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Journal of Chromatography A, 943 (2001) 47–54

JOURNAL OF CHROMATOGRAPHY A

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Analysis of polynitrophenols and hexyl by liquid chromatography–mass spectrometry using atmospheric pressure ionisation methods and a volatile ion-pairing reagent

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Received 12 June 2001; received in revised form 17 October 2001; accepted 22 October 2001

Abstract

An LC–MS method for the determination of picric acid (2,4,6-trinitrophenol), its reductive transformation products picramic acid (2-amino-4,6-dinitrophenol) and iso-picramic acid (4-amino-2,6-dinitrophenol) and hexyl (2,2′,4,4′,6,6′-hexanitrodiphenylamine) has been developed. The analytes were separated using ion-pairing chromatography with a volatile ion-pairing reagent suitable for subsequent MS detection. The performance of an atmospheric pressure chemical ionisation (APCI) and an electrospray ionisation (ESI) interface was compared. ESI-MS is more sensitive for the analytes, especially for hexyl and picric acid, APCI-MS delivered more fragments necessary for unequivocal identification. With LC–ESI-MS limits of detection using single ion monitoring (SIM) mode are 4 ng (iso-picramic acid), 800 pg (picramic acid), 400 pg (picric acid) and 80 pg (hexyl). For quantification, ^{15}N-picric acid was used as an internal standard. Using this new method, the degradation of picric acid in soil was monitored in a laboratory study. Furthermore, the presence of picramic acid was for the first time verified in soil samples from a former ammunition plant.

Keywords: Environmental analysis; Nitrophenols; Hexyl; Picric acid; Picramic acid; Isopicramic acid

1. Introduction

Due to their toxic and carcinogenic properties, nitroaromatic compounds (NACs) have a high hazardous potential to humans and the environment [1,2]. They are used as basic chemicals for paints, agrochemicals, plastics and pharmaceuticals [3] and their high consumption leads to a considerable release of NACs into the environment. Numerous ammunition plants, ordnance works, firing ranges and army depots are further potential sources of contamination for soil and ground water with NACs [4,5]. Many ammunition plants were built next to ground water reservoirs, which are nowadays used for drinking water purposes, for example in Stadtallendorf (Hessen, Germany) or Elsnig (Saxony, Germany).

Microbial degradation of NACs in the environ-

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0021-9673/02/$ – see front matter
PII: S0021-9673(01)01430-3

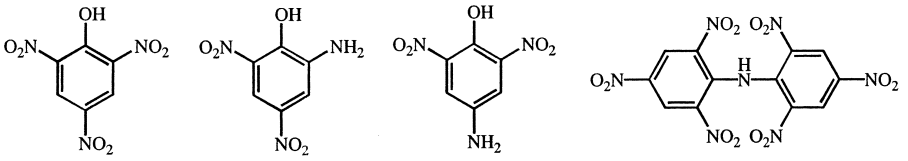

Fig. 1. Chemical structures of the investigated compounds.

ment is frequently studied [6]. Reduction of the nitro group(s) ultimately leads to the corresponding amino compounds, which are often even more harmful to humans and the environment than their precursors [1,2,6]. Furthermore, the increased polarity of the degradation products makes them more mobile in aqueous systems, resulting in an overall higher threat to ground water receptors and soil. Widespread use, mobility and harmfulness of NACs and their degradation products make their analysis in environmental matrices essential.

For monitoring of the most common explosive, trinitrotoluene (TNT), numerous methods have been developed which include its major reductive metabolites, mono- and diamino nitrotoluenes [7,8]. In contrast, degradation products of picric acid (2,4,6-trinitrophenol) have been neglected so far although picric acid is used extensively in explosives and dyes and also occurs as major by-product in the production of several other NACs [9]. Microbial reduction of picric acids leads to picramic acid (2-amino-4,6-dinitrophenol) [10] and possibly also iso-picramic acid (4-amino-2,6-dinitrophenol). Another nitroaromatic compound for which sensitive and selective determination methods are largely lacking is 2,2′,4,4′,6,6′-hexanitrodiphenylamine (hexyl).

