Nitroaromatic compounds (nitrobenzene, p-nitrotoluene, p-nitrochlorobenzene, 2,6-dinitrotoluene, o-dinitrobenzene, 1-nitronaphthalene, 2-nitronaphthalene, 4-nitrobiphenyl)

Application	Determination in plasma
Analytical principle	Capillary gas chromatography
Completed in	August 1990

Summary

With this method the most important industrially used nitroaromatic compounds can be specifically determined in one single operation. The detection limits are sufficiently low to enable their determination in the range which is relevant to occupational medicine. Moreover, the practicability of the method makes it suitable for routine health surveillance. One of the merits of this methodic development is that a large number of industrially important organic substances were tested for interference. Among other substances a range of well known chlorinated aromatic hydrocarbons can also be determined on this basis (see Section 9.4).

Nitroaromatic compounds present in blood samples in their free forms are extracted from the separated plasma and simultaneously concentrated using 2,2,4-trimethylpentane (iso-octane). The quantitative separation of the assay substances from the rest of the plasma components and from each other is carried out by gas chromatography in a fused silica capillary with a chemically bonded stationary phase. An electron capture detector serves as a detection system.

Plasma samples of persons who were not previously exposed to nitroaromatic compounds are used for calibration. Specific amounts of nitroaromatic compounds which are relevant to occupational medicine are added to the plasma samples and they are processed and analysed as described. For quantitative evaluation, calibration curves are obtained by plotting the peak areas as a function of the concentrations used.

If necessary, an internal standard can be selected from the numerous substances which can be determined by this method.

Nitrobenzene

Within-series imprecision:	Standard deviation (rel.)	s_w = 1.7 %
	Prognostic range	u = 3.8 %
	At a concentration of 20 µg nitrobenzene per litre blood and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 10.4 %
	Prognostic range	u = 21.7 %
	At a concentration of 20 µg nitrobenzene per litre blood and where n = 20 days	
Inaccuracy:	Recovery rate	r = 78–119 %
Detection limit:	10 µg Nitrobenzene per litre blood	

p-Nitrotoluene

Within-series imprecision:	Standard deviation (rel.)	s_w = 2.2 %
	Prognostic range	u = 4.9 %
	At a concentration of 20 µg p-nitrotoluene per litre blood and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 14.7 %
	Prognostic range	u = 30.7 %
	At a concentration of 20 µg p-nitrotoluene per litre blood and whre n = 20 days	
Inaccuracy:	Recovery rate	r = 85–107 %
Detection limit:	15 µg p-Nitrotoluene per litre blood	

p-Nitrochlorobenzene

Within-series imprecision:	Standard deviation (rel.)	s_w = 7.4 and 5.0 %
	Prognostic range	u = 16.7 and 11.3%
	At concentrations of 5 and 20 µg p-nitrochlorobenzene per litre blood and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 11.2 and 11.3 %
	Prognostic range	u = 23.5 and 23.6 %
	At concentrations of 5 and 20 µg p-nitrochlorobenzene per litre blood and whre n = 20 days	
Inaccuracy:	Recovery rate	r = 90–118 %
Detection limit:	1 µg p-Nitrochlorobenzene per litre blood	

2,6-Dinitrotoluene

Within-series imprecision:	Standard deviation (rel.)	s_w = 3.6 and 4.8 %
	Prognostic range	u = 8.1 and 10.8 %
	At concentrations of 5 and 20 µg 2,6-dinitrotoluene per litre blood and where n = 10 determinations	

Between-day imprecision:	Standard deviation (rel.)	s = 10.1 and 6.8 %
	Prognostic range	u = 21.1 and 14.2 %
	At concentrations of 5 and 20 µg 2,6-dinitrotoluene per litre blood and where n = 20 days	
Inaccuracy:	Recovery rate	r = 78–119 %
Detection limit:	2.0 µg 2,6-Dinitrotoluene per litre blood	

o-Dinitrobenzene

Within-series imprecision:	Standard deviation (rel.)	s_w = 11.5 %
	Prognostic range	u = 26.0 %
	At a concentration of 20 µg o-dinitrobenzene per litre blood and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 17.5 %
	Prognostic range	u = 36.6 %
	At a concentration of 20 µg o-dinitrobenzene per litre blood and where n = 20 days	
Inaccuracy:	Recovery rate	r = 80–116 %
Detection limit:	5 µg o-Dinitrobenzene per litre blood	

1-Nitronaphthalene

Within-series imprecision:	Standard deviation (rel.)	s_w = 10.5 and 9.2 %
	Prognostic range	u = 23.7 and 20.8 %
	At concentrations of 5 and 20 µg 1-nitronaphthalene per litre blood and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 14.2 and 15.7 %
	Prognostic range	u = 29.7 and 32.8 %
	At concentrations of 5 and 20 µg 1-nitronaphthalene per litre blood and where n = 20 days	
Inaccuracy:	Recovery rate	r = 92–112 %
Detection limit:	2.5 µg 1-Nitronaphthalene per litre blood	

2-Nitronaphthalene

Within-series imprecision:	Standard deviation (rel.)	s_w = 16.7 and 10.3 %
	Prognostic range	u = 37.7 and 23.3 %
	At concentrations of 5 and 20 µg 2-nitronaphthalene per litre blood and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 18.2 and 15.4 %
	Prognostic range	u = 38.0 and 32.2 %
	At concentrations of 5 and 20 µg 2-nitronaphthalene per litre blood and where n = 20 days	
Inaccuracy:	Recovery rate	r = 91–108 %
Detection limit:	2.5 µg 2-Nitronaphthalene per litre blood	

