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ORIGINAL ARTICLE

Simple and fast headspace-gas chromatographic determination of formic acid in urine: application to the assessment of occupational exposure to formaldehyde

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ABSTRACT

Formaldehyde (FA) is a chemical widely used in industry, especially in the manufacture of resins and adhesives, with important exposures occurring in the occupational setting. Currently in Brazil, there is no official biomarker to evaluate the occupational exposure to FA. However, urinary formic acid concentrations have been proposed as a possible biomarker and a headspace gas chromatographic method for its determination was developed and validated. The method is based in the automated headspace sampling of the methyl-derivative of formic acid produced *in situ*, followed by separation in a CPWAX 52CB (30 m x 0.25 mm, 0.25 µm) column and flame-ionization detection. Total analytical run time was 9 min. Precision assays showed CV % lower than 6.0 % and accuracy in the range of 101.0 to 102.7 %. Lower limit of quantitation was 5 mg L^{-1}. The method was successfully applied to 51 urine samples obtained from workers of a furniture manufacturing industry, with overall mean of urinary formic acid concentrations of 21.1 ± 9.9 mg L^{-1}. Among the groups of workers distributed according to their working divisions, the one with the higher environmental FA exposure (air concentration of 0.09 ppm), presented urinary formic acid concentrations significantly different from the others (33.9 ± 5.4 mg L^{-1} vs 20.0 ± 9.4 mg L^{-1}), what could indicate a trend to higher urinary formic acid concentration at higher FA exposures, even at low environmental concentrations.

Keywords: Formaldehyde, biomonitoring, formic acid, headspace, gas chromatography

1. Introduction

Formaldehyde (FA) is a chemical widely used in industry, especially in the manufacture of resins and adhesives. Besides its ubiquitous presence in indoor air, important exposures to FA occur in the occupational setting (1).

The major route of absorption of FA is by inhalation and the intensity of the physiological responses depends on FA concentrations in air (2). The biological half-life is extremely short, about 1 min (3). The metabolism of FA is mostly based on formaldehyde dehydrogenase activity, responsible to its conversion to formic acid, which is excreted in urine (4). Cellular toxicity of FA is related to the saturation of the metabolic detoxification pathways (5). FA can react with deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and proteins forming reversible or irreversible adducts. FA form DNA-protein crosslinks and the incomplete repair of these connections can lead to mutations, especially chromosomal mutations and micronuclei in proliferating cells (6). An increased concern with FA toxicity has been raised by the recent report of the *International Agency for Research on Cancer* (IARC) that classified FA as a human carcinogen, based on mortality studies of workers exposed to FA with an increased incidence of nasopharyngeal cancer (6).

Urinary formic acid concentrations have been used as a biomarker of exposure to FA (4). However, considering the formation of formic acid from other metabolic sources, there is a wide variability in reported baseline urinary concentrations, being described as 12.5 mg L^{-1} (7) 13.0 mg L^{-1} (8) or 20.0 mg L^{-1} (9). Currently in Brazil there is not official biomarker for the occupational exposure to FA and the exposition on labor is evaluated by the measurement of environmental concentrations.

The aim of this study was to validate a methodology for the measurement of urinary formic acid using headspace sampling associated to gas chromatography with flame ionization detection

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and to evaluate the concentrations of formic acid in the urine of workers of a furniture manufacturing industry.

2. Experimental

2.1 Chemicals

Formic acid and acetonitrile (HPLC grade) were purchased from Merck KGaA (Darmstadt, Germany). Sulfuric acid 96% was purchased from Panreac PA (Barcelona, Spain). Methanol (HPLC grade) was purchased from Merck KGaA (Darmstadt, Germany). Ultrapure water was obtained through an Elga Purelab Ultra® apparatus from Elga LabWater (Lane End, UK).

2.2 Preparation of solutions and standards

The formic acid stock solution was prepared at the concentration of 1 mg mL^{-1} by dilution with ultrapure water. Working solutions was prepared combining aliquots of stock solution and proper volumes of ultrapure water at the concentrations of 100 mg L^{-1}. From the working solutions, 6 analytical solutions were prepared containing formic acid at the concentrations of 10, 20, 40, 60, 80 and 100 mg L^{-1}. The working internal standard solution was acetonitrile 2 mg mL^{-1}.