Recently, liquid chromatography with mass spectrometric detection (LC–MS) has been introduced in the analysis of nitrophenols. Mass spectrometry is highly sensitive and selective due to the possibility of single ion monitoring (SIM). As interfaces for coupling LC and MS, thermospray ionisation (TSP) [11], electrospray ionisation (ESI) [11] and atmospheric pressure chemical ionisation (APCI) [11–13] have been applied to the analysis of nitrophenols. All of these are soft ionisation methods, which produce few fragments. For acidic compounds like the analytes, mainly $[M-H]^-$ anions are generated. These can be detected sensitively in the SIM mode. Hexyl was also determined with LC–APCI-MS–MS [14] and LC–ESI-MS–MS [15]. The two isomeric picramic acids have not been investigated with LC–MS so far.

The aim of this study was (i) to develop a method capable of separating and sensitively determining the highly acidic analytes picric acid, its potential degradation products and hexyl using LC–MS and quantification with a ^{15}N-labeled internal standard, (ii) to study the formation of picramic acids as degradation products of picric acid in soil and (iii) to apply this method to the analysis of contaminated soil from a former ammunition plant. Structures and properties of the investigated analytes are given in Fig. 1 and Table 1, respectively.

Table 1
Properties of investigated analytes

Compound	Molecular formula	CAS No.	Molecular mass (g/mol)	pK_a
Picric acid	$C_6H_3N_3O_7$	88-89-1	299	0.38 [31]
iso-Picramic acid	$C_6H_5N_3O_5$	17973-92-1	199	4.32*
Picramic acid	$C_6H_5N_3O_5$	96-91-3	199	4.10*
Hexyl	$C_{12}H_5N_7O_{12}$	131-73-7	439	2.81 [32]

*Calculated according to Ref. [33] based on the pK_a of phenol.

2. Experimental

2.1. Chemicals and materials

Chemicals were obtained from Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany) in the highest purity available and used without further purification unless otherwise stated. Methanol (LC grade), acetonitrile (LC grade) and dichloromethane (for residue analysis) were purchased form Promochem (Wesel, Germany). ^{15}N-Nitric acid was obtained from Iostec (A Matheson, USA Company, 99 atom% ^{15}N at least, 5 g acid in 7.3 g solution), picramic acid (as sodium salt hydrate) from Sigma (Steinheim, Germany). Cartridges for solid-phase extraction were LiChrolut RP 18, 3 ml, 200 mg from Merck.

Iso-picramic acid was synthesised from 4-acetamidophenol following a method by Dabney [16]. In order to purify the purchased picramic acid it was dissolved in water, adjusted to pH 4 with HCl (17%), and extracted three times with dichloromethane. ^{15}N-Picric acid was synthesised from phenol with ^{15}N-nitric acid according to Ref. [17].

2.2. Sample preparation

Stock solutions of the analytes were prepared in concentrations of 1 g/l (picric acid, iso-picramic acid) and 0.2 g/l (picramic acid, hexyl) in methanol and stored at 4°C.

2.2.1. Soil samples

For spiking experiments, a clay garden soil was taken and dried at 50°C before use. Three samples of 100 g were taken and each was mixed thoroughly with 1 g glucose to facilitate degradation. Aqueous picric acid solution (30 ml) was added, so that final concentrations of picric acid were 100, 50 and 10 mg/kg, respectively. As negative control, 100 g of soil was mixed with 1 g glucose and 30 ml water. Moisture was maintained at about 30% by daily weighing and adding a quantity of water equal to the evaporated amount, followed by thorough mixing. Samples of approximately 1 g were taken every 24 h, dried at 50°C and weighed. For extraction, 5 ml methanol was added. The samples were placed in an ultrasonic bath for 15 min and then left for 12 h. The suspension was centrifuged (20 min, 450 *g*) and the supernatant removed with a pipette. The concentration of picric acid in the extraction solution was estimated by colorimetry and, if necessary, diluted with mobile phase or concentrated under nitrogen flow at 50°C and then dissolved in 1 ml mobile phase.

Three soil samples from the former ammunition plant Hirschhagen (Germany) were available for an investigation of real samples. An aliquot of about 7 g was dried at 50°C. Extraction was carried out twice with 5 ml methanol in an ultrasonic bath for 15 min. After centrifugation (20 min, 450 *g*) the supernatant was removed with a pipette, concentrated to dryness under nitrogen flow at 50°C and diluted in 1 ml mobile phase.

