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Trace determination of free formaldehyde in DTP and DT vaccines and diphtheria–tetanus antigen by single drop microextraction and gas chromatography–mass spectrometry

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ABSTRACT

An immersed single drop microextraction (SDME) method was successfully developed for the trace enrichment of formaldehyde from DTP and DT vaccines and diphtheria–tetanus antigen. The formaldehyde was derivatized by means of the Hantzsch reaction. The dehydropyridine derivative was extracted into a microdrop of chloroform that suspended in a 4 ml sample solution for a preset time. The microdrop was then retracted into the microsyringe and injected directly into a gas chromatography–mass spectrometry (GC–MS) injection port. Effects of different parameters such as the type of solvent, extraction time, stirring rate, and temperature were studied and optimized. The limit of detection was 0.22 ng/l and relative standard deviation (RSD) value was 6.2% ($n = 5$). The regression coefficient was satisfactory ($r^2 = 0.992$) and linear range was obtained from 1 to 500 ng/l.

1. Introduction

Formaldehyde, the simplest of carbonyl compounds, is ubiquitous in the environment and is commonly found in air, water, and industrial products. The compound is known to be mutagenic and carcinogenic. Because of the adverse health effects, ambient, occupational, and consumer exposures to formaldehyde have been an issue of serious concern. Analytical chemistry and toxicology of this specific compound have been extensively discussed [1].

Formaldehyde is one of common substances found in vaccines inactivating bacterial products. It is also used to kill unwanted viruses and bacteria that might be found in cultures used to produce vaccines. To assure the safety of vaccines, the Centers for Disease Control and Prevention (CDC), the Food and Drug Administration (FDA), the National Institutes of Health (NIH), and other national and international agencies routinely monitor and conduct research to examine any new evidence that would suggest possible problems with the safety of vaccines [2]. Only a few works can be found where formaldehyde is directly determined without any previous derivatization reaction [3,4]. In general, derivatization reactions are normally used for the determination of this compound. A variety of reagents such as 2,4-dinitrophenylhydrazine[5–9], chromotropic acid, 3-methyl-2-benzothiazolone hydrazone [9], pararosaniline [10,11], 2-diphenylacetyl-1,3-indandione-1-hydrozone [12], dimedone [13] and lutidine [14,15] have been employed to chemically modify formaldehyde.

One of the most important methods for the determination of formaldehyde is the lutidine method, which uses the Hantzsch reaction to derivatize formaldehyde to 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine, also called 3,5-diacetyl-1,4-dihydrolutidine (DADHL). This method is relatively simple, rapid and sensitive.

Gaseous formaldehyde was sampled by derivatization with o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) adsorbed onto poly (dimethylsiloxane)/divinylbenzene SPME fibers. The oxime product was analyzed by GC [16].

The derivatization of formaldehyde by means of Hantzsch reaction is characterized by cyclization of two acetyl acetone and formaldehyde in the presence of ammonia to form DADHL [15,17,18].

Most of the methods for the determination of formaldehyde were reviewed in 2001 [19]. Formaldehyde has been measured in biological samples by GC–MS, GC-flame ionization detection (FID) [20,21] and high performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection [22,16]. To achieve the necessary levels of sensitivity, an enrichment step is needed prior to the chromatographic analysis.

Supercritical fluid extraction and solid-phase microextraction are the most commonly used techniques for extraction of formaldehyde [16,23–25]. Recently, a solvent-minimized sample pretreatment procedure, known as SDME, has gained lots of attentions

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[26–28]. An important additional feature of SDME is the integration of extraction and injection in a microsyringe, making it possible to employ this miniaturized medium for extraction as well as an injection device for the GC [27–30]. This methodology is not only fast and inexpensive, but also needs only common laboratory equipment and does not suffer from carry-over between extractions that may be experienced using SPME. Due to the need for small volume of solvent, there is minimal exposure to toxic organic solvents. Solvent microextraction, in combination with GC, has been shown to be quite efficient for the determination of phenol and chlorophenols [30], s-triazine herbicides [31], chlorobenzenes [32], pesticides [33] and nitroaromatic explosives [34] in water samples as well as for the screening of cocaine and cocaine metabolites in biological fluid [35].