The 2,4-dinitrophenylhydrazine derivative of FA was prepared according to SKC Operating Instructions and analytical solutions were prepared in the range of 0.078 µg to 2.5 µg mL^{-1}.

2.3 Equipment and chromatographic conditions

The CG-FID system consisted of a Varian CP-3800 gas chromatograph and a CombiPAL autosampler, both controlled by Galaxie Workstation program from Varian (Midlesburg, Holland). Separation was performed on a CP WAX 52 CB column (30 m x 0.25 mm x 0.25 µm). Injector temperature was 220ºC. The carrier gas was helium at a constant flow of 2 mL min^{-1}. Detector temperature was 250ºC. Injection was in split mode, with split ratio of 1:10. The oven temperature program was set at 30ºC for 6 min, followed by an increase of 35ºC min^{-1} to the final temperature of 135ºC. The duration of the chromatographic analysis was 9 min.

2.4 Determination of environmental concentrations of FA

Enviromental concentrations of FA were determined using UMEX-100 passive samplers (SKC Inc., Eighty Four, USA) according to EU ISO 16000-4-2004. The concentrations of the 2.4-dinitrophenylhydrazone derivative of FA were determined using an Accela UHPLC® coupled to an Accela® Photodiode Array Detector, both from Thermo Scientific (San Jose, USA). The separation was performed on a Hypersil Gold® C18 column (150 x 4.6 mm, particle diameter 5 µm), from Thermo Scientific (San Jose, USA). Mobile phase consisted of 33 % water in methanol. The flow rate was set at 1 mL min^{-1}. Column temperature was set at 30ºC. Total run time was 8 minutes. Spectra were acquired in the range of 230 to 370 nm and the quantitation wavelength was 365 nm.

2.5 Sample preparation

To 10 mL vials, 2 mL of either calibration, quality control or patient's urine samples were added, followed by 0.1 mL of IS working solution (acetonitrile 2 mg mL^{-1}), 500 µL de H_2SO_4 99% e 100 µL de methanol. Vials were capped quickly to avoid loss of analyte by volatilization and transferred to the autosampler. Automatic incubation of the samples occurred at 50ºC for 10 min, with intermittent stirring at 500 rpm. Injection volume was 1.000 *µL*.

2.6 Selectivity

Selectivity was tested by evaluating the mass spectra of 6 urine samples submitted to the sample preparation described above and submitted to GC-MS analysis to check for the presence of other compounds than methyl-formate. Spectra were recorded on a Focus GC coupled to an ISQ single quadrupole mass spectrometer, both from Thermo Scientific (San Jose, USA), operating at EI mode (70 eV). Mass spectra were acquired at full scan mode, from m/z 30 to 300. The chromatographic conditions were the same as described for GC-FID.

2.7 Linearity

The linearity of the calibration models was evaluated in 6 analytical solutions containing 10, 20, 40, 60, 80 and 100 mg L^{-1} of formic acid. Replicates (n=6) at each concentration were analyzed as described above. Calibration curves were constructed by calculating the ratios of the peak area of the analyte to the peak area of the internal standard and relating these ratios with nominal concentrations of the calibration samples. Curves were fitted by using several weighting factors (1/x, $1/x^2$, 1/y, $1/y^2$). The calibration models were evaluated by their correlation coefficients (r) and Cumulative Percentage Relative Error (∑%RE) (10). Daily calibration curves using the same concentrations (single measurements per concentration) were prepared with each batch of validation or clinical samples.

2.8 Formic acid stability

Formic acid stability was evaluated at three concentration levels: quality control low level (15 mg mL^{-1}, QCL), quality control low medium level (50 mg mL^{-1}, QCM) and quality control high level (90 mg mL^{-1}, CQA), which were analyzed in triplicate on the day of preparation and after 1, 6, 9 and 13 days of storage, at the temperatures of 25º*C, 4ºC and -20ºC*. Stability was evaluated as the percentage of the average concentrations measured on the day of the preparation of the QCs. The acceptance criterion for stability was mean values within ±15% of the concentrations measured on the day of the preparation of the QCs.

