Journal of Chromatography B, 870 (2008) 17–21

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of cyanide and volatile alkylnitriles in whole blood by headspace solid-phase microextraction and gas chromatography with nitrogen phosphorus detection

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ARTICLE INFO

Article history:
Received 29 October 2007
Accepted 6 May 2008
Available online 24 May 2008

Keywords:
Headspace
Headspace-SPME
GC-NPD
Volatile alkylnitriles
Cyanide
Crotononitrile
Allylnitrile

ABSTRACT

Simultaneous determination of cyanide and volatile alkylnitriles such as acetonitrile, *cis*- and *trans*-crotononitrile, allylnitrile and butyronitrile at low ppb concentration on whole blood (rat and mice) by headspace solid-phase microextraction (HS-SPME) followed by gas chromatography (GC) with nitrogen phosphorus detection has been achieved for the first time. SPME extraction time and temperature were optimized using a star experimental design. Optimum conditions for cyanide extraction were chosen to analyze unspiked blood samples containing alkylnitriles as that analyte occurs at the lowest concentrations. For all analytes, the developed methodology yielded good quality parameters. In all cases, good reproducibility (relative standard deviation ≤12%), detection limits (<3 ng mL^{-1}) and quantification limits (<4 ng mL^{-1}) were recorded.

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1. Introduction

Alkylnitriles are widespread organic compounds of both natural and synthetic origin being used in many technical applications. Nevertheless, a variety of toxic effects in mammalian species have been reported. Indeed, the metabolic breakdown of the parent nitrile by enzymatic activities within the organism releases free cyanide. Then cyanide impairs the energy metabolism of cells causing acute lethality [1,2]. In addition, neurotoxic properties have been reported for a number of nitriles, including the unsaturated 4-carbon alkylnitriles namely, allylnitrile (ALN) [3], *cis*-crotononitrile (CCN) [4] and *trans*-crotononitrile (TCN) [5,6] whereas butyronitrile (BTN) does not share those effects. The kind of toxic effects caused by nitriles may thus vary greatly as a function of their precise chemical structure, so their toxicological evaluation requires understanding of the structure–activity relationship. Major issues in this relationship are the rate at which nitriles are metabolized to cyanide *in vivo*, and the identity of the enzymes catalyzing that metabolism [7,8]. Elucidation of these questions often requires obtaining blood concentration data for both the parent compound and its metabolite.

Cyanide has been analyzed in blood using different analytical techniques. One earlier and widely used method is based on the Conway microdiffusion cells where the acidified cyanide migrates to an alkaline solution which is determined by colorimetric [8,9], fluorimetric [10] or electrometric methods [11]. More recently, it has been carried out including a separation step coupled to a detection technique such as high performance liquid chromatography coupled to a fluorimetric detection [12] or capillary electrophoresis coupled to an ultraviolet detector [13]. Moreover, gas chromatography (GC) coupled to electron capture detector has been increasingly used following a derivatization step [14,15], headspace GC coupled to electron capture detector [16,17] or nitrogen-phosphorus detector (NPD) [18,19] and solid-phase microextraction (SPME) GC combined with NPD [19,20] or mass spectrometry [21]. Nevertheless, only a limited number of studies have been carried out for alkylnitriles determination in whole blood and to the best of our knowledge only one work deals with the simultaneous determination of cyanide and volatile alkylnitriles using headspace-GC-NPD [22]. However, in order to understand the metabolic pathways for different alkylnitriles, trace level determination of the parent compound and its metabolites should be undertaken.

The aim of this work was to optimize the determination of free cyanide and different alkylnitriles using SPME GC-NPD in whole blood matrix for use in experimental toxicity studies. SPME technique has proven to be a suitable technique for the determination

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doi:10.1016/j.jchromb.2008.05.031

of volatile compounds from different chemical classes in a variety of matrices including those of environmental and biological origin. The preconcentration of the target analytes in the SPME sorbent ensures improved limits of detection if compared to conventional headspace-GC. In this work, the main SPME parameters were optimized using a star experimental design. Furthermore, the developed methodology was evaluated analyzing whole blood samples from mice that had been administered with the target analytes.