Following our research on SDME of organic pollutants from aqueous media [30,31,36] and sol–gel-based SPME of some drugs from plasma samples [37,38], for the first time, an immersed SDME-based technique for the determination of formaldehyde in DTP, DT vaccines and diphtheria–tetanus antigen was developed. A microdrop of chloroform solvent was found to be a suitable extraction medium, while the extraction temperature (as high as 20 °C) could be controlled using a water-jacketed vessel. Influences of other important parameters along with matrix effect were also studied.

2. Experimental

2.1. Reagents

Naphthalene, chloroform, toluene, butyl acetate, cyclohexane, carbon tetrachloride, ammonium acetate, trichloroacetic acid (100%w/v) methanol (HPLC-grade) and sodium chloride (99.5% minimum) were purchased from Merck (Darmstadt, Germany). Formaldehyde (37% stabilized with 10% methanol) and acetyl acetone (99.5%) were prepared from Riedel-de-Haen (Seelz, Germany). DTP, DT Vaccines and diphtheria and tetanus antigen were kindly provided by Razi vaccine and serum research institute (Karaj, Iran).

Prior to the derivatization process it was necessary to prepare ammonium acetate and acetyl acetone solutions Ammonium acetate solution was prepared by dissolving 15 g of ammonium acetate in water, adding 0.3 ml glacial acetic acid and diluting to 100 ml in water. This solution could be used within one week of preparation. The acetyl acetone solution was made by adding 0.2 ml of acetyl acetone to 100 ml of ammonium acetate solution.

DTP, DT Vaccines and diphtheria and tetanus antigens were provided by Razi vaccine and serum research institute (Karaj, Iran) and stored at 4 °C.

2.2. Apparatus

A Hewlett-Packard (HP, Palo Alta, USA) HP 6890 series GC equipped with a split/splitless injector and a HP 5973 mass-selective detector system were used. The MS was operated in the EI mode (70 eV). Helium (99.999%) was employed as carrier gas and its flow rate was adjusted to 1 ml/min. The separation of dehydropyridine derivative was performed on a 28.5 m × 250 μm i.d. fused-silica capillary column coated with a 0.25 μm bonded film of HP-1 MS. The GC column temperature was programmed at 40 °C for 2 min and then raised to 260 °C at 10 °C/min, 10 min hold at 260 °C. The injector temperature was set at 280 °C, and all injections were carried out on the splitless mode. The ion source and quadrupole temperatures were set at 230 and 150 °C, respectively. The GC–MS interface was maintained at 280 °C. The MS was operated using total ion current (TIC) mode, scanning from m/z 40 to 300. For quantitative determination, the MS was operated in time-scheduled selective ion monitoring (SIM) mode. Quantitation was performed by calculating peak areas relative to the IS (naphthalene).

2.3. Derivatization of formaldehyde

Stock solutions (20 μg/ml) of formaldehyde were prepared in water, and stored at −20 °C. Eventually, to 1 ml of water containing 20 μg/ml of formaldehyde 4 ml of water and 5 ml of acetyl acetone reagent were subsequently added. The final solution was heated at 40 °C using a circulating water bath and allowed to stand for 40 min.

2.4. Extraction apparatus and SDME procedure

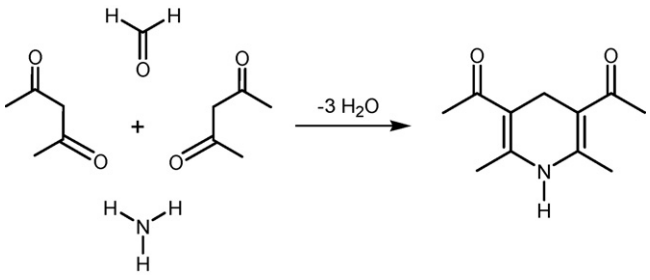
The details of the developed device have been, already, explained in elsewhere [33,34]. In this technique, the analytes are distributed between the bulk aqueous phase and a microdrop of organic solvent, suspended directly at the tip of a microsyringe needle that is immersed in a stirred aqueous sample solution. After a certain time, when sufficient amounts of analytes are transferred into the organic extractor, the microdrop is retracted into the microsyringe, and subsequently part or all of the organic solvent is injected into the gas chromatographic system. After SDME of dehydropyridine derivative of formaldehyde in a certain time, the extract was finally injected into the GC–MS system.