4-Nitrobiphenyl

Within-series imprecision:	Standard deviation (rel.)	s_w = 12.7 %
	Prognostic range	u = 28.7 %
	At a concentration of 20 µg 4-nitrobiphenyl per litre blood and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 19.8 %
	Prognostic range	u = 44.7 %
	At a concentration of 20 µg 4-nitrobiphenyl per litre blood and where n = 20 days	
Inaccuracy:	Recovery rate	r = 85–109 %
Detection limit:	10.0 µg 4-Nitrobiphenyl per litre blood	

Nitroaromatic compounds

Nitroaromatic compounds have played a key role in organic chemistry for more than 100 years [1]. They are prepared by nitration, when one or more of the hydrogen atoms in the aromatic ring are replaced by nitro groups. Nitroaromatic compounds can undergo numerous substitution reactions to introduce e.g. the alkyl, sulfonic acid, amino, hydroxyl and halogen groups into the molecule. Thus, they form the starting material for the synthesis of an almost infinite number of substances which can be used to produce dyes, herbicides, pharmaceutical products and plastics. Most of the nitroaromatic compounds are initially reduced to the corresponding amine before undergoing further treatment [1]·

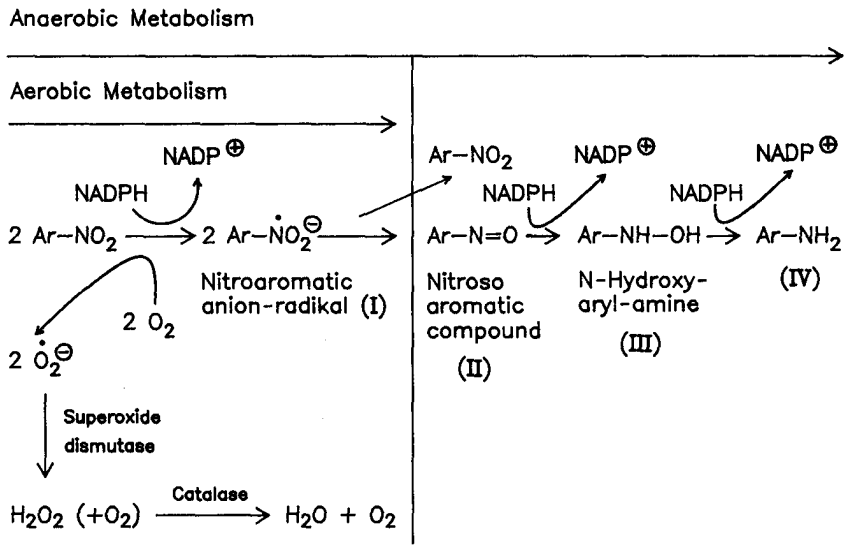
As a rule, unsubstituted nitroaromatic compounds are rapidly and intensively resorbed through the skin. It is important to note that they have been assigned the letter "H" (i.e. danger of cutaneous absorption) in the list of MAK values [5]. Even slight contamination of clothing can lead to the absorption of toxic amounts of nitroaromatic substances when the exposure is sufficiently long. Cutaneous absorption and toxicity generally fall when nitroaromatic compounds are substituted as described above. At the same time there is an increase in the excretion rate [2–4]. For example, when the nitroaromatic and amino-aromatic compounds are sulfonated their toxicity is decisively reduced and, in general, no formation of methaemoglobin occurs [7].

Amino and nitroaromatic compounds exhibit comparable toxic effects [3, 4]. From the point of view of occupational medicine it should be noted, however, that despite the similarity in their chemical structures the biological effects produced by the amine are different to those caused by the corresponding nitro compound [6]. Thus, all three isomers of dinitrobenzene belong to the most potent methaemoglobin-generating substances, but of the corresponding diaminobenzenes only p-diaminobenzene can produce anything like the same effect.

Methaemoglobin formation as a result of nitrobenzene poisoning is one of the classical cases of occupational medicine [7]. Nitroaromatic compounds which are absorbed unchanged are reduced by nitro-reductases in the liver to form the real methaemoglobin-inducing substances, i.e. the nitroso derivatives and N-hydroxyl aryl amines [8]. All the intermediate products are further reduced to the corresponding amino aromatic compounds [9], which are excreted in the urine after acetylation [10].

As a whole, the metabolism of the nitroaromatic compounds proceeds somewhat more slowly than that of the aromatic amines. However, after a certain latent period, methaemoglobin levels rise at least as high, if not even higher, than after exposure to aromatic amino compounds. This is due to the fact that the availability of nitrobenzene for methaemoglobin and haemoglobin conjugate formation is generally higher than after exposure to aniline because the availability of the former is independent of the state of acetylation [13]. The immediate symptoms of intoxication, in particular the methaemoglobin level, especially at a low degree of acetylation, subside more slowly than after comparable exposures to aromatic amines [14]. It need only be pointed out that aromatic amino conjugates are still detectable in the erythrocytes weeks after absorption of nitroaromatic compounds [13, 15, 16].

The cellular reduction of nitroaromatic compounds is catalysed by NADPH-cytochrome P-450 reductases. The reaction proceeds via one radical (I) and two non-radical intermediates (II and III) to give the corresponding aryl amines (IV). The following diagram briefly illustrates the enzymatic reduction of the nitroaromatic compounds in the presence and absence of oxygen [17].



In addition, hydroxylation of the aromatic ring system of both the nitro and the amino aromatic hydrocarbons occurs. Renal excretion of o-, m-, and p-nitro and o-, m- and p-amino phenols are observed following exposure to nitroaromatic compounds [11, 12]. Further metabolic derivatives of the nitroaromatic compounds can be formed by -N-N-dimerization of the reduced intermediates [18, 19].