2. Experimental methods

2.1. Chemicals and reagents

CCN and TCN were obtained by fractionated distillation from a commercially available isomeric mixture (99%, *cis*:*trans* ratio of approximately 60:40, Aldrich Química, Alcobendas, Spain) as described previously [4]; fractions with an isomeric purity greater than 97% were used in the present series of experiments. Analytical grade potassium cyanide was purchased from Fluka (Buchs, Switzerland), ALN, BTN and ACN (Lichrosolv®) were purchased from Merck (Darmstadt, Germany).

All solvents used, analytical grade, were purchased from Merck. Stock solutions (3000 μg mL^{-1}) were prepared in methanol. Standard solutions (60 μg mL^{-1}) were prepared on a monthly basis and were stored at −24 °C.

SPME fibers were obtained from Supelco (Bellefonte, PA, USA). In this work, the tested fibers were 100 μm polydimethylsiloxane, 75 μm carboxen-polydimethylsiloxane and 85 μm polyacrylate. All of them were conditioned prior to use following the manufacturer conditions.

2.2. Animals

The care and use of animals were in accordance with the 5/1995 Law and 214/1997 Act of the Autonomous Government (Generalitat) of Catalonia, and approved by the Ethics Committee on Animal Experiment of the University of Barcelona.

Adult male Sprague–Dawley rats (n = 2) were anaesthetized with 400 mg kg^{-1} chloral hydrate and 10–20 mL of blood were obtained from each rat by terminal cardiac puncture, to be used in spiking experiments. For *in vivo* experiments, 10 mice of the 129S1/SvImJ strain were dosed by intragastric intubation with a CCN (97%)–TCN (3%) mixture (2.25 mmol kg^{-1}, n = 5, females) or ALN (0.75 mmol kg^{-1}, n = 5, males) dissolved in 6 mL kg^{-1} of corn oil. After appropriate times, the mice were anaesthetized, and 0.6 mL of blood was obtained from each mouse by terminal cardiac puncture.

2.3. Apparatus and procedures

GC analyses were carried out on a MFC 500 chromatograph from Carlo Erba (Milan, Italy) coupled to NPD 800 detector from Fisons with a J&W (Folsom, CA, USA) GasPRO column (60 m × 0.32 mm ID). Helium from Abello Linde (Barcelona, Spain) was used as carrier gas at 190 kPa (1.4 mL min^{-1}). Injection was performed in the splitless mode (1 min 40 s) and injector and detector temperatures were kept at 260 °C. Oven temperature was programmed from 130 °C (1 min) ramped at 2 °C min^{-1} to 150 °C (0 min), and then at 8 °C min^{-1} to 260 °C (15 min).

2.3.1. Headspace procedure

The GC headspace experiments were performed with a model 2t® Static Headspace Sampler (Teknokroma, Sant Cugat del Vallès, Spain) at 90 °C with an equilibration time of 10 min. Air volumes of the headspace ranging from 200 to 500 μL were injected in the GC-NPD system using a thermal insulated syringe. Samples (600 μL whole rat blood spiked with the target analytes (TCN 2.0 μg, CCN 2.0 μg, BTN 2.5 μg, ALN 3.0 μg and HCN 4.5 μg) were transferred into 20 mL vials containing 1 mL of acetic/acetate buffer 0.1 M (pH 4.3) in order to avoid blood coagulation and preserve the samples.

2.3.2. Headspace SPME procedure

After fiber selection, extraction parameters for this fiber were further optimized using 600 μL of rat whole blood spiked with the target analytes (TCN 0.04 μg, CCN 0.04 μg, BTN 0.05 μg, ALN 0.06 μg and HCN 0.09 μg) and 400 μL buffer acetic/acetate 0.1 M in a 6 mL vial containing a magnetic stirrer. Prior to the analysis, 30 μL of HCl (25%) were added to dissociate HCN from methemoglobin [11]. During extraction, sample temperature was controlled using a heater-magnetic stirrer Ikamag® RCT Basic with heating and electronic contact thermometer Ikatron® ETS-D4 fuzzy provided by Ika (Staufen, Germany). SPME fiber was kept 1 cm below the vial septum during the extraction. In order to ensure a proper interanalysis fiber cleansing and to avoid sample cross-contamination SPME fiber was kept in the injection port during 15 min after the start of the determination.