3. Results and discussion

SDME is based on the partition of analyte between two immiscible liquid phases; extracting polar organic compounds, i.e. dihydropyridine derivative, from aquatic media becomes rather more difficult as they tend to stay in aqueous media. Furthermore, headspace SDME seems to be an inefficient method due to the low volatility of analytes with polar characteristics. However, it has been shown that SDME can be used as an efficient method for the extraction of phenol and chlorophenols [30] and s-triazine herbicides in aquatic media [31]. The feasibility of an immersed SDME method was, therefore, considered in order to bring the extracting phase in direct contact with the analyte, enhancing the overall mass-transfer coefficient with respect to the organic phase, β_o, an influential factor affecting observed rate constant (k) given by:

$$k = \frac{A_i}{V_o}\tilde{\beta}_o\left(\kappa\frac{V_o}{V_{aq}} + 1\right) \quad (1)$$

where A_i is the interfacial area, κ the distribution constant, and V_{aq} and V_o are the volumes of aqueous and the organic phases, respectively. Clearly, the higher β_o value is an indication of higher efficiency for the extraction process. According to this equation and the film theory convective–diffusive mass transfer [27], an immersed SDME method for preconcentration of dihydropyridine derivative from aqueous samples appeared to be quite promising. This is especially true when the mechanism of the derivatization is in a way that a rather large fluorescent derivative can be formed [18] (Scheme 1).



Scheme 1. The derivatization process of formaldehyde.

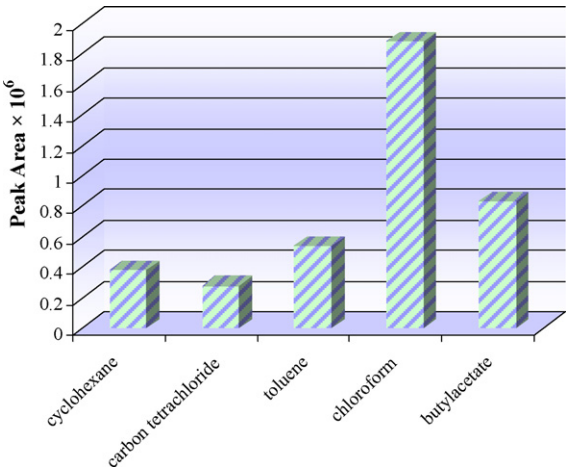


Fig. 1. Extraction efficiencies obtained for different organic solvents.

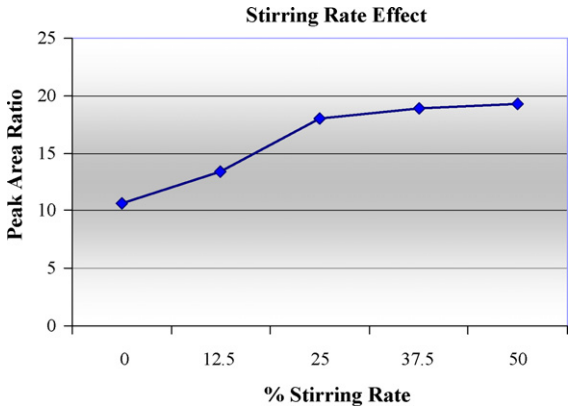


Fig. 2. Effect of stirring rate on the extraction efficiency of dihydropyridine derivative of formaldehyde from aquatic medium.

3.1. Optimization of immersed SDME

A univariate approach was employed to optimize influential factors in this method. Various parameters affecting the SDME efficiency including the type of solvent, stirring rate, extraction time, temperature of sample solution, and ionic strength were optimized. In order to correct any possible changes on the microdrop, naphthalene was added to the organic solvent as internal standard. The ratio of peak area of dihydropyridine derivative and that of naphthalene was used to assess the extraction efficiency under various conditions.

