

Determination of Diphenylamine Residue in Fruit Samples Using Dispersive Liquid-Liquid Microextraction Coupled with Ion Mobility Spectrometry

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ABSTRACT: In this study, a simple, fast, and inexpensive method is introduced to extract and Determine Remaining Diphenylamine (DPA) in fruit samples by the combination of Dispersive Liquid-Liquid MicroExtraction (DLLME) and Ion Mobility Spectrometry (IMS). The main parameters such as the type and volume of extraction solvent, volume of dispersive solvent, salt addition, centrifugation time, and sample pH that affected the extraction efficiency were evaluated by performing single-factor variable experiments. Chlorobenzene was selected as the extraction solvent, which was dispersed into samples with methanol as the dispersive solvent. In the optimized experimental conditions, the suggested technique showed good linearity in the range of 25–550 $\mu\text{g/L}$ with a correlation coefficient (R^2) 0.997. The detection limits were obtained based on S/N of 3 as 7.5 $\mu\text{g/L}$ in the standard solution and 17 $\mu\text{g/kg}$ in apple and pear samples. The repeatability and reproducibility of the method expressed as intraday ($n=5$) and interday ($n=3$) relative standard deviations were 6.0 and 7.5%, respectively at a concentration level of 150 $\mu\text{g/L}$ DPA, and the enrichment factor was 36.0. Analysis of fruit samples for measurement of DPA showed that the introduced method has great potential to extract and determine the DPA in real samples.

KEYWORDS: Dispersive liquid–liquid microextraction; Ion mobility spectrometry; Diphenylamine; Fruit samples.

INTRODUCTION

The high quality of the fruit is very important in their marketing and selling overseas. Scald is a physiological disorder caused by prolonged exposure of apples and pears to low temperatures in stores, the disorder symptom, which is the browning of the apple or pear skin, appears and develops during the storage stage. As a result, the economic value of the fruit in the fresh market is lost, leading to severe financial losses to the farmer [1]. Nowadays, the use of

antioxidants immediately after harvest is the most common way to control scalding. Diphenylamine (DPA) is one of the most commonly used agents in increasing apple post-harvest quality [2]. Antioxidant properties of DPA lead to its anti-scald activity, which protects fruit skin against alpha-farnesene oxidation products during storage [3]. Therefore, during the past few decades, there has been an increasing interest in its application all over the world. But recent

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studies have revealed that DPA can target the red blood cell system [4] and even the liver and kidneys after prolonged exposure to DPA [5]. These findings have raised worries about the use of DPA during the post-harvest period of fruits [6].

The Maximum Residual Levels (MRLs) for diphenylamine in apples and pears, according to EU regulations in foodstuffs, are 5 and 10 mg/kg, respectively [7]. Especially with new world concerns about food contamination and the higher interest of consumers to use organic fruits and vegetables, the determination of the DPA residues on fruit with an accurate, easy, low-cost, and fast method is essential. Several methods of DPA analysis in apples have been developed worldwide using solvent extraction such as HPLC with fluorescence detection [8]; fluorimetric method [9]; Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS) [10]; headspace solid-phase microextraction and gas chromatography/mass spectroscopy [11]; Thin-film electrochemical sensor for diphenylamine detection using molecularly imprinted polymers [2]; supercritical fluid extraction followed by HPLC [12]; Molecularly Imprinted Polymer with UV-Vis spectrometry [13]. After extraction, chromatographic methods to clear the interfering substances usually require clean-up steps. On the other hand, in comparison with chromatographic methods, fluorescence-based methods are very sensitive and simple. However, problems related to interference in complex food matrices may occur during the analysis [3]. Therefore, it seems necessary to establish a simple and reliable method for determining DPA in fruit samples.

Recently, a method based on the mobility of gas-phase ions in an electric field to identify and detect different compounds has been developed as an instrumental analytical method called Ion Mobility Spectroscopy (IMS). The ions are detected using a Faraday plate detector at the end of the drift tube. The separation of ionic species with different masses, charges, and cross-sections based on the velocity difference and reaching the detector at different times will be done [14]. The shape, mass, and charge distribution of the ions are factors that affect the ion travel time of the drift tube, which is called ion-drift time. The ion drift time is used to identify the ion and the height of the peak or peak area is proportional to the concentration of the analyte ion. The ionization source such as Ni^{63} or Corona Discharge (CD) is a critical element of IMS. This method is suitable for measuring compounds such as

amines that have high proton affinity and, because of high sensitivity, simplicity, speed, and low cost, it has been developed in recent years [15].