2.4. Experimental design

An experimental design was performed on spiked rat whole blood in order to optimize the headspace SPME most important extraction parameters, namely SPME extraction time and sample temperature [23]. Thus, a factorial experimental design (two factors composite design, n = 11) was carried out, with time and temperature as independent variables (Fig. 1). This experimental design has been chosen, as it has been reported to be appropriate for the optimization of a limited number of variables [24]. The central point was measured in triplicate in order to evaluate method reproducibility. Tested temperatures ranged from 25 to 70 °C and time ranged from 5 to 55 min. Response was statistically tested with a polynomial regression model using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and surface plots were generated using Matlab 7.0 (Mathworks, Natick, MA, USA). The method suitability was assessed by analyzing real whole blood samples collected from mice which had been orally administered with the target analytes. Samples were quantified with an external calibration using acetonitrile as

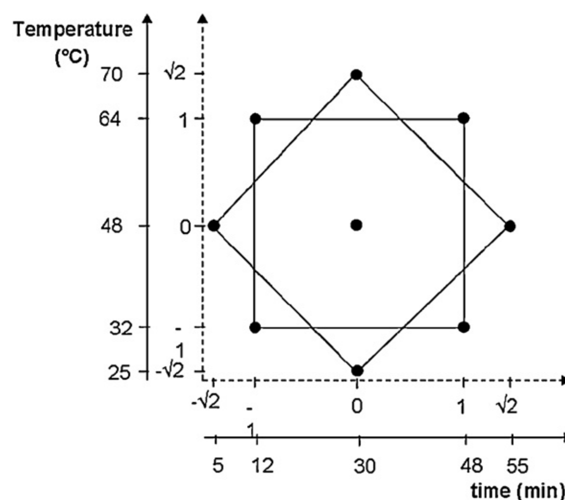

Fig. 1. Two factor composite experimental design considering temperature and time as independent variables. The central experimental point was determined in triplicate. Axis values were plotted both in the real (continuous line) and normalized scale (dotted line).

internal standard. All target analytes showed a good linearity ($R^2 > 0.99$) as determined using 6 different concentrations between 1.4 and 400 ng mL^{-1}. Detection limits were calculated as the procedural blank concentrations added to three times their standard deviation [25]. This calculation method is very useful when target analytes are present in the procedural blank. Quantification limit was considered to be the lowest point of the calibration plot; with this method, the resulting limits are more conservative than the ones obtained by the use of the 10 times signal to noise ratio method, which is more frequently chosen. In addition, this method for determining the quantification limit imposes further requirements on abundance of the analyte and linearity of the response that strengthens the analytical technique.

3. Results and discussion

Chromatographic conditions were optimized in order to achieve a baseline separation of the different target analytes (HCN, ACN, CCN, TCN, allylnitrile and BTN) in 30 min. Due to the matrix complexity (whole blood), a blank (water + reagents) and a procedural blank (whole blood + reagents) were carried out in order to confirm that no interfering matrix components would coelute with the target analytes. Fig. 2 shows a chromatogram of a spiked rat blood sample where NPD selectivity is fully appreciated with only the presence of a few matrix peaks, which do not interfere with target analyte determination. A small peak with the retention time of HCN was found in both the blank (water + reagents) and the procedural blank (whole blood + reagents) chromatograms (Fig. 2). This peak was identified as HCN by identical retention times in several different chromatographic conditions. This background HCN concentration was fairly constant, small enough to not significantly interfere with HCN quantification in the samples, and was included when determining the detection and quantification limits for this analyte. Presence of volatile analytes in the procedural blanks is not an uncommon finding when highly sensitive detection techniques are used.