3.1.1. Solvent selection

Five water-immiscible solvents with different polarity and water solubility including chloroform, butyl acetate, toluene, cyclohexane and carbon tetrachloride were examined in order to find the most suitable solvent for extraction. Solvent selectivity was evaluated for the extraction of 4 ml of sample containing 1 μg/ml of formaldehyde, already derivatized into its corresponding dihydropyridine derivative, in deionized water. The stirred solution (at 10% of maximum stirring efficiency) was sampled at 25 °C for 15 min using 3 μl of each organic solvent. Since these solvents have various water solubilities, longer sampling times, higher sample temperature and faster stirring rates were, therefore, avoided. The results are given in Fig. 1. The extraction efficiency was based on the average peak area of each analyte for three replicate analyses. Apparently, chloroform shows higher extraction efficiency in comparison with other solvents. The primary reason could be attributed to the higher polarity of chloroform ($\log K_{ow} = 1.89$, the octanol–water partition coefficient), which favors interaction with polar compounds. Clearly, the higher value of octanol–water partition coefficient indicates the less hydrophilic character for the substance of interest. In the mean time, the chloroform microdrop could be more easily manipulated preventing the drop loss even when faster stirring rates were used. Other solvents including butyl acetate ($\log K_{ow} = 2.06$), toluene ($\log K_{ow} = 2.454$), cyclohexane ($\log K_{ow} = 2.588$) and carbon tetrachloride ($\log K_{ow} = 2.6$) were, therefore, excluded from further investigation.

3.1.2. Stirring rate effect

Sample agitation enhances extraction efficiency and reduces extraction time, especially for higher molecular mass analytes [27]. For the purpose of the present study three replicate analyses were taken at four different stirring rates: 0 (no agitation), 12.5%, 25%, 37.5% and 50% of maximum stirring efficiency. Faster stirring rates were avoided as they resulted in dislodgement of the organic drop from the needle tip. In all cases, the 3 μl chloroform drop was exposed at 25 °C for 15 min to a 4 ml of sample containing 1 μg/ml of formaldehyde. Fig. 2 shows that the agitation improves the extraction efficiencies of dihydropyridine significantly. This is in agreement with the expected behavior of solvent microextraction based on the film theory convective–diffusive mass transfer [27,28] and our recent results on phenols [30] and s-triazine herbicides [31].

Although high stirring rates increase the enrichment factors considerably, the stability of a microdrop at the tip of the needle could be dramatically affected when a high stirring rate is used. This is especially true when prolonged sampling times are applied. Thus, for all further experiments a stirring rate at 37.5% of maximum stirring efficiency was used. Using a small magnet with consistent stirring rate and avoiding any temperature convection was quite essential for achieving an acceptable precision.

3.1.3. Drop size effect

The solvent drop size was another important parameter, which was investigated. Fig. 3 shows that the GC–MS responses increase with chloroform drop volume in the range of 2–4 μl and then decrease when the drop size was increased further. This decrease in response after using 4 μl microdrop could be resulted from the dilution of analyte in higher amount of organic solvent. Moreover, the speed of extraction is influenced by observed rate constant (s^{-1}) given by Eq. (1) and our previous results on s-triazine herbicides [31] supports this phenomenon. The influence of drop size, therefore, originates from the integrated influence of two factors, justifying why the GC–MS response enhances with increasing drop size up to 4 μl and decreases afterward.

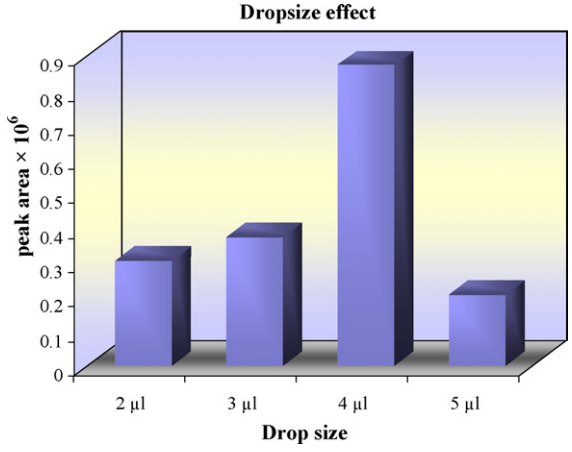


Fig. 3. Effect of different drop size on the extraction efficiency.