2.3. LC conditions

LC was carried out with a P 580 A HPG (high pressure gradient) pump, an on-line degasser DG-2410, an auto sampler GINA 50, a column oven (set to 30°C), and a diode array detector UVD 340; processing and data analysis were done with Chromeleon software (LC equipment and software all from Dionex, Germering, Germany). The detector was equipped with a pressure stable microflow cell UZ-GT-MIC (volume 0.14 μl; LC Packings, Amsterdam, The Netherlands) to allow the in-series coupling of the diode array detection (DAD) and MS systems. The column was a Nucleosil 120-3 C_{18}, 5 μm, 250×3 mm (Macherey-Nagel, Düren, Germany) with a pre-column of the same material (10× 3 mm). The flow-rate was set to 1 ml/min for ion-suppression chromatography and 0.4 ml/min for ion-pairing chromatography. Details of gradients are given in the text. The injection volume was 40 μl. UV spectra were recorded in the range of 200 to 600 nm. Chromatograms were recorded at three different wavelengths (230, 360 and 420 nm).

2.3.1. Preparation of solutions for mobile phases

Tributylammonium formate (TBAF) solution was prepared by dissolving 2.9 ml tributyl amine (20 mmol) in 10 ml methanol and mixing this with a solution of 1.2 ml formic acid (20 mmol) in 800 ml water. After adjusting to pH 7.0 with ammonium hydroxide solution, the liquid was filled up with

water in a 1-l measuring flask. Solid-phase extraction over an RP18 cartridge was used to remove impurities from this mobile phase stock solution. The desired concentrations of ion-pairing reagent were obtained by diluting the stock solution.

2.4. MS conditions

MS spectra were recorded with a HP5989B (Hewlett-Packard, Waldbronn, Germany) equipped with an APCI or ESI interface (Analytica of Branford, Branford, CT, USA). The following settings were used: capillary exit voltage −100 V (unless otherwise stated), corona voltage (for APCI only) −1500 V, vaporiser heater temperature 350°C, drying gas heater temperature 350°C, quadrupole temperature 100°C, dwell time 5 ms. The negative ion mode was used throughout all experiments. In the scan mode masses were detected from m/z 100 to 600 with 0.39 scans/s. In SIM measurements the $[M-H]^-$ anions of the analytes were monitored: m/z 198 (picramic acid and iso-picramic acid), 228 (picric acid), 231 (^{15}N-picric acid) and 438 (hexyl).

3. Results and discussion

3.1. Optimisation of the chromatographic separation

All analytes are acidic and are hardly retained on a reversed-phase column due to their predominant presence in ionic form. To overcome this problem, two different approaches can be applied: (a) ion-suppression chromatography with an acidic mobile phase and (b) ion-pairing chromatography.

Separation of the analytes with ion-suppression chromatography was achieved using formic acid at different pH values. However, peaks were broad and hence limits of detection rather high. This method was therefore not further investigated.

Common ion-pairing reagents like tributylammonium-hydrogensulfate (TBAHS) are not feasible for coupling of LC–MS, as they precipitate in the LC–MS interface and contaminate the ionisation source [18,19]; a volatile ion-pairing reagent had to be found instead. Recently, Schmidt et al. investigated the separation of nitrobenzoic acids and nitrotoluenesulfonic acids with several volatile ion-pairing reagents [20]. They obtained best results with TBAF, which was chosen for our study as well. Our investigation showed that as little as 0.5 mmol/l provided sufficient retention of the analytes. Adequate chromatographic separation was obtained with a Nucleosil RP 18 column and a gradient of 90% to 5% TBAF solution within 20 min and then isocratic for 20 min with 5% TBAF solution (Fig. 2).

3.2. Optimisation of MS detection

For the optimisation of MS conditions, solutions of the analytes in methanol (c=10 mg/l) were injected directly into the APCI and ESI interfaces, and m/z values were scanned in the negative ion mode. $[M-H]^-$ anions resulted in the predominant signals in all cases. These are listed in Table 2 together with some further detected fragment ions. ESI turned out to be much more sensitive for the detection of the present analytes, especially for hexyl