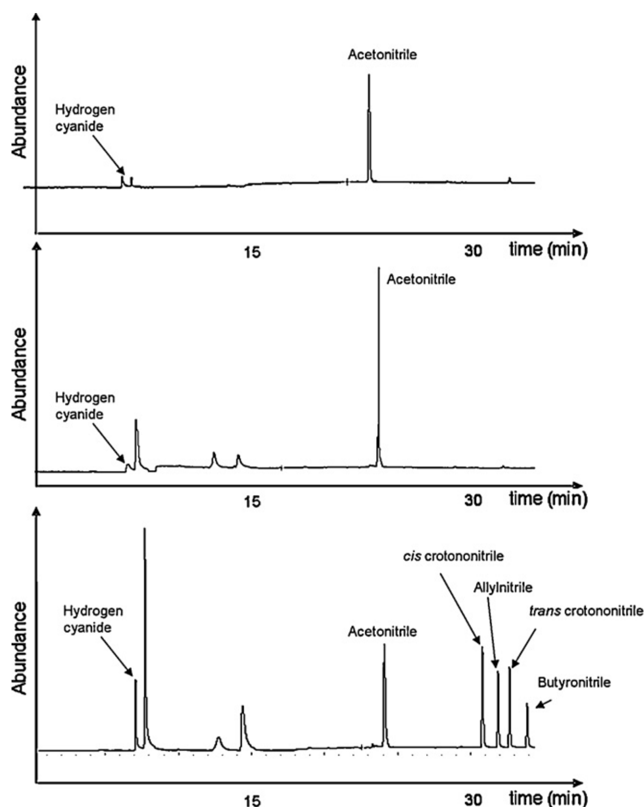

Fig. 2. Top: GC-NPD chromatogram from blank (water + reagents) spiked with ACN. Middle: procedural blank (whole blood + reagents) spiked with ACN. Bottom: sample spiked with nitriles and cyanide mixture ranging from 0.07 to 1.10 μg mL^{-1}.

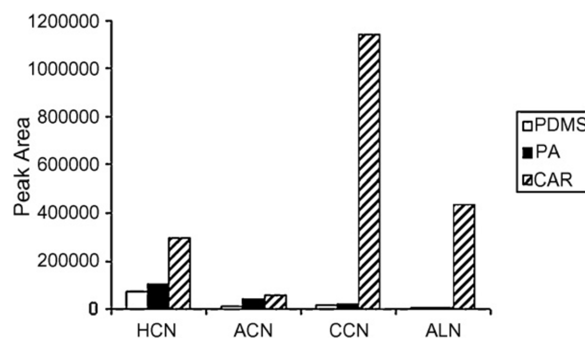

Fig. 3. Recorded GC-NPD peak areas using three different SPME fibers: polydimethylsiloxane (PDMS), polyacrylate (PA) and carboxen/polydimethylsiloxane (CAR) on the same mixture of nitriles spiked in a rat sample.

Simultaneous data on alkylnitriles and cyanide have only been reported using headspace-GC-NPD [22]. Therefore, this technique was firstly used in this work for the detection of nitriles and cyanide in *in vivo* experiments. However, the obtained cyanide detection limit (5 μg mL^{-1} considering 3 times the standard deviation of the procedural blank) was too high for many toxicological studies and far from the reported in the previous headspace-GC study which was calculated using another methodology (0.7 ng mL^{-1}, 3 signal to noise ratio) since hydrogen cyanide is always in the blank [22].

In order to improve these results, and considering that SPME is a good alternative to headspace, the suitability of this technique for simultaneous determination of nitriles and cyanide was evaluated using spiked rat whole blood.