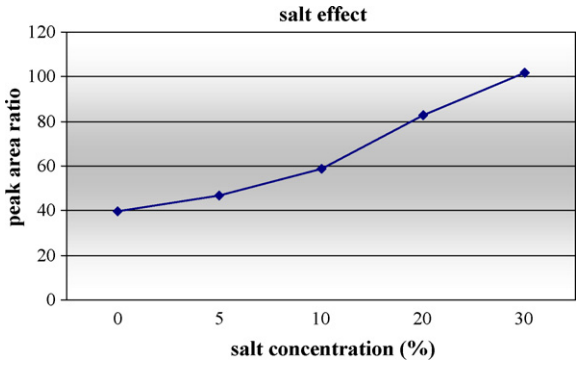


Fig. 4. Effect of ionic strength on the extraction efficiency.

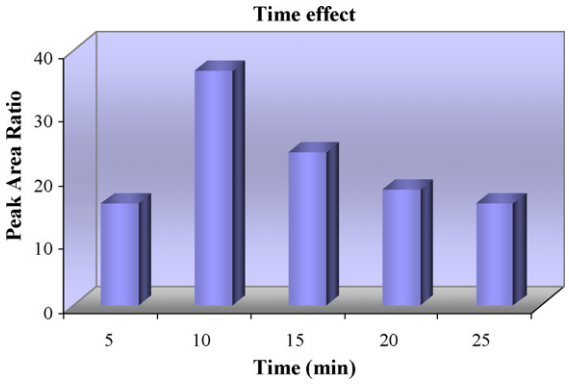


Fig. 6. SDME time profiles obtained for the studied formaldehyde.

3.1.4. Ionic strength effect

The influence of salt addition on the efficiency of SDME was also investigated. Usually, the presence of salt increases the ionic strength of aqueous solution and would affect the solubility of organic solutes. This can be explained by the engagement of water molecules in the hydration spheres around the ionic salt. These hydration spheres reduce the concentration of water available to dissolve solute molecules. This should, then, drive additional solutes into the organic extractant. This effect is rather important for SPME and addition of more than 1% of sodium chloride to enhance the extraction efficiency of the fibers have been reported [39–41]. Our results, however, show an increase in efficiency for dihydropyridine derivative (Fig. 4). Thus, for all further experiments a salt concentration of 30% was used.

3.1.5. Temperature effect

Temperature is a major parameter affecting extraction efficiency. Increasing the reaction temperature by 10 K approximately doubles the rate of reaction. This part of work was carried out using a temperature range of 5–25 °C employing a laboratory-made device. As Fig. 5 shows the extraction efficiency increases as the solution temperature is enhanced. This is expected behavior, since at higher temperatures; the mass transfer coefficients along with the rate constants are enhanced. However, the microdrop tends to become depleted as temperature is raised. This is due to the fact that the boiling point of chloroform is low and consequently extraction efficiency is decreased.

3.1.6. Extraction time effect

Extraction time is a major parameter affecting the extraction efficiency. This effect was studied in the range of 5–25 min at room temperature keeping the stirring rate constant at 38% of maximum stirring efficiency. Fig. 6 shows that the analytical signal increases quickly with sampling time in the range of 5–10 min, and after 10 min extraction efficiency decreases. This might be attributed to the reduction of the microdrop size when longer extraction times are used.

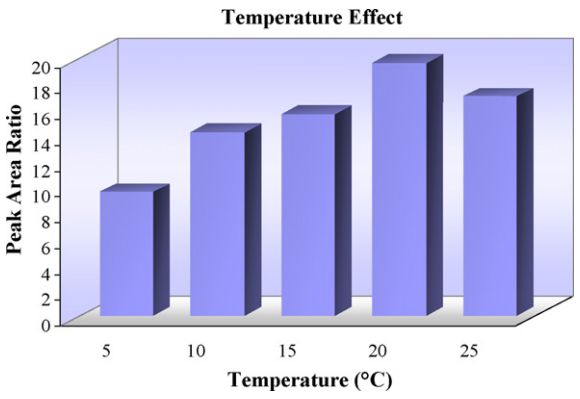


Fig. 5. Extraction efficiencies obtained at various extraction temperatures.