2.9 Accuracy and precision

The quality control samples (QCL, QCM and QCL, as described in the stability evaluation) were analyzed as described

above in triplicate on each of 5 days. Within-assay precision and between-day precision were calculated by one-way ANOVA with the grouping variable "day" and were expressed as CV%. Accuracy was defined as the percentage of the nominal concentration represented by the concentration estimated with the calibration curve. The acceptance criterion for accuracy was mean values within ±15% of the theoretical value and for precision a maximum CV of 15% was accepted (11).

2.10 Lower limit of quantitation

An independent quality control sample at the concentration of the lowest point of the calibration curve (5 mg L^{-1}) was included in the accuracy and precision experiments (quality control at the limit of quantitation, QCLOQ) and was tested in triplicate in three different days. The acceptance criteria established for the limit of quantification was accuracy within 100 ± 20% of the nominal value and a maximum CV of 20% (11).

2.11 Method application

The study was approved by the Institutional Review Board of Universidade Feevale (349.445). A group of 51 workers of a furniture manufacturing industry, potentially exposed to formaldehyde, were recruited in August 2013. Written informed consent was obtained from all participants. End-of-shift urine samples were obtained and kept at 4ºC until analysis. Environmental concentrations of FA were obtained at each one of the 7 different working divisions of the group of workers (machining centers and drilling, metal cutting, packaging, lamination edges, lamination/press, painting edges, painting UV), using passive UMEX-100 samplers as previously described.

2.12 Statistical analysis

The results are expressed as mean ± standard error of the mean. Significant differences between the experimental groups were determined for each measurement using a one-way ANOVA followed by the Student *t* test. The comparison between groups will be carried out using the Student *t* test. Values of $p < 0,05$ were considered as significant.

3. Results and discussion

3.1. Sample preparation and chromatography

There are few methodological alternatives for the determination of formic acid concentrations in urine. An enzymatic method has been described where formic acid was quantitatively oxidized in the presence of nicotinamide adenine dinucleotide (NAD+) by formate dehydrogenase (12). However, the use of gas-chromatographic (GC) methods is the most common approach. Sophisticated GC methods were described including the use of in-tube extraction gas chromatography–mass spectrometry (GC-MS-ITEX), which requires sophisticated instrumentation and consumables (13). Another reported approach is the use of solid-phase microextraction (SPME). Lee *et al.* used headspace-SPME of formic acid in urine after its derivatization to methyl formate under acidic conditions, associated to gas-chromatographic analysis (14) Another study reported the use of headspace-SPME coupled to GC using boron trifluoride as derivatizing agent for formic acid (15).

A more straightforward approach is the use of headspace sampling of a volatile derivative of formic acid followed by GC analysis. Such procedure has several advantages by dispensing the use of solvents (15), automated and unattended processing and the injection of clean samples into the chromatographic column, associated with high sensitivity (14). In the present study, methyl-formate was formed in the vial trough the reaction of methanol with formic acid, catalyzed by sulfuric acid and the derivative was volatile enough to be sampled in the headspace, at mild temperatures. Overall, the sample preparation procedure was simple and fast. Chromatographic separation of methyl-formate and acetonitrile (internal standard) was obtained in a polar capillary column, with a total run time of 9 min. The retention time of the formic acid derivative was 3.5 min and the retention time of the internal standard was 7.3 min (Figure 1). To confirm that the detected peaks at 3.5 min were methyl-formate, we measured electron impact mass spectra from 6 different urine samples from non-exposed subjects. In all samples the obtained spectra were almost identical to those of the authentic compound, with the ions 60, 44, 31 (base peak) 29 and 15, confirming the identity of the analyte.

3.2. Method validation

There was no indication of instability of formic acid in any of the tested conditions, as shown in Table 1. The quality-control samples tested in the stability test presented the following percentage concentrations of the values measured on the day of the preparation of the QCs: QCL - 87.6 to 105.9% at 25ºC, 94.0 to 108.2 at 4ºC and 90 to 108.3 at -20ºC; QCM - 93.2 to 106.1 at 25ºC, 93.2 to 106.9 at 4ºC and 89.4 to 106.3 at -20ºC; QCA - 94.5 to 113.3 at 25ºC, 93.3 to 111.7 at 4ºC and 94.1 to 111.5 at -20ºC. Overall, formic acid concentrations were stable for 13 days under the different evaluated conditions, with measured concentrations in the range of 87.6 to 113.3 % of those measured at the day of preparation of the QCs, with exception of a single point (122.7%), what can be attributed to experimental error.