The toxic effects of various nitroaromatic compounds are attributed to formation of free reactive nitroaromatic anion radicals as a result of enzyme activity [19]. Their most important reactions are with the cellular macromolecules, especially with proteins [18, 20–22] and nucleic acids [23]. These reactions may explain the mutagenic and

carcinogenic effects of certain nitroaromatic compounds independent of the effects of the corresponding aromatic amines [24,25]. Specialized literature must be consulted for the molecular biological details of the metabolism as well as the clinical features of the nitroaromatic compounds [7, 10].

Nitrobenzene

Nitrobenzene (123,1 g/mol, fp. 6°C, bp. 211 °C) is a colourless, oily liquid which smells of bitter almond oil. Nitrobenzene serves as the starting material for the manufacture of aniline, nitrochlorobenzene and dinitrobenzene. In addition it is used as a solvent. In animal studies a variety of damage to organs was observed after exposure to 300 ppm nitrobenzene. The spleen, liver and blood were particularly affected. The LD_{50} for the rat is 650 mg/kg (oral administration). A dose of 35 mg per kg body weight is lethal to humans. Nitrobenzene shows no mutagenic properties in the Ames test. No sister chromatid exchanges were found. Exposure to nitrobenzene causes increased formation of methaemoglobin. Longer periods of exposure may cause anaemia. Nitrobenzene is primarily taken up by inhalation at the workplace. The MAK value list [5] points out the danger of cutaneous absorption.

The BAT (biological tolerance value for working materials) value is based on the haemoglobin-bound aniline which is formed by reduction of nitrobenzene in the human body. The BAT value [5] (100 µg aniline per litre blood) is equivalent to the aniline released from its haemoglobin conjugate.

p-Nitrotoluene

At room temperature p-nitrotoluene (137.15 g/mol, fp. 51.9 °C, bp. 238 °C) is a yellow crystalline substance. It is used for the production of its chlorinated derivatives and p-toluidine. It is the starting material in the manufacture of dyes, aroma substances, polyurethane and optical whitening agents.

No irritation was caused by p-nitrotoluene in animals studies. The LD_{50} for rats is 2250 mg/kg (oral administration). Tests for mutagenic effects proved negative. There is no evidence to indicate a carcinogenic effect on animals. p-Nitrotoluene and its positional isomers have been assigned a MAK value of 5 mL/m^3 [5]. Intake of p-nitrotoluene causes increased methaemoglobin formation.

p-Nitrochlorobenzene

p-Nitrochlorobenzene (157.6 g/mol, fp. 83 °C, bp. 242 °C) forms yellow crystals. It is an intermediate product in the manufacture of dyes, pharmaceutical products and pesticides such as parathion.

In animal experiments p-nitrochlorobenzene causes irritation to the eyes and the mucous membranes [7]. A dose of 550 mg/kg is lethal to rats. There is no evidence of mutagenic or carcinogenic effects in animals. In contrast, toxic doses are known to have a teratogenic effect. In humans p-nitrochlorobenzene has a strongly sensitizing effect. The substance is converted to p-chloroaniline and nitrochlorophenols in the human organism. Methaemoglobin formation is one of the classical symptoms following the increased intake of this nitroaromatic compound [7]. Its MAK value is 1 mg/m^3 in total dust [5].

DinitrotoIuenes (mixture of isomers)

The dinitrotoluenes (182.1 g/mol, fp. 52–93 °C, bp. 300 °C with decomposition [2,4-dinitrotoluene]) are mainly used as a technical mixture of isomers. The individual isomers form yellow to orange needles, rhombohedrons or prisms. The mixture is an oily liquid at room temperature. Dinitrotoluenes are used to manufacture dyes, isocyanates as well as explosives.

The intake of dinitrotoluene leads to neuromuscular damage in dogs. In mutagenicity tests the dominant-lethal test and DNA repair proved negative, while the lymphoma test proved positive in mice. Dinitrotoluenes can cause tumors of the liver (hepatocellular carcinomas) and other organs in rats. A variety of tumors was also observed in mice. Therefore the dinitrotoluenes were placed in Category III A 2) of the substances which have proved to be carcinogenic in animal experiments by the Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area. For this reason no MAK value was assigned to them [5]. Human exposure to an industrial mixture, largely consisting of 2,4- and 2,6-dinitrotoluene causes a kind of anaemia. There is no evidence to suggest that fertility is affected by dinitrotoluenes. Similar to all nitroaromatic compounds dinitrotoluene causes the formation of methaemoglobin.

Dinitrobenzene

Dinitrobenzenes (168.1 g/mol, fp. 90–118 °C, bp. 303 °C) form colourless to yellowish needles with a strong odour. Dinitrobenzenes are used to manufacture phenylenediamines, nitroanilines, dyes, fuels as well as celluloid. They are also employed as corrosion inhibitors.

Animal studies have shown that dinitrobenzene is absorbed to a large degree through the skin. Anaemia, swelling of the spleen and damage to the liver result. The LD_{50} for rats ranges between 5 and 80 mg/kg (oral administration). Although animal studies have not shown evidence of carcinogenic effects, dinitrobenzenes have been assigned to category III B, i.e. compounds which are justifiably suspected of having carcinogenic potential, by the Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area [5]. One of the characteristic symptoms of the intake of dinitrobenzene is the increased formation of methaemoglobin.