3.2. GC–MS determination

After sample extraction, an aliquot of 4 μl of chloroform containing the extracted dihydropyridine derivative was directly injected into the GC–MS system. To obtain the highest possible sensitivity, the MS detection was operated using time-scheduled SIM based on the selection of two mass peaks of the highest intensity. Table 1 lists the retention time, selected masses and the start scan time for formaldehyde studied by GC–MS. The EI mass spectrum of compound was already obtained by the direct injection of a standard solution of the derivatized analyte into the GC–MS. Mass spectrum of dihydropyridine derivative (Fig. 7) confirms the derivatization process as it contains a peak at m/z 191, which is due to being aromatic by elimination of two hydrogen ($C_{11}H_{15}NO_2$, m/z 193) and base peak at m/z 176, which is due to elimination of methyl group.

3.3. Quantitative evaluation

The optimized method was examined for the extraction and determination of formaldehyde in Vaccine samples. The SDME–GC–MS analysis was performed using a 10 min extraction time at 20 °C, samples were stirred at 37.5% of maximum stirring efficiency, sodium chloride was 30% and the drop size of chloroform was 4 μl. A typical chromatogram obtained under these conditions is shown in Fig. 8.

All vaccine samples were spiked with different concentrations of formaldehyde solution. After extraction and GC–MS analysis, calibration curve for vaccines were plotted. A linearity range of 1–500 ng/l with the equation of $y = 140.67x + 77213$ for vaccines was obtained. The regression coefficient was satisfactory ($r^2 > 0.99$). the limits of detection and quantification, based on a signal-to-noise ratio of S/N = 3 and 10, were at 0.22 and 0.55 ng/l, respectively. In addition, the precision of method was determined based on five replicate analysis and the RSD% value of 6.2% was obtained.

Table 1
Retention times, selected ions and start time of compounds studied by GC–MS.

Compound	Retention time (min)	Selected ions (m/z)	Start scan time (min)
NP (IS)	10.6	127, 128	3
Formaldehyde	15.4	176, 191	13.5

[a] Formaldehyde was derivatized prior to extraction and subsequent determination.

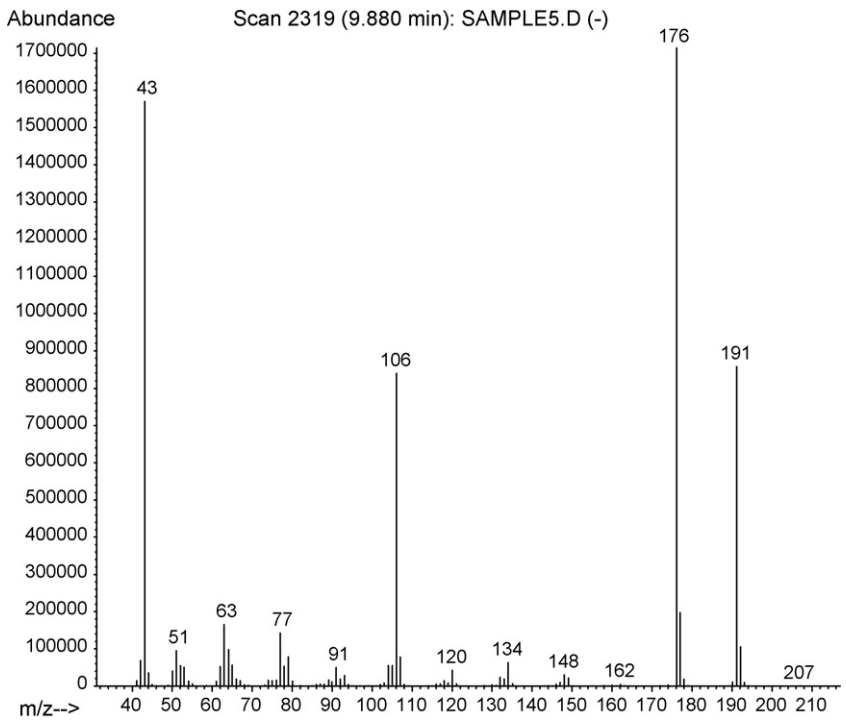


Fig. 7. Electron impact mass spectrum of dihydropyridine derivative of formaldehyde.