Headspace SPME extraction depends on different parameters such as temperature, extraction time, pH, headspace volume and fiber choice. Different fibers were tested working on the headspace mode and better results were obtained with the carboxen-polydimethylsiloxane fiber (Fig. 3), confirming the results already reported for hydrogen cyanide [21]. Sample pH is an important parameter as cyanide could occur both in its ionic form, non-volatile, or protonated, a gas at room temperature (bp = 26 °C). To keep cyanide protonated (pK_a 9.2) and dissociate it from matrix components, acidic conditions were established by adding 30 μL of HCl (25%) to the extraction vials prior to analysis. Extraction time (t) and temperature (T) were optimized using the experimental design described in Fig. 1 and their influence on cyanide and alkylnitrile extraction was assessed. Data were modeled using a multiple linear regression, including the first-order interaction and the quadratic terms. The general equation for the polynomial regression was as follows:

$$R_x = \beta_0 + \beta_1 t + \beta_2 T + \beta_3 tT + \beta_4 t^2 + \beta_5 T^2 \quad (1)$$

where R_x is the NPD absolute area obtained for the target analyte x, t and T were the extraction time and temperature, respectively and β_x the different obtained coefficients.

A surface response was obtained for each analyte as a function of extraction time and temperature (Fig. 4). The resulting equations were statistically tested accounting for more than the 80% of the target analytes variance (Table 1). Both temperature and extraction time were found to be significant parameters for CCN, TCN, ALN and BTN showing better extraction yields at higher temperature and longer extraction times. In this way, β_1 was found to be positive and statistically significant for all these analytes being