1-Nitronaphthalene

1-Nitronaphthalene (173.16 g/mol. fp. 59 °C, bp. 304 °C) forms yellow crystals. It is employed industrially as a starting material for the manufacture of 1-naphthylamine. The LD_{50} for rats is 86–350 mg/kg (oral administration). 1-Nitronaphthalene causes slight irritation to the skin and eyes in animal experiments. There is no evidence of a higher incidence of tumors in mice or rats as a result of application of 1-nitronaphthalene. Nor is there any indication that 1-nitronaphthalene causes cancer in humans. Despite this, there is reason to suspect the substance of carcinogenic potential on account of its metabolism. It is therefore placed in Category III B [5]. The substance is reduced to 1-naphthylamine in the organism. 1-Nitronaphthalene does not cause increased formation of methaemoglobin.

2-Nitronaphthalene

2-Nitronaphthalene (173.16 g/mol. fp. 78.7°C, bp. 312°C) forms yellow crystals. 2-Nitronaphthalene is not used industrially.

The substance exhibits only very slight acute toxicity, the LD_{50} for rats is 4400 mg/kg (oral administration). The Ames test is positive for 2-nitronaphthalene. Tumors of the bladder were found in dogs and papillomas were determined in monkeys. Thus, 2-nitronaphthalene belongs to the substances which have been unequivocally proven carcinogenic in animal experiments. One case of a bladder tumor was observed in a person who was exposed to high doses of 2-nitronaphthalene (and other nitroaromatic compounds) in the 1950s. It was recognized as an occupational disease. Methaemoglobin formation is only slightly increased by 2-nitronaphthalene, if at all.

4-Nitrobiphenyl

4-Nitrobiphenyl (199.2 g/mol. fp. 113.8 °C, bp. 340 °C) forms white needles which are insoluble in water. The substance is not manufactured industrially, but it is formed as a by-product of the technical distillation of nitrobenzene. It is also found in cigarette smoke, etc.

The substance shows only slight acute toxic effects. The LD_{50} for rats is 2230 mg/kg (oral administration). 4-Nitrobiphenyl proved to be mutagenic in various tests. Tumors of the bladder were caused in dogs. Thus, 4-nitrobiphenyl is included in the list of substances which have been unequivocally proven carcinogenic in animal studies. The substance is also suspected of causing cancer in humans. However, this suspicion has not yet been conclusively confirmed. 4-Nitrobiphenyl is reduced to the carcinogen 4-aminodiphenyl in the human organism. It does not cause increased formation of methaemoglobin.

Table 1 summarizes some of the characteristic data for the above mentioned nitroaromatic compounds.

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Table 1: Characteristic data for several nitroaromatic compounds which are relevant to occupational medicine.

Substance	MAK value mg/m^3	Cutaneous Absorption	Danger of Sensitization	Carcinogen Group	Methaemoglobin formation	BAT value
Nitrobenzene	5	+	–	–	+	100 µg/L (aniline released from Hb conjugate)
Nitrotoluene (all isomers)	30	+	–	–	+	–
p-Nitrochlorobenzene	1 G	+	–	–	+	–
Dinitrotoluenes (isomer mixture)	–	+	–	III A 2	+	–
Dinitrobenzene (all isomers)	–	+	–	III B	+	–
1-Nitronaphthalene	–	(+)	–	III B	–	–
2-Nitronaphthalene	–	(+)	–	III A 2	(+)	–
4-Nitrobiphenyl	–	(+)	–	III A 2	–	–

G: total dust

Nitroaromatic compounds (nitrobenzene, p-nitrotoluene, p-nitrochlorobenzene, 2,6-dinitrotoluene, o-dinitrotoluene, 1-nitronaphthalene, 2-nitronaphthalene, 4-nitrobiphenyl)

Application	Determination in plasma
Analytical principle	Capillary gas chromatography
Completed in	August 1990

Contents

1 General principles

Nitroaromatic compounds present in blood samples in their free forms are extracted from the separated plasma and simultaneously concentrated using 2,2,4-trimethylpentane (iso-octane). The quantitative separation of the assay substances from the rest of the plasma components and from each other is carried out by gas chromatography in a fused silica capillary with a chemically bonded stationary phase. An electron capture detector serves as a detection system.

Plasma samples of persons who were not previously exposed to nitroaromatic compounds are used for calibration. Specific amounts of nitroaromatic compounds which are relevant to occupational medicine are added to the plasma samples and they are processed and analysed as described. For quantitative evaluation, calibration curves are obtained by plotting the peak areas as a function of the concentrations used.

If necessary, an internal standard can be selected from the numerous substances which can be determined by this method.

2 Equipment, chemicals and solutions

2.1 Equipment

Gas chromatograph with capillary equipment, preferably with an autosampler, electron capture detector (ECD) and chart recorder or integrator
Gas chromatographic column: Fused silica capillary; length 60 m; inner diameter 0.25 mm; stationary phase DB 1701, film thickness 0.25 µm (e.g. from ict)
alternatively: Fused silica capillary; length 50 m; inner diameter 0.32 mm; stationary phase SE 54, film thickness 0.52 µm
Disposable syringes containing an anticoagulant (e.g. Monovetten® from Sarstedt containing potassium EDTA)
Centrifuge tubes with ground glass stoppers (contents e.g. 10 mL)
Centrifuge
10, 20 and 100 mL Glass volumetric flasks
Automatic microlitre pipettes, adjustable for dosages between 2 and 10 µL, 10 and 100 µL as well as 100 and 1000 µL (e.g. from Eppendorf)
8 mL Transfer pipettes
Glass injection flasks (contents e.g. 5 mL)
Shaker (e.g. Vortex from Cenco, the Netherlands)
0.3 mL Microvials or rolled-edge flasks with crimp caps (e.g. from WGA)

2.2 Chemicals

Nitrobenzene, p.a.
p-Nitrotoluene, p.a.
p-Nitrochlorobenzene, p.a.
2,6-Dinitrotoluene, p.a.
o-Dinitrobenzene, p.a.
1- and 2-Nitronaphthalene, p.a.
4-Nitrobiphenyl, p.a.
Methanol, p.a.
2,2,4-Trimethylpentane (iso-octane) for spectroscopy
Ethanol, p.a.
Sodium chloride, p.a.
Physiological saline (154 mmol/L) (e.g. isotonische Kochsalzlösung from Fresenius) or 0.9 % aqueous NaCl solution prepared in the laboratory
Helium (99.889 %)
Purified nitrogen (99.999 %)
Preserved blood, or animal blood or plasma (e.g. from GMN,Walldorf)
Before using these kind of pooled blood samples they should be tested to determine whether they contain substances which interfere with the analysis.