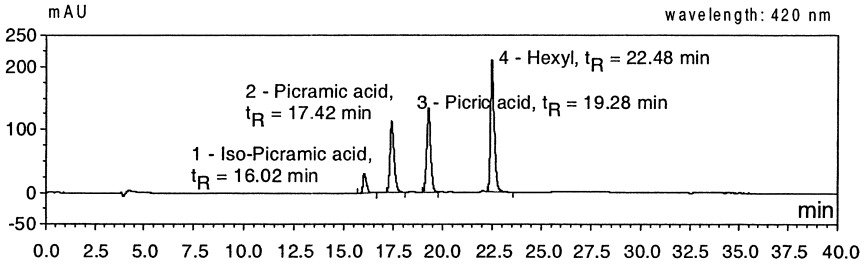

Fig. 2. Optimised chromatographic conditions with ion-pairing reagent TBAF and diode array detection, λ=420 nm; eluent (A) TBAF (c=0.5 mmol/l) and (B) methanol: linear gradient from 90% (A) to 5% (A) in 20 min, 20 min hold; analyte concentrations: 10 mg/l.

Table 2
Observed ions and optimised CapEx voltage for analytes in ESI-MS

Compound	m/z	Ion assignment	CapEx voltage optimum (V)
iso-Picramic acid	199	$[M]^-$	−95
	198	$[M-H]^-$	−95
	182	$[M-H-O]^-$	−240
Picramic acid	199	$[M]^-$	−100
	198	$[M-H]^-$	−100
	182	$[M-H-O]^-$	−145
Picric acid	228	$[M-H]^-$	−105
	212	$[M-H-O]^-$	−105
	196	$[M-33]^-$*	−130
Hexyl	439	$[M]^-$	−135
	438	$[M-H]^-$	−135
	393	$[M-NO_2]^-$	−125

*Structure of fragment unknown.

and picric acid. This is in accordance to studies of other small relatively polar analytes [21] and explained by the fact that, in ESI, ionisation mainly occurs through ion-evaporation, compared to gas-phase reactions in APCI. Hence, analytes already charged in solution are expected to show high sensitivity in ESI. This is clearly the case for the highly acidic picric acid and hexyl and explains the great sensitivity of these analytes in negative ion mode measurements with ESI-MS. The lower sensitivity for both picramic acid and iso-picramic acid can be explained by the presence of the basic amino groups which somewhat disfavours the abundance of $[M-H]^-$ anions.

Fragmentation can be controlled via adjustment of the capillary exit voltage (CapEx-voltage) [22]. To obtain maximum sensitivity, the signal intensity of the $[M-H]^-$ anions and two further anions was scanned against the CapEx-voltage in the SIM mode. The voltages generating highest intensity are shown in Table 2. An example of a CapEx-sweep is given for the detection of picramic acid with ESI-MS (Fig. 3). To obtain maximum sensitivity for the $[M-H]^-$ anions, a CapEx voltage of −100 V was used in the following measurements, unless otherwise stated. Other MS parameters like lens and skimmer voltages were optimised by injecting a mixture of the four analytes with concentrations of 1 mg/l (picric acid, picramic acid, iso-picramic acid) and 200 μg/l (hexyl) in methanol.

3.3. LC–MS coupling with the ESI interface

For coupling of LC and MS the ion-pair chromatographic method with TBAF as described above and

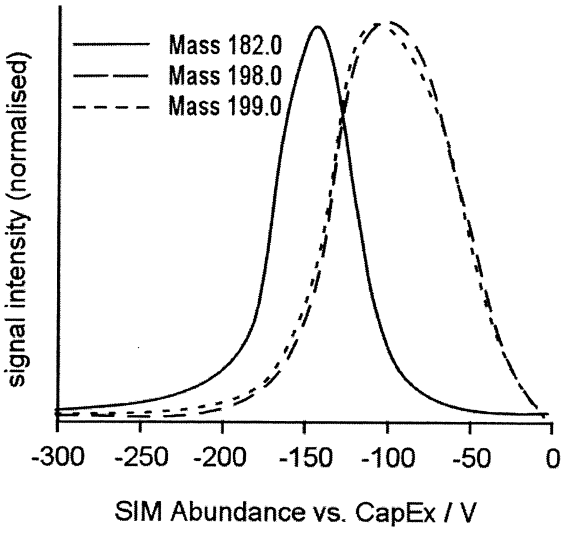

Fig. 3. Ramp over capillary exit voltage (CapEx) for picramic acid.