Considering the heteroscedasticity of the calibration data, several weighting factors were evaluated in order to obtain a proper regression model in the linearity assay. Among all tested weighting factor, 1/x was selected as the most appropriate, presenting the Σ% RE of - 0.17^{-13}. Daily calibration curves presented *r* always higher than 0.995. QC samples for accuracy and precision experiments were prepared at 3 concentrations (QCL, QCM, and QCH) covering the calibration range. The results of the accuracy and precision experiments are given in Table 2. All accuracy values fulfilled the acceptance criteria for this parameter, lying within the range of 101.0 to 102.7% of the nominal concentrations of the analyte. With-assay precision (CV %) was in the range of 2.3 to 4.8% and between-assay precision of 2.7 to 6.0%, also in accordance to the acceptance criteria. The lower limit of quantitation was 5 mg L^{-1}, presenting an intra-assay

Table 1. Values of stability testing at 25, 4 and - 20 °C.

Day	Percentage of the concentration measured at day 0								
	25°C			4°C			-20°C		
	QCL	QCM	QCA	QCL	QCM	QCA	QCL	QCM	QCA
1	105.4	100.4	103.7	99.8	102.7	111.7	94.3	106.3	105.7
	102.1	103.2	113.3	101.3	106.9	109.4	90.0	105.4	102.7
	104.9	104.2	100.8	108.2	105.2	109.7	107.7	105.1	94.1
6	105.6	101.2	103.0	98.0	99.6	100.9	92.8	91.3	105.3
	100.0	106.1	110.8	96.1	100.5	105.9	97.3	101.2	111.5
	101.2	122.7	109.6	101.9	98.8	110.1	103.1	101.2	104.3
9	104.0	103.5	104.1	98.6	101.9	99.3	108.3	89.5	100.8
	105.4	93.2	94.5	101.2	101.5	98.0	95.2	89.4	96.2
	105.9	100.7	102.8	100.0	98.7	94.5	92.6	93.7	99.1
13	87.6	96.3	104.1	94.8	97.5	93.3	106.8	92.9	94.8
	93.7	100.2	100.0	98.0	98.4	97.4	102.0	93.3	95.4
	90.5	98.1	94.7	94.0	93.2	97.9	99.6	93.6	95.2

CV% of 2.6 and an inter-assay CV% of 5.5. Assay sensibility was adequate for biological monitoring of formaldehyde exposure, being able to measure endogenous levels.

3.3. Method application

The developed method was apply for the determination of formic acid on end-of-shift samples obtained from workers potentially exposed to FA at a furniture manufacturing industry. The 51 workers group was divided in 7 working divisions, where passive samplers for FA were placed for 8 h, according to the manufacturer's instructions. Environmental concentrations of FA and urinary concentrations of formic acid are presented in Table 3.

Environmental concentrations of FA were on the range of 0.03 to 0.09 ppm, being below those considered as acceptable by the US Occupational Safety and Health Agency (OSHA) of 0.75 ppm (16), but above the US National Institute for Occupational Safety and Health (NIOSH) established threshold of 0.016 ppm (17).

Currently, there is no officially recommended biomarker for the occupational exposure to FA. However, Coelho reported that urinary concentrations of formic acid in urine of workers of a FA producing factory (17.14 ± 5.41 mg L^{-1}), were statistically significantly higher than those measured at a control group (8.94 ± 2.92 mg L^{-1}) (14).

In our study group, the overall mean of formic acid concentrations was 21.1 ± 9.9 mg L^{-1} (range 3.8 to 41.9 mg L^{-1}). Among the groups of workers distributed according to their working divisions, only one presented urinary formic acid concentrations significantly different from the others (33.9 ± 5.4

Table 2. Precision and accuracy of the method.

QC Sample	Concentration (mg L^{-1})	Precision		Accuracy (%)
		Between--assays (CV%)	Within--assays (CV%)	
QCLOQ	5	5.5	2.6	101.2
QCL	15	4.3	4.8	102.7
QCM	50	2.7	3.4	101.0
QCA	90	6.0	2.3	101.2

Table 3. Environmental concentrations of FA and formic acid concentrations in exposed workers.