Recently, the combination of IMS and extraction techniques has been used to detect varied compounds in the foodomics field and the food composition analysis such as volatile compounds in pig fat [16], melamine in milk [17], histamine in tuna fish [18], determination of bisphenol A in canned food [19], organophosphorus pesticide residues in rice samples [20], and quality assessment of apple essence [21].

One of the most important steps of analytical procedures is sample preparation because it is so time-consuming and polluting step. Liquid-Liquid Extraction (LLE) and Solid-Phase Extraction (SPE), which are two of the most common methods of sample preparation, are time-consuming, expensive and require the use of large amounts of organic solvents [22, 23]. This is in contrast to green chemistry, which has received a great deal of attention these days. Therefore, there is a critical need to develop simpler and inexpensive methods for analyzing samples. At present, trends in analytical chemistry are towards simplifying and minimizing sample preparation, as well as minimizing organic solvents. Dispersive Liquid-Liquid Microextraction (DLLME) is a sample preparation method with a high enrichment factor. In this technique, the acceptor-to-donor phase ratio significantly is reduced [24]. DLLME prevents the volatilization of extractants compared to the conventional Liquid Phase Microextraction Methods (LPME) and, there is a possibility to simplify the extraction procedure, reduce the consumption of organic solvents and increase the sensitivity, and reach rapid equilibration [25-27]. In DLLME, an appropriate mixture of the extraction and dispersive solvents is rapidly injected into the aqueous sample to produce a cloudy solution and then the fine droplets settle at the bottom of the conical test tube by centrifuging. Finally, the determination of analytes in sediment can be performed by proper instrumental analysis [28-32].

Achieving high-speed data, relatively low technical cost, and high sensitivity are some of the advantages of combining ion mobility spectroscopy with microextraction techniques [33-37].

DLLME method has been expanded to detect various analytes in recent years, such as pollutants in water [38], lead in water [39], Copper and Nickel in Marine Brown Algae [40], diazepam [41], doxepin [42] and formaldehyde in seawater samples [43].

In this paper, the combination of DLLME and Ion Mobility Spectrometry (DLLME-IMS) was evaluated for the first time to determine diphenylamine in fruit samples. Some effective parameters on extraction efficiency were studied, such as the type and volume of extracting solvent type and volume of disperser solvent, pH, and salt addition. The proposed method was successfully applied to determine diphenylamine in real samples.

EXPERIMENTAL SECTION

Chemicals and Reagents

The diphenylamine (DPA) >99% was purchased from Merck company (Darmstadt, Germany). Chlorobenzene, dichloromethane, dichloroethane, chloroform, and methanol with the analytical grade, also were obtained from Merck. An ultra-pure water purification system is used for the purification of water (Nanopure, USA).

Stock standard solution of 1000 $\mu\text{g/mL}$ DPA was prepared by dissolving an appropriate amount of the compound in methanol and then stored at 4 $^{\circ}\text{C}$ in refrigerator. Later to prepare working standard solutions; daily, the stock standard solution was diluted using ultra-pure water to the required concentrations. NaOH and H_3PO_4 (0.1 mol/L) were used for pH adjustment.

Apparatus

A laboratory designed ion mobility spectrometer was used to detect and quantification DPA. It is working in positive mode with a ^{63}Ni ionization source. The samples were injected into the spectrometer through a hand-made injection port with a digital temperature-controlled heater. The optimized experimental conditions for obtaining the ion mobility spectra of the compound are listed in Table 1.

For the phase separation, a Rotofix 32A (Hettich, Germany) centrifuge was used. A Metrohm model 692 pH/ion meter (Herisau, Switzerland) with combined glass-calomel electrode was used for pH measurements.