2.3 Calibration standards

The starting and stock solutions, and the calibration standards containing the nitroaromatic compounds must be transported and stored only in glass vessels.

Starting solution:
10 mg of each nitroaromatic compound are dissolved in methanol in a 20 mL volumetric flask. After mixing the flask is filled up to the mark with methanol.

Stock solution A:
1 mL of the starting solution is pipetted into a 100 mL volumetric flask. The flask is subsequently filled up to the mark with physiological saline.
Content of each nitroaromatic compound 5 mg/L (5 ng/µL)

Stock solution B:
200 µL of the starting solution are pipetted into a 100 mL volumetric flask. The flask is subsequently filled up to the mark with physiological saline.
Content of each nitroaromatic compound 1 mg/L (1 ng/µL)

Preparation of the calibration standards in plasma:
The calibration standards are prepared by spiking pooled plasma samples (each 8 mL) as shown in Table 2.
All the calibration standards in plasma as well as the starting and stock solutions can be stored in the refrigerator at + 4 °C for only a limited period. They must be freshly prepared after 7 days at the latest.

3 Specimen collection and sample preparation

The blood specimen (3–5 mL) is withdrawn from the arm vein using a disposable syringe containing the anticoagulant EDTA. Plasma is immediately separated from the blood specimen by centrifugation at 1500 g for 10 min. The plasma is transferred to a glass injection flask. Assay samples must be stored only in glass vessels. In this state it is transportable and can be stored in a cool place until the analysis is carried out. If longer storage is necessary, the sample should be deep frozen.

A pinch of NaCl is added to 1 mL of plasma. Then 100 µL ethanol are added to cause deproteinization. The mixture is vigorously shaken with 200 µL 2,2,4-trimethylpentane for 60 sec on the shaker. After brief centrifugation, the organic phase is taken up and transferred to 0.3 mL rolled-edge flasks. The organic extracts can be stored in sealed sample flasks at +4°C for up to three months.

Each analytical series includes a reagent blank in which ultrapure water is substituted for plasma. This reagent blank is processed and analysed as described.

4 Operational parameters for gas chromatography

Nitroaromatic compounds were successfully determined under the following gas chromatographic conditions:

Gas chromatographic column:	Material:	Fused silica	alternatively:
	Length:	60 m	50 m
	Inner diameter:	0.25 mm	0.32 mm
	Stationary phase:	DB 1701, cross-linked	SE 54
	Film thickness:	0.25 µm	0.52 µm
Detector:		ECD	ECD
Temperatures:	Column:	5 min at 50 °C, then increase of 8 °C per min to 280 °C, then 10 min isotherm	1 min at 80°C, then increase of 8 °C per min to 270 °C, then 10 min isotherm
	Injection block:	300 °C	280 °C
	Detector:	340 °C	300 °C
Carrier gas:		Helium at a column pressure of 2 bar (2000 hPa)	Helium, 2 ml/min

Make up gas:	Nitrogen, 50 mL/min	Argon/methane (95:5), 60 mL/min
Septum purging:	5 mL/min	
Sample volume:	1 µL	2 µL
Injection technique:	splitless injection for 10 sec, then with split of 60 mL/min	split injection, ratio 1:30

Figure 1 shows a gas chromatogram of a processed blood sample spiked with 20 µg nitroaromatic compounds per litre blood.

5 Analytical determination

For the gas chromatographic analysis, 1 µL of each extract is injected into the gas chromatograph, preferably by means of an autosampler. The nitroaromatic compounds are detected with an ECD.

If the peak areas of the injected sample does not he within the linear range of the calibration curves the organic extract must be appropriately diluted with 2,2,4-trimethylpentane.

6 Calibration

Calibration standards are prepared according to Section 2.3. They are processed and gas chromatographically analysed as described in Sections 3, 4 and 5. The peak areas are plotted against the corresponding concentrations used. The resulting calibration curves are linear between the detection limit and 400 µg/L (cf. Section 9.4).

It is not necessary to plot a new calibration curve for each analytical series. As a rule it is sufficient to analyse two standards in plasma. However, if large discrepancies occur between the peak areas of these standards and those in the original calibration curve then it must plotted anew.

7 Calculation of the analytical result

When the peak areas of each nitroaromatic compound have been obtained their concentrations (in µg per litre plasma) can be directly read off from the calibration curves. If necessary, the reagent blank value must be taken into account.

8 Standardization and quality control

Quality control of the analytical results is carried out as stipulated in TRgA 410 of the German Arbeitsstoffverordnung (Regulation 410 of the German Code on Hazardous Working Materials) [26]. As no quality control material is commercially available at present, it must be prepared in the laboratory. Preparation is carried out as described in Section 2.3. Plasma is spiked with known quantities of nitroaromatic compounds, divided into aliquots and stored at -20 °C. A quality control sample is included in each analytical series. Every fourth analytical series includes a sample containing a known quantity of nitroaromatic compounds to check the accuracy of the method.