Group	n	Formaldehyde in air (ppm)	Formic acid in urine (mg L^{-1})*
Group 1	7	0.07	16.3 ± 6.7 [a] (3.8 – 23.2)
Group 2	4	0.09	33.9 ± 5.4 [b] (30.1 – 41.8)
Group 3	8	0.03	18.5 ± 7.6 [a] (8.3 – 30.5)
Group 4	3	0.04	18.9 ± 5.5 [a] (15.3 – 25.3)
Group 5	11	0.03	20.7 ± 11.6 [a] (5.9 – 40.7)
Group 6	13	0.06	21.3 ± 10.6 [a] (4.6 – 41.9)
Group 7	5	0.03	23.0 ± 10.4 [a] (8.5 – 36.5)
51		P	0.15

* Average ± SD.
Range ANOVA (α = 0.05).
([a,b] same letters do not differ in test t 5% significance).

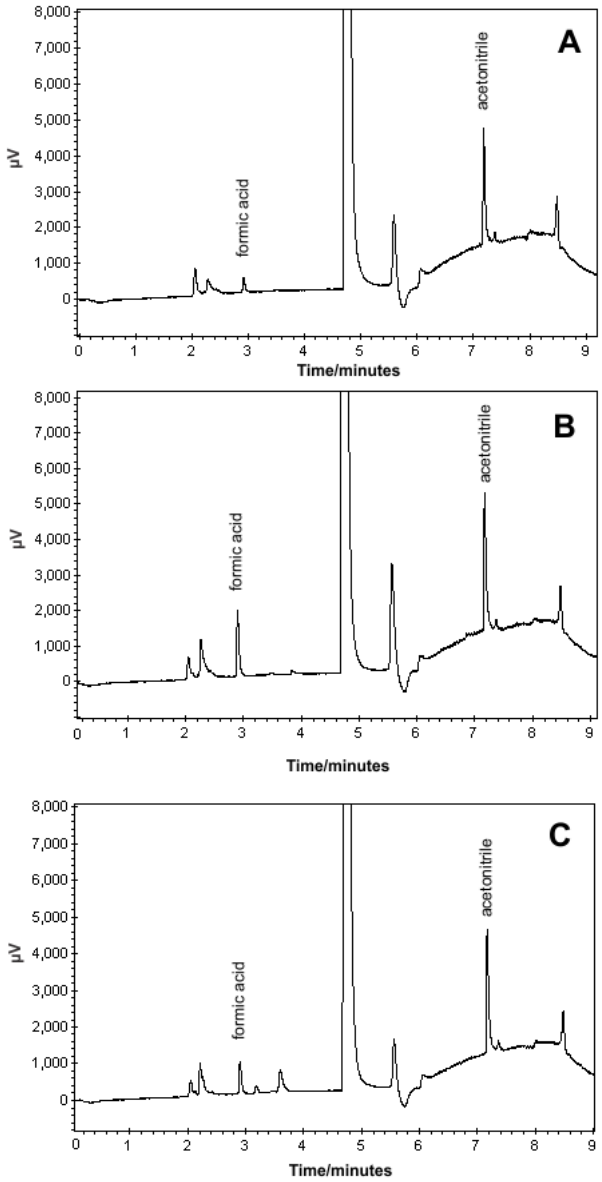

Figure 1. A) Chromatogram obtained from an analytical solution containing formic acid at 4 mg L^{-1}. **B)** Chromatogram obtained from an analytical solution containing formic acid at 18 mg L^{-1}. **C)** Chromatogram obtained from a urine sample of a worker exposed to FA, with formic acid concentration of 8,36 mg L^{-1}.

mg L^{-1} vs 20.0 ± 9.4 mg L^{-1}). This group with higher mean formic acid concentration was also the one with the higher environmental FA exposure (air concentration of 0.09 ppm), what could indicate a trend to higher urinary formic acid concentration at higher FA exposures, even at low environmental concentrations.

4. Conclusions

A simple, rapid and easily automated for the determination of formic acid in urine using headspace sampling and GC-FID was validated, presenting suitable analytical performance suitable for its routine use. Formic acid was stable in urine up to 13 days in room temperature. The method was successfully applied to 51 urine samples obtained to workers exposed to low environmental levels of FA. The group of workers exposed to higher FA concentrations presented significantly higher urinary formic acid concentrations), what could indicate a trend to higher urinary formic acid concentration at higher FA exposures, even at low environmental concentrations.

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