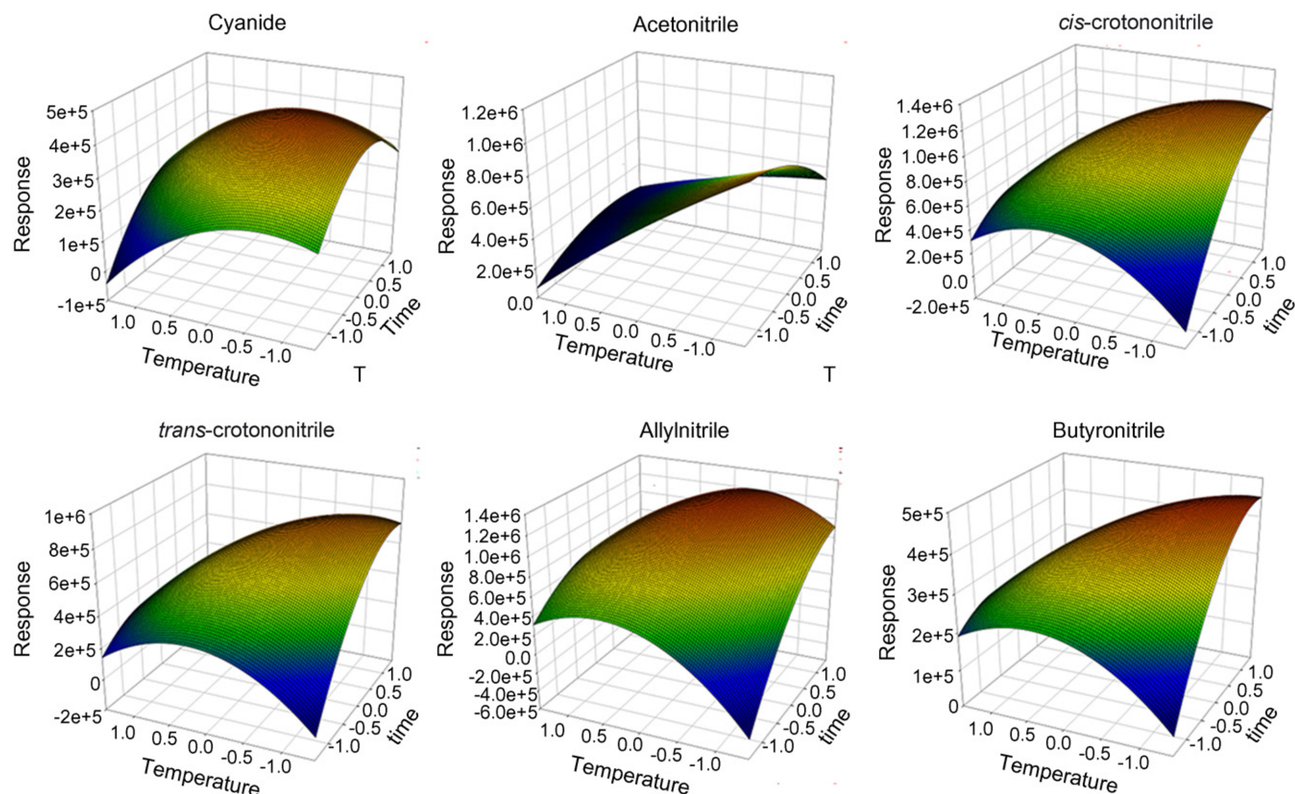

Fig. 4. Surface plots showing the influence of extraction time and temperature on the target analytes response. Note that temperature (25–70 °C) and extraction time (5–55 min) have been normalized.

the most relevant coefficient (Table 1). These trends are related to kinetic controlled SPME mechanisms. On the other hand, for HCN and ACN, which are the most volatile analytes, extraction time is not so relevant and better extraction yields are obtained at lower temperatures being β_2 coefficients negative and statistically significant (Table 1). In this case, the SPME preconcentration seems to be thermodynamically controlled. Therefore, at higher temperatures the partition coefficients between the fiber and the headspace analyte concentrations decrease. As global optimum conditions, 35 min and 37 °C were chosen because HCN is the most toxic compound and showed the worse detectability. Furthermore, it should be pointed out that in alkylnitrile toxicity studies, HCN, as a metabolite, may occur at lower concentrations than the parental compounds.

In order to evaluate the matrix effects, Milli Q water was spiked with alkylnitriles at 0.07 and 1.10 μg mL^{-1} and the results obtained were compared with those from a whole blood sample spiked at the same concentration. Since similar results were obtained, no matrix effect was observed and samples could be quantified by the external calibration method. The suitability of the developed methodology has been assessed by analyzing whole blood of mice which have ingested nitriles. ACN was chosen as internal standard (IS) as its volatility is intermediate between gaseous HCN and the less volatile alkylnitriles (CCN, TCN, ALN, BTN). The developed methodology yielded good quality parameters for determination of all analytes (Table 1), including reproducibility, detection limit and quantification limit. Reproducibility has been calculated using the SPME conditions of the experimental design central point (30 min and 48 °C; $n = 3$) which was carried out at different days. In Table 2, the limit of detection reported in the literature using different techniques are compared, showing the lack of uniformity in methods used to calculate this parameter. Many of the reported

Table 1
Coefficients of the polynomial regression (β_x), explained variance and quality parameters from the method at optimal conditions

	HCN	ACN	CCN	ALN	TCN	BTN
β_1	47,439	−57,807	149,696*	262,47*	103,21*	42.344*
β_2	−58,835*	−215,67*	−120,75	49,103	−103,71*	−49,658*
β_3	18,882	81,229	−176,37*	−140,69	−122,23	−69,782*
β_4	−71,237*	−46,426	−173,17*	−146,75	−125,32*	−59,712*
β_5	−59,587	−22,780	−187,36*	−236,55*	−138,18*	−56,497*
% explained variance	80	83	88	87	87	83
LOD (ng mL^{-1})[a]	2.8	1.5	1.1	1.4	1.1	1.2
LOQ (ng mL^{-1})[b]	3.1	1.9	1.5	1.9	1.4	1.6
Relative standard deviation[c]	12	–	7	5	7	12

* Statistically significant ($p < 0.05$).

[a] LOD (limit of detection) was calculated as the procedural blank concentration added to three times its standard deviation.

[b] LOQ (limit of quantification) was considered to be the lowest point of the calibration plot.

[c] Relative standard deviation calculations were carried out using acetonitrile as internal standard.

