standard deviations) of each individual quality-control material

are determined in a pre-analytical period (Bader et al. 2010). The quality-control materials can be stored at –20 °C for at least six months without analyte loss.

Additionally, a reagent blank, consisting of 2 ml of ultra-pure water, is included in each analytical run.

11 Evaluation of the method

The reliability of the method was confirmed by comprehensive validation as well as by replication and verification of the method in a second, independent laboratory.

11.1 Precision

Within-day precision

To determine within-day precision, pooled urine was spiked with the analyte at low, medium and high analyte concentrations. The samples were each processed five times in one day. The results are summarised in Table 4.

Tab. 4 Within-day precision for the determination of 2,6-DFBA in urine (n = 5)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
2,6-DFBA	2.5	7.74	21.5
	20	4.15	11.5
	100	2.93	8.13

Day-to-day precision

Day-to-day precision was also determined at low, medium, and high analyte concentrations. For the determination of day-to-day precision, the spiked urine samples were each investigated on five different days. The precision data thus calculated are presented in Table 5.

Tab. 5 Day-to-day precision for the determination of 2,6-DFBA in urine (n = 5)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
2,6-DFBA	2.5	8.92	24.8
	20	2.06	5.73
	100	1.58	4.40

11.2 Accuracy

The accuracy of the method was calculated based on the data used to ascertain day-to-day precision. The urine used for method validation displayed a 2,6-DFBA background concentration of 1.5 µg/l. Accounting for this background level, Table 6 shows the relative recovery rates.

Tab. 6 Relative recovery rates for the determination of 2,6-DFBA in urine (n = 5)

Analyte	Spiked concentration [µg/l]	Measured concentration [µg/l]	Relative recovery rate *r* [%]	
			Mean	Range
2,6-DFBA	2.5	3.97	99.2	92.7–106
	20	22.4	101	99.5–104
	100	104	101	99.6–103

Additionally, recovery experiments using individual urine samples were performed to determine the accuracy of the method. To this end, six individual urine samples were spiked with the analyte at concentrations of 2.5 µg/l, 20 µg/l, and 100 µg/l, and then analysed. The relative recovery rates in the individual urine samples are presented in Table 7.

Tab. 7 Relative recovery rates for the determination of 2,6-DFBA in individual urine samples

Urine	Creatinine level [g/l]	Relative recovery rate *r* [%]		
		2.5 µg/l	20 µg/l	100 µg/l
1	0.51	101	89.8	92.3
2	0.93	101	92.5	92.2
3	1.40	87.5	110	101
4	1.57	84.5	94.7	87.0
5	1.95	115	111	93.2
6	2.20	118	88.6	85.5

To check the method for matrix effects, calibration standards were prepared in ultra-pure water or in pooled urine, processed, and analysed. The slopes of the calibration curves are shown in Table 8 ($R^2 > 0.996$). A comparison of the slopes of the calibration curves suggests that the sample matrix has a negligible impact on the analytical results.

Tab. 8 Comparison of the slopes of the calibration curves in water or pooled urine for the determination of 2,6-DFBA in urine

Analyte	Calibration-curve equation (water)	Calibration-curve equation (pooled urine)	Difference [%]
2,6-DFBA	y = 0.0445x	y = 0.0438x	1.6

11.3 Limits of detection and quantitation

The limits of detection and quantitation for 2,6-DFBA were determined in accordance with DIN 32645 (DIN 2008). To this end, an 11-point calibration in the concentration range from 0 to 10 µg/l was established in pooled urine and analysed. Table 9 shows the limits of detection and quantitation for the analyte thus calculated.

Tab. 9 Limits of detection and quantitation for the determination of 2,6-DFBA in urine (n = 3)

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
2,6-DFBA	0.416	1.32

11.4 Sources of error

No interferences occur in the 2,6-DFBA detection when solvents and reagents of the specified purity grade are used. The separation of 2,6-DFBA from the positional isomers (2,3-DFBA; 2,4-DFBA; 2,5-DFBA; 3,4-DFBA; 3,5-DFBA) was investigated by the developers of this method and was verified for the method presently described (see Figures 3 and 4).

In an interlaboratory comparison, six urine samples from workers occupationally exposed to diflubenzuron were analysed by the developers of the method, the external verifiers of the method, and in a third laboratory. For this

comparison, the third laboratory worked with a different, independent method based on GC-MS/MS. Comparison of the results between the laboratories yielded good consistency of the determined 2,6-DFBA concentrations, which lied between 5 µg/l and 150 µg/l.

The external verifier of the method also subjected the six urine samples of the exposed workers to acid hydrolysis prior to solid-phase extraction (addition of 200 µl of 37% hydrochloric acid, 1 h at 75 °C). There was no evidence of higher 2,6-DFBA concentrations due to hydrolysis.

12 Discussion

The method described herein allows for the selective and sensitive quantitation of 2,6-DFBA in urine. The method has been designed for purposes of occupational health and is only partially suitable for the reliable quantitation of background levels up to 2 µg/l. This limitation is due to the fact that, at concentrations up to about 2 µg 2,6-DFBA per litre of urine, the verification of the analyte via the second ion trace (qualifier, $m/z = 141$) is not possible. The external verifiers of the method found low 2,6-DFBA concentrations in the unspiked urine used for the calibration standards and in two individual (unspiked) urines used for validation. These concentrations lied between the limit of quantitation calculated by the method verifiers of 0.6 µg/l up to about 2 µg/l, and relied on only one ion trace. In any case, the compound cannot be conclusively identified at these low concentrations for which verification using the qualifier was not possible; as such, even an unspecific interference might yield these results.

If necessary, the use of the most modern analytical instrumentation may increase the detection sensitivity of the method. The linear working range of the method reaches up to 100 µg/l and may need to be extended depending on the circumstances of exposure. For example, 2,6-DFBA contents of up to 150 µg per litre of urine were quantified in a diflubenzuron-exposed collective (see Table 1).

5-Bromo-2-fluorobenzoic acid proved to be a suitable ISTD, whereby the external verifier of the method additionally tested 2,5-DFBA as an ISTD. The use of 2,5-DFBA also allows for a sensitive and precise quantitation of the analyte. If necessary, using the deuterated 2,6-DFBA as an ISTD may further improve the precision of the method.

Instruments used Gas chromatograph Agilent 8890 GC with autosampler (7693A) and mass-spectrometric detector (5977B MSD); evaluation software (Enhanced ChemStation) (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

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