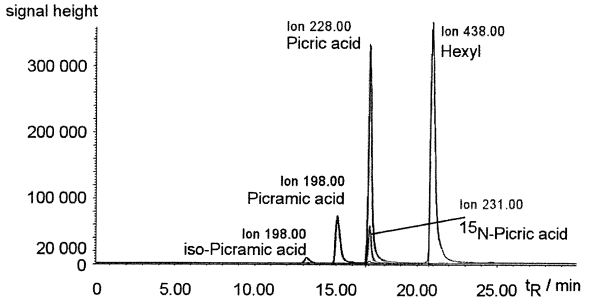

Fig. 4. LC–ESI-MS, single-ion detection of $[M-H]^-$ anions of analytes, m/z 198 (iso-picramic acid, picramic acid), 228 (picric acid), 231 (^{15}N-picric acid) and 438 (hexyl); eluent (A) TBAF (c=0.5 mmol/l) and (B) methanol: linear gradient from 80% (A) to 5% (A) in 30 min, 10 min hold; analyte concentration: 1 mg/l for picric acid and hexyl; 2 mg/l for picramic acid and iso-picramic acid.

the ESI interface were used. The initial aqueous fraction of the mobile phase had to be lowered from 90 to 80% to ensure complete evaporation of the solvent in the interface chamber. Hence, the gradient was 80% to 5% TBAF solution (c=0.5 mmol/l) within 20 min and then isocratic with 5% TBAF solution (Fig. 4). In agreement with Refs. [18] and [20], use of the volatile ion-pairing reagents led to no observable contamination of the ionisation source. Detection in the MS system was set to SIM of the $[M-H]^-$ anions of the four analytes to obtain maximum sensitivity.

Limits of detection (LODs) were determined by running a dilution series of four different concentrations in methanol. Concentrations were 200, 100, 20 and 10 μg/l (picric acid, picramic acid, iso-picramic acid). The hexyl concentrations were a fifth of those of the other analytes. Signal/noise (S/N) ratios of three measurements were averaged. Picric acid and hexyl could be determined in the most diluted solution with $S/N>10$, resulting in LODs <10 and <2 μg/l, respectively. With an injection volume of 40 μl this equals injected masses of <400 and <80 pg, respectively. For picramic and iso-picramic acid, detection with $S/N>3$ was only possible in the more concentrated solutions, leading to LODs of 20 and 100 μg/l, respectively.

The sensitivity of the developed method is better than that given in previous papers. Astratov et al. [11] reported LODs of 4 and 1 ng for picric acid and hexyl, respectively, with LC–thermospray (TSP)-MS. LODs for picric acid and hexyl given for other methods such as LC–UV [23], high-performance thin-layer chromatography (HPTLC) [24] and LC with electrochemical detection [25] were also higher than for our method.

Whilst determining the LODs, it was observed that day-to-day reproducibility of the signal intensity was rather poor with deviations of up to 40%. This is a well-known drawback of LC–MS [26]. A common approach to avoid these problems is the use of an internal standard and determination of the signal areas in relation to the area of this standard. We chose ^{15}N-labeled picric acid, which is chemically similar to the investigated analytes, can be detected selectively in the MS system as $[M-H]^-$ anion and has a natural abundance of only 0.04%.

For quantification of the analytes a one point calibration was performed every day. The calibration mixture consisted of 200 μg/l internal standard (^{15}N-picric acid) and 500 μg/l of picric acid, picramic acid and iso-picramic acid as well as 100 μg/l of hexyl. To validate the repeatability of the developed method, two analyte mixtures with different concentrations were measured (see Table 3). The obtained standard deviations were between 1 and 6% for the higher concentrated mixture and considerably higher for the lower concentrated mixture. The reason for the poorer accuracy in the latter is a deviation from a linear response assumed with the

Table 3
Repeatability of quantification via one-point calibration with ^{15}N-picric acid as internal standard: expected and experimental values with standard deviation

	Expected value (μg/l)	Experimental value (μg/l)	RSD (%)*
iso-Picramic acid	503	475	6
	101	86	22
Picramic acid	515	473	6
	103	78	21
Picric acid	535	537	1
	107	117	4
Hexyl	100	101	10
	20	19	18

*n=3 for each level.

use of a one-point calibration, a phenomenon well known in ESI. However, for the purpose of our study the results were satisfactory. Best results were obtained for picric acid, probably due to its similarity to the internal standard.