9 Reliability of the method

On one hand, the analytical reliability criteria were tested with plasma samples, to which specific amounts of nitroaromatic compounds had been added as described above. On the other, in order to simulate normal clinical practice and reproduce in vivo conditions as closely as possible, nitroaromatic compounds were also added to whole blood, from which plasma samples were prepared by centrifugation. Figure 2 shows the different calibration curves obtained as a result of the two addition procedures. Both curves are linear and highly significantly correlated. They are divergent, but by a constant value. Investigations have shown that part of the nitroaromatic compounds added to whole blood diffuses into the erythrocytes.

The analytical reliability criteria for the precision based on the spiked whole blood samples are, however, valid for the determination in plasma samples.

9.1 Precision

To determine the within-series imprecision, pooled blood was divided into two aliquots and spiked with different amounts of nitroaromatic compounds. After spiking the two blood pools contained 5 µg/L or 20 µg/L of each nitroaromatic compound. Each of these spiked blood samples was analysed ten times. Relative standard deviations ranging from 1.7 to 16.7 %, equivalent to prognostic ranges between 3.8 and 37.7 %, were calculated (cf. Table 3).

To determine the between-day imprecision, the two blood samples which contained 5 or 20 µg of each nitroaromatic compound per litre blood, were processed and analysed on 20 different days. The relative standard deviations lay between 6.8 and 19.8%, the corresponding prognostic ranges were between 14.2 and 44.7% (cf. Table 3).

9.2 Accuracy

Recovery experiments were carried out using the spiked blood samples described above. These samples which contained 5 or 20 µg/L of each nitroaromatic compound were analysed ten times. Recovery rates of 78 to 119 % were found (cf. Table 4). To determine the losses which occur during sample treatment, the results for the nitroaromatic compound solutions prepared as described were compared with results obtained from solutions which did not undergo the same treatment. No blank values were observed for the unspiked plasma samples.

9.3 Detection limit

Under the analytical conditions described here the following concentrations of the nitroaromatic compounds per litre blood could be detected with a probable error of 10 %: 10 µg/L nitrobenzene; 15 µg/L p-nitrotoluene; 1.0 µg/L p-nitrochlorobenzene; 2.0 µg/L 2,6-dinitrotoluene; 5.0 µg/L o-dinitrobenzene; 2.5 µg/L 1-nitronaphthalene; 2.5 µg/L 2-nitronaphthalene and 10.0 µg/L 4-nitrobiphenyl (cf. Table 4).

9.4 Sources of error

Matrix components which cause interference are separated by extraction with 2,2,4-trimethylpentane in the purification step described here. It is important to ensure that the chemicals used do not contain contaminants which cause background interference in the chromatogram. In particular, the 2,2,4-trimethylpentane should be tested for accompanying substances. If the 2,2,4-trimethylpentane is not sufficiently pure hexane can also be used for the extraction.

The solutions or samples containing the nitroaromatic compounds should be collected, transported and stored only in glass vessels, as absorption problems can arise in plastic vessels.

Tests were carried out to investigate whether interference occurs between the nitroaromatic compounds themselves and whether substances which are commonly found in the chemical industry, such as o-, m-, p-dichlorobenzene; 1,2,4-trichlorobenzene; 1,2,4,5-tetrachlorobenzene; pentachlorobenzene and hexachlorobenzene interfere with the gas chromatographic determination (cf. Table 5). No interference was caused in all these cases. Investigation of plasma samples of persons who had not been occupationally exposed to nitroaromatic compounds showed no interfering peaks.

If the calibration curves between the detection limit and 400 µg/L do not prove to be linear as a result of the properties of the ECD, further calibration standards must be determined and new calibration curves plotted. In that case evaluation using a calibration factor is impossible.

10 Discussion of the method

No gas chromatographic methods for the determination of nitroaromatic compounds in body fluids have been published. The method presented here is convenient because the various nitroaromatic compounds can be simultaneously analysed without using complicated procedures for concentrating them.

Moreover, it has proved to be practicable and suitable for routine laboratories because it renders time-consuming preparation techniques and complicated instruments unnecessary. Approximately 50 samples can be processed in two hours. Thus, it is suitable as a routine method of surveillance for occupational medicine.

The whole method shows little susceptibility to interference. When investigations were carried out to test whether other substances which occur at the workplace affect the determination no significant interference could be observed.

The range of nitroaromatic compound concentrations which is relevant to occupational medicine can be reliably determined with this method. The acute toxic concentration range can also be determined. However, due to the limited linearity of the ECD, smaller volumes of the biological material must be used or additional dilution steps are necessary.

Detection limits lower than those given in Section 9.3 can be achieved, but more time-consuming preparation is necessary. Larger quantities of the samples must be extracted, the extracts must be purified and subsequently concentrated.

In principle, it is also possible to use an internal standard. However, it is necessary to know which substances occur at the workplace. In this case an internal standard can be selected from those substances which have been tested for interference (see Table 5). The chemical and physical properties of the internal standard should resemble those of the investigated substances as closely possible. This standard is added to the biological material before sample treatment.

Many more nitroaromatic compounds and, if appropriate, chlorinated aromatic hydrocarbons could basically be determined using this method, but no reliability criteria have been established for them. With regard to chlorinated aromatic hydrocarbons the method "Determination of chlorinated aromatic hydrocarbons" should be consulted in this volume.

This method for plasma analysis can also be used for examining whole blood samples. The distribution of the absorbed nitroaromatic compounds in the extracellular and intracellular compartments of the human organism and their significance for occupational medicine still requires scientific clarification.

In contrast to the method described here, split injection can also be used. The examiner obtained good results with a split ratio of 1:30. However, when low detection limits are desired it is advisable to use the splitless technique suggested here.