3.4. Matrix effect evaluation

As it has been already mentioned by many researchers in this field, SDME is a non-exhaustive extraction procedure and for this reason the relative recovery, defined as the ratio of GC–MS peak areas of analyte in natural and deionized water sample, spiked with the same amount of analyte, was used [30–33] to asses the relative recovery. To determine the relative recovery in diphtheria and tetanus antigens prior to derivatization, protein contents must be removed. For this propose, 0.5 ml of antigen with small amount of trichloroacetic acid was centrifuged at a rate of 8000 rpm in 5 min. However, there was no need to centrifuge vaccine samples as the stability of microdrop was not influenced by the matrix effect. The solutions were subsequently derivatized and extracted using developed method. Then, the samples were spiked with 1 μg/ml of formaldehyde and similar procedure was performed as well. The same procedure was carried out for the deionized water. The relative recovery values were found to be 88%, 85%, and 95% for DTP, DT,

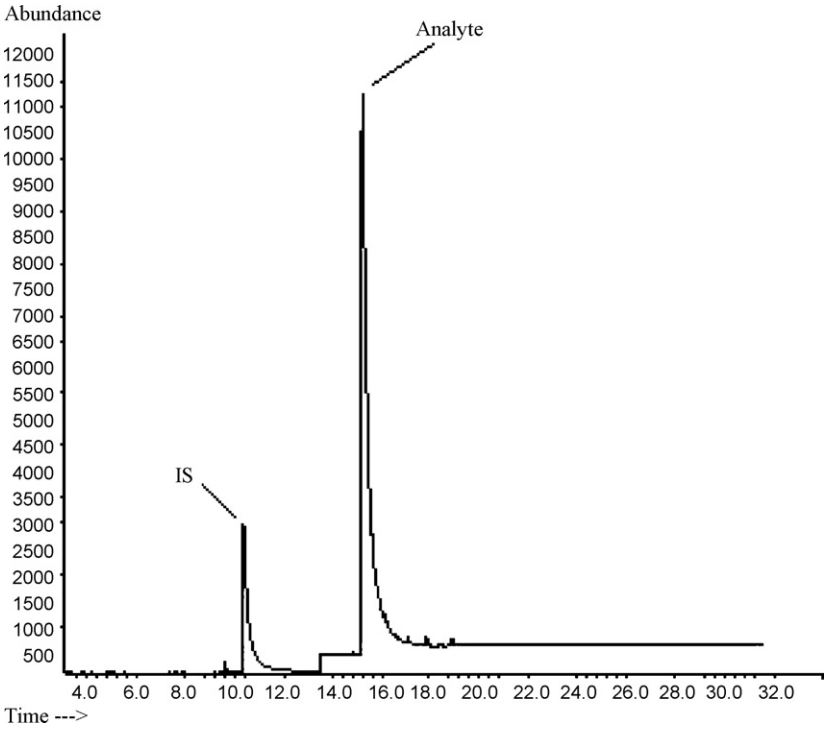


Fig. 8. Mass chromatograms obtained after SDME of formaldehyde at concentration level of 1 μg/ml.

and tetanus antigen samples, respectively. Moreover, the amount of formaldehyde in these samples were 23.5 and 44.0 and 365.0 ng/l, respectively.

4. Conclusion

In this work, a rather convenient and much easier approach was developed to determine formaldehyde in vaccine and antigen samples. A microdrop of chloroform was shown to be an efficient medium for SDME of derivatized formaldehyde from these samples which was subsequently injected directly into a GC–MS system. The method was based upon direct contact of the extracting microdrop with the vaccine and antigen samples. Influential parameters such as type of solvent, solvent drop size, extraction time, stirring rate, temperature, and ionic strength were optimized. The developed method is rather rapid, simple, linear, and reproducible. It is easy to use for the qualitative and quantitative analysis of free formaldehyde in DTP, DT vaccines and diphtheria and tetanus antigen while small volumes of sample and micro-scale size of organic extracting solvent are required.

The present work provides acceptable precision and sensitivity with a simple one step procedure. The method conveniently overcomes the difficulties encountered in other procedures and can be used in the analysis of free formaldehyde in vaccines.

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