Table 2
Comparison of the detection limits obtained for the determination of cyanide in blood using different analytical techniques

Technique	LOD ($ng\,mL^{-1}$)	Simultaneous determination[a]	Method of LOD calc.	Reference
Colorimetric, barbituric acid-pyridine reagent	4.1	–	2 S.D. background blood level	[27]
Fluorescence	0.8	–	3 S.D. (determined in water)	[10]
Amperometry (silver electrode)	2.2	–	2 S.D. background level (standard solutions)	[28]
IC-UV/fluorescence	2.3	–	3 S.D. background level	[12]
Headspace-GC-ECD	5	–		[16]
Headspace-GC-NPD	1	–	10 S.D. background level	[18]
Headspace-GC-NPD	13.8	–		[19]
Headspace-GC-NPD	0.7	+	3 S.D. background level	[22]
SPME-GC-MS	6	+[b]	3 S.D. (determined in water)	[21]
SPME-GC-NPD	2.8	+	3 S.D. procedural blank	This work

[a] + technique used to simultaneously detect alkylnitriles. – technique not used to simultaneous analyze alkylnitriles.
[b] ACN was used as internal standard but no other AN is determined.

Table 3
Concentrations of HCN and alkylnitrile concentrations ($ng\,mL^{-1}$) in blood of mice administered with CCN (97%, plus 3% TCN) or ALN

Dose group	Mouse number	HCN	CCN	TCN	ALN	BTN
CCN	C1	19.3	75.8	8.2	<LOD	<LOD
	C2	53.5	333.9	28.5	<LOD	<LOD
	C3	53.1	65.7	10.4	<LOD	<LOD
	C4	103.2	16.7	11.6	<LOD	<LOD
	C5	72.9	246.5	11.1	<LOD	<LOD
	Mean	60.4	147.7	14.0	<LOD	<LOD
ALN	A1	45.1	<LOD	<LOD	68.5	<LOD
	A2	26.1	<LOD	<LOD	42.9	<LOD
	A3	34.1	<LOD	<LOD	94.1	<LOD
	A4	26.7	<LOD	<LOD	99.5	<LOD
	A5	23.6	<LOD	<LOD	98.3	<LOD
	Mean	31.1	<LOD	<LOD	80.6	<LOD

methods do not take into account the presence of trace levels of cyanide already in the procedural blanks. Moreover, to the best of our knowledge only headspace-GC and SPME-GC are suitable techniques for the simultaneous determination of cyanide and alkylnitriles. It should be added than when working in SPME, the extraction temperature needed is lower than when working with headspace, which will help in avoiding both coextraction of semivolatile compounds and target analyte degradation.

NPD has shown to be a sensitive and selective detector perfectly suited for the determination of HCN and alkylnitriles in whole blood showing lower limits of detection than the ones of a mass spectrometry based technique [21]. Moreover, the methodology was tested in real blood samples from five mice administered with a CCN (97%)–TCN (3%) mixture ($2.25\,mmol\,kg^{-1}$) and five mice administered with ALN ($0.75\,mmol\,kg^{-1}$). Blood was extracted after 30 min for the ALN group and after 6 h for the CCN group. A summary of the results is presented in Table 3. HCN was identified in all samples from both CCN–TCN and ALN administered mice, confirming its relevance in the metabolism of these alkylnitrile. Furthermore, some samples were reanalyzed after 1 month showing no significant differences. Therefore, the developed methodology was shown to be suitable to analyze HCN and alkylnitriles in blood samples within experimental toxicology studies.

4. Conclusions

In this work, an analytical methodology based on headspace-SPME combined with GC-NPD detection has been developed for the simultaneous determination of cyanide and alkylnitriles in mice and rat whole blood. SPME optimization results are coherent with the physicochemical properties of the target analytes, the more volatile ones appear to be controlled by a thermodynamic process while the others on kinetics. Nevertheless, the developed method shows acceptable quality parameters and suitability for alkylnitrile toxicology studies where a better understanding of nitrile metabolism is needed. Furthermore, the obtained results indicate that this technique should be preferred to headspace GC-NPD in terms of selectivity and sensitivity. This technique has already been used in the study of the role of metabolism by the CYP2E1 enzyme in the toxicity of CCN in the mouse [26].

Acknowledgements

Research funding was obtained from the Spanish Ministry of Science and Education through the projects CGL2005-02846/BOS and BFU2006-00343/BFI.

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