As a whole, the reliability and practicability of this method fulfil the requirements of occupational medicine for the assessment of internal stress due to working materials.

Instruments used:
Gas chromatograph 3700 and electron capture detector from Varian, integrator 3388 A and autosampler 7672 A from Hewlett Packard

Gas chromatograph HP 5890 and electron capture detector, data system 3350 and autosampler 7673 A from Hewlett Packard

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Table 2: Preparation of the calibration standards in plasma.

Volume of the stock solution		Final volume of the plasma calibration standards	Concentration of the plasma calibration standards
A 5 ng/µL	B 1 ng/µL		
µL	µL	mL	µg/L
–	–	8	0
–	40.0	8	5
–	80.0	8	10
–	160.0	8	20
80.0	–	8	50
160.0	–	8	100
320.0	–	8	200
640.0	–	8	400

Table 3: Precision of the gas chromatographic determination of the nitroaromatic compounds in blood.

Nitroaromatic compounds	Expected value	Within-series imprecision (n = 10)				Between-day imprecision (n = 20 days)			
		Actual value	Standard deviation		Prognostic range	Actual value	Standard deviation		Prognostic range
	µg/L	µg/L	µg/L	%	%	µg/L	µg/L	%	%
Nitrobenzene	20	12.9	0.22	1.7	3.8	15.4	1.6	10.4	21.7
p-Nitrotoluene	20	15.5	0.34	2.2	4.9	14.3	2.1	14.7	30.7
p-Nitrochlorotoluene	5	6.3	0.47	7.4	16.7	6.3	0.71	11.2	23.5
	20	15.4	0.77	5.0	11.3	17.3	1.96	11.3	23.6
2,6-Dinitrotoluene	5	10.8	0.39	3.6	8.1	8.0	0.81	10.1	21.1
	20	15.6	0.75	4.8	10.8	16.2	1.1	6.8	14.2
o-Dinitrobenzene	20	6.1	0.70	11.5	26.0	7.8	1.37	17.5	36.6
1-Nitronaphthalene	5	4.2	0.44	10.5	23.7	3.8	0.54	14.2	29.7
	20	8.9	0.82	9.2	20.8	10.2	1.6	15.7	32.8
2-Nitronaphthalene	5	5.7	0.96	16.7	37.7	3.0	0.54	18.2	38.0
	20	10.1	1.04	10.3	23.3	10.4	1.6	15.4	32.2
4-Nitrobiphenyl	20	10.8	1.37	12.7	28.7	10.6	2.1	19.8	44.7

Table 4: Recovery experiments and detection limits for the gas chromatographic determination of nitroaromatic compounds in blood.

Nitroaromatic compounds	Expected value	Accuracy Recovery rate	Losses during preparation*	Detection limit
	µg/L	%	%	µg/L
Nitrobenzene	20	78–119	0–21	10.0
p-Nitrotoluene	20	85–107	0–30	15.0
p-Nitrochlorobenzene	5	90–118	0–12	1.0
	20	94–107	0–22	
2,6-Dinitrotoluene	5	78–119	5–40	2.0
	20	86–108	9–36	
o-Dinitrobenzene	20	80–116	63–75	5.0
1-Nitronaphthalene	5	92–116	0–2	2.5
	20	93–104	0–3	
2-Nitronaphthalene	5	97–108	0–2	2.5
	20	91–108	0–2	
4-Nitrobiphenyl	50	85–109	13–50	10.0

* The losses during preparation will be compensated by working up the calibration standards in plasma in the same manner.

Table 5: Substances which have proved to cause no interference with this method.

2,4-; 2,6-; 3,4-dichlorotoluene	3,5-dichloro-1,2-dinitrobenzene
o-chlorobenzaldehyde	4,6-dichloro-1,3-dinitrobenzene
2-; 4-chlorobenzylchloride	pentachloronitrobenzene
benzylchloride	2,6-dichlorobenzylchloride
o- ,m- ,p-dichlorobenzene	3,4-dichlorobenzylchloride
2,3-; 3,4-dinitrotoluene	3,4-dichlorobenzotrichloride
o-,m-,p-nitrochlorobenzene	3-; 4-nitrobenzylchloride
trichloromethylbenzene	2,4-dinitrochlorobenzene
1,2,3-trichlorobenzene	2,3,7,8-TCDD
2,4,5-trichloronitrobenzene	2,3,7,8-TCDF
1,2,3,5-; 1,2,3,4-tetrachlorobenzene	Lindane
1-; 2-chloronaphthalene	epichlorohydrin
2-chloro-4-nitrotoluene	monochlorohydrin
4-chloro-3-nitrotoluene	dichloromethane
4-chloro-2-nitrotoluene	chloroform
6-chloro-2-nitrotoluene	carbon tetrachloride
2,3-dichloro-l-nitrobenzene	1,2-dichloroethane
2,4-dichloro-1 -nitrobenzene	trichloroethane
2,5-dichloro-l-nitrobenzene	tetrachloroethylene
3,4-dichloro-l-nitrobenzene	1,2,4-trichlorobenzene
3,5-dichloro-l-nitrobenzene	1,2,4,5-tetrachlorobenzene
pentachlorobenzene	hexachlorobenzene