3.4. Picric acid occurrence and degradation in soil

To investigate the proposed degradation of picric acid to picramic acid and iso-picramic acid, soil was spiked with picric acid and processed as described in the Experimental section. The concentration of extractable picric acid declined rapidly in all three samples. In the two soil samples spiked with 100 and 50 mg/kg low concentrations of picramic acid were found. The presence of picramic acid was unequivocally verified by variation of the CapEx-voltage in measurements of the sample and a reference standard, which resulted in the same fragmentation pattern. The detected amounts of picric acid and picramic acid for the soil spiked with 50 mg/kg picric acid are shown in Fig. 5. The amount of picramic acid is only about 1% of that of picric acid, thus a complete mass balance is not possible yet. This might be due to the formation of metabolites not included in our study and/or to irreversible sorption (i.e., not extractable with our method) of picric acid or, more likely, its reduction products. The latter is a well-known phenomenon for aromatic amines that may form covalent bonds with various functional groups in soil organic matter [27]. Iso-picramic acid was never found during these experiments. Probably, picric acid is regioselectively reduced in the nitro group in ortho position as was previously reported for 2,4-dinitrophenol under various conditions [28,29].

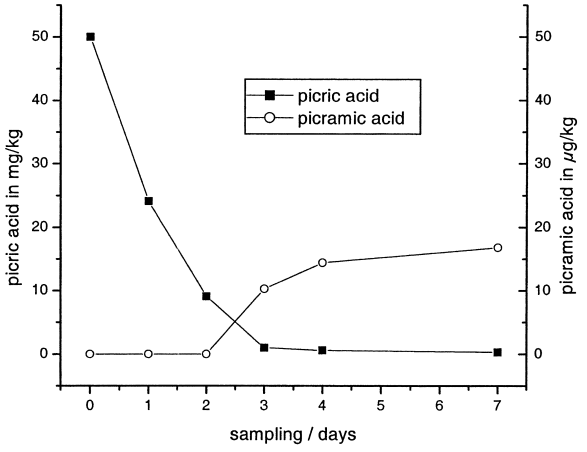

Fig. 5. Degradation of picric acid and formation of picramic acid in spiked soil.

Since this study was designed as a first test of picric acid degradability in soil, LODs were not determined for the whole analytical procedure. Taking into account the LODs for the developed LC–MS method and assuming complete recovery in the extraction step concentrations of 20 μg/kg (iso-picramic acid), 4 μg/kg (picramic acid), 2 μg/kg (picric acid) and 0.4 μg/kg (hexyl) could be determined.

Picric acid was found in all three soil samples from the former ammunition plant in Hirschhagen, Germany at concentrations of 16, 260 and 1420 μg/kg, respectively. Iso-picramic acid and hexyl were not found, whereas picramic acid was present at 8.5 μg/kg in the sample with the highest concentration of picric acid. Although limited in sample numbers, these preliminary results have shown the need to monitor picric acid degradation products in contaminated soil and, presumably, ammunition waste water.

4. Conclusions

LC–ESI-MS has been found a valuable tool for the analysis of acidic nitroaromatic compounds. Separation was achieved with a volatile ion-pairing reagent, reliable quantification attained with ^{15}N-labeled picric acid as internal standard. The developed method was successfully applied to soil analysis and picramic acid was, for the first time, identified as a reductive metabolite of picric acid in soil. In environmental samples, NACs are found at concentrations from ng/l to several hundred μg/l for water samples and from ng/kg to mg/kg for soil samples. A toxicological evaluation of NACs [30] states 0.1 μg/l as precautionary value for water and 1 mg/kg for soil. The indicative LODs of the present study are well below these requirements. For routine analysis of soil samples, the overall method, including the sample preparation steps, needs to be val-

idated thoroughly in order to ensure that environmental quality criteria can be controlled.

Due to the hazardous properties of amino nitroaromatics the degradation of picric acid and its metabolites in soil and water should be investigated further. Future studies shall concentrate on the regioselectivity of picric acid reduction and completion of a mass balance for picric acid degradation. The latter includes the identification of other metabolites and the elucidation of irreversible sorption processes for the reduced species. ^{14}C- and/or ^{15}N-enriched picric acid might be a helpful tool in addressing these research needs.

Acknowledgements

Thanks to Kerstin Heck for technical assistance and Michael Berg and Jan Eijkel for helpful comments on the manuscript.

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