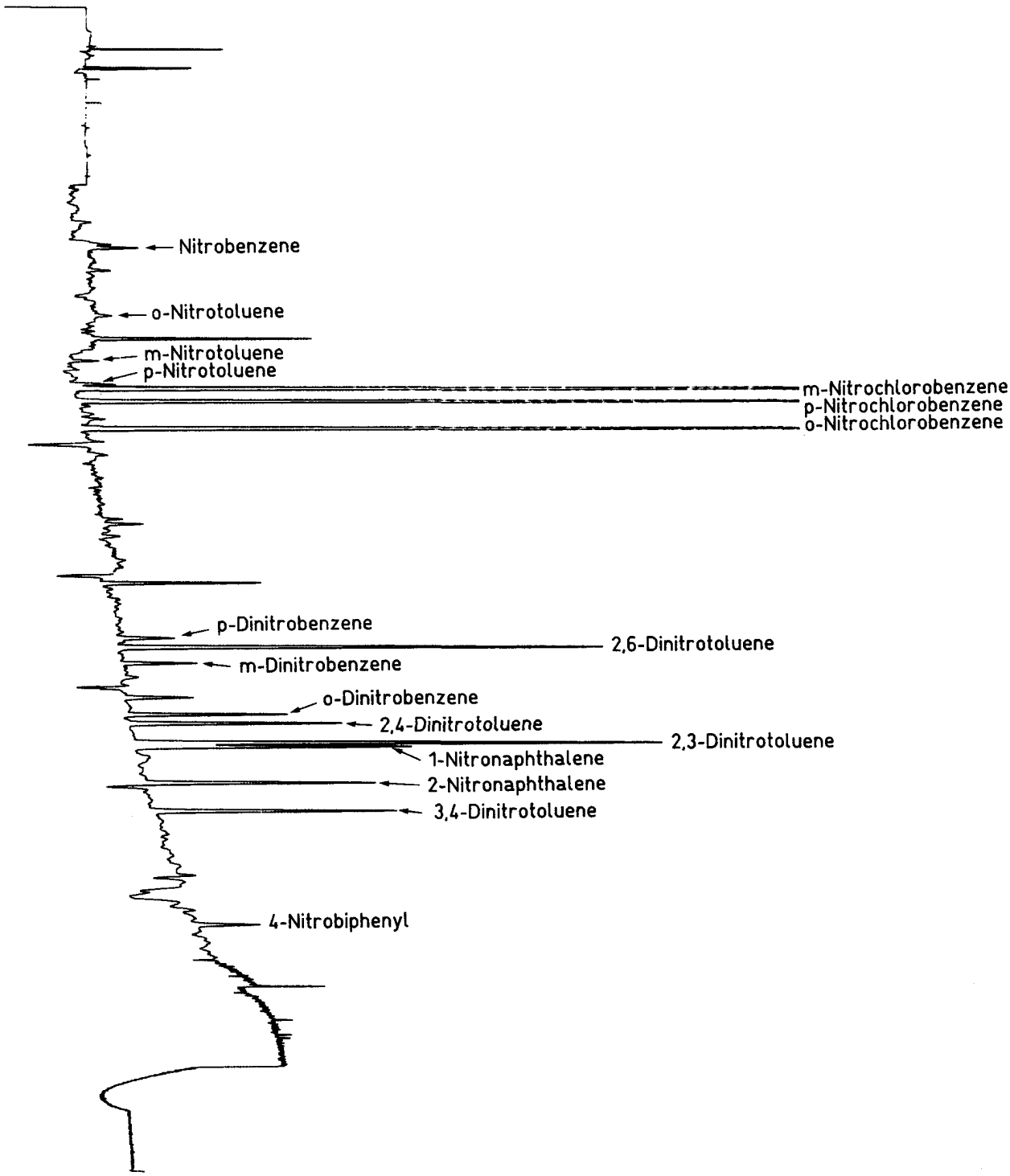

Fig. 1: Gas chromatogram of a prepared blood sample spiked with 20 µg of each nitroaromatic compound per litre blood, attenuation 28.

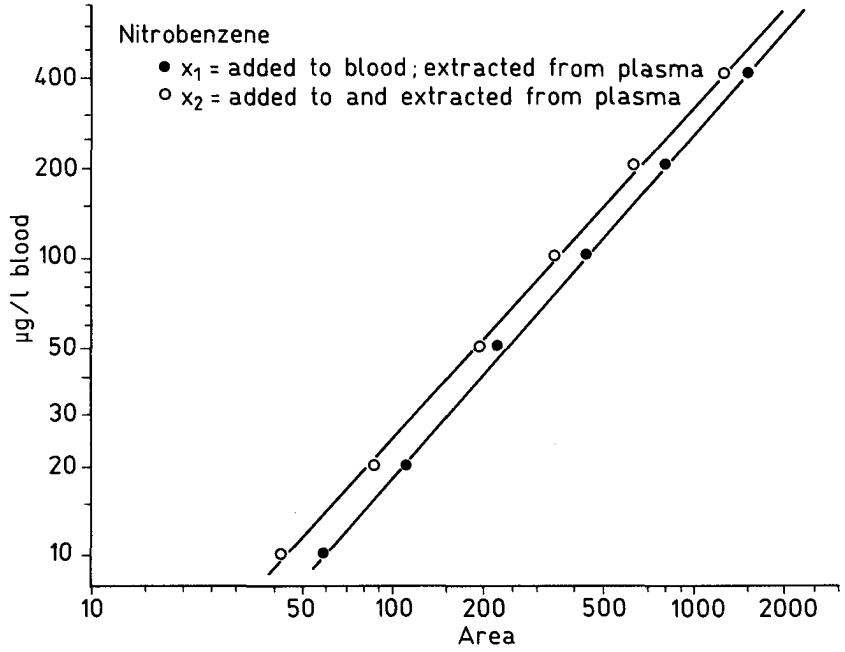

Fig. 2: Comparison of the calibration curves obtained by the different preparation techniques described in Section 9, using nitrobenzene as an example.
• Nitrobenzene was added to blood, plasma is prepared and analysed as described.
◦ Nitrobenzene was added to plasma and the sample was analysed as described.