Sample treatment

Apple and Pears samples were purchased from different fruit markets in Tehran-Iran. One kilogram of each fruit sample collected randomly was chopped and homogenized using a high-speed blender. Then, 25 g portions of these samples were taken and placed in a centrifuge tube; after 10-min centrifuging at 5000 rpm, the content was filtered through a nylon filter for removing

Table 1. The optimized experimental ion mobility spectrometry (IMS) conditions for DPA

Parameter	Value
Drift field voltage (V)	5500
Drift gas flow, air (mL/min)	500
Carrier gas flow, air (mL/min)	150
Drift tube temperature ($^{\circ}\text{C}$)	150
Injection temperature ($^{\circ}\text{C}$)	200
Drift tube length (cm)	12
Shutter grid pulse (μs)	200

the solid particles. Finally, the filtrate was diluted 5 times with purified water and used as an analytical sample.

Dispersive liquid-liquid micro-extraction procedure

An appropriate volume (1mL) of methanol as disperser solvent containing 100 μL of chlorobenzene as extraction solvent was injected rapidly into a 5 mL sample solution spiked with 500 $\mu\text{g/L}$ DPA that was placed in a 10 mL centrifuge glass tube with conical bottom. Thereby, a cloudy state was formed; consequently, DPA was extracted into the fine droplets of chlorobenzene dispersed into the aqueous phase. The mixture was centrifuged for 7 min at 5000 rpm and, the organic phase was sedimented at the bottom of the test tube (65 μL); then, 2 μL of the organic phase was injected into IMS for quantitation. In the optimization experiments, 5 mL of purified water spiked with 500 $\mu\text{g/L}$ DPA was used. The enrichment factor was calculated using the Eq. (1):

$$EF = \frac{C_{sed}}{C_0} \quad (1)$$

Where C_{sed} is the concentration of the analyte in the organic phase, C_0 is the initial concentration in the aqueous sample, and EF defined as the enrichment factor. C_{sed} was calculated from the calibration graph. The extraction recovery (ER) was defined as the percentage of the total analyte amount (n_0), which was extracted into the organic phase (n_{sed}), Eq. (2).

$$ER = EF \left(\frac{V_{sed}}{V_{aq}} \right) \times 100 \quad (2)$$

Where V_{sed} and V_{aq} are the volumes of organic and sample solutions, respectively [28, 44].

RESULTS AND DISCUSSION

Reduced mobility constants and mass assignments

In ion mobility spectrometry, the ion mobility constant,

Table 2: Reduced mobilities (K_0) and Proton affinity for DPA and MA

Compound	MW	K_0 (cm ² V s)	Proton affinity (kJ/mol)
DPA	169	1.61	910 [46]
MA	31	2.65	896 [47]

K (cm²/Vs), is used to identify the analyte from the ion peaks observed, like retention time in chromatography. For determination of mobility constants using the relationship (Eq. (3)):

$$k = \frac{d}{tE} \quad (3)$$

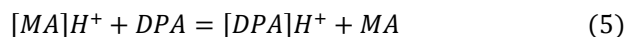
In this equation d is the distance an ion will drift in the measured time t and E is the electric field.

The ion mobility through the drift region is affected by the electric field force, the geometry and the electrostatic resistance of the drift gas molecules. Ion mobility (K) varies as a function of drift gas temperature and pressure so for the temperature and pressure standardization reduced ion mobility (K_0) is defined (Eq. (4)):

$$K_0 = K \left(\frac{273}{T} \right) \left(\frac{P}{101.325} \right) \quad (4)$$

Where T is the temperature (in K) and P is the pressure (in kPa). In practice, for determination of K_0 a reference ion (the ‘‘calibrant’’) is used.

In the positive mode the drift gas contains trace amounts of methylamine (MA) used as reactant. According to the following proton transfer reaction, the analyte molecule gets ionized:



Ion lifetimes and the proton affinity of the sample molecule must be considered as variables for optimum response. If the proton affinity of the sample molecule is greater than that of MA the reaction proceeds. Where response is not observed for an analyte can be attributed to low proton affinity or ion instability [45, 46]. There is a comparison between DP and MA in their proton affinity and mobility constant in Table 2.

Effect of type and volume of extraction solvent

One of the most critical steps in dispersive liquid–liquid microextraction is the selection of the appropriate extraction. It must have a high extraction capability of the target analytes, low solubility in water, and also, it should be easily dispersed in water. Based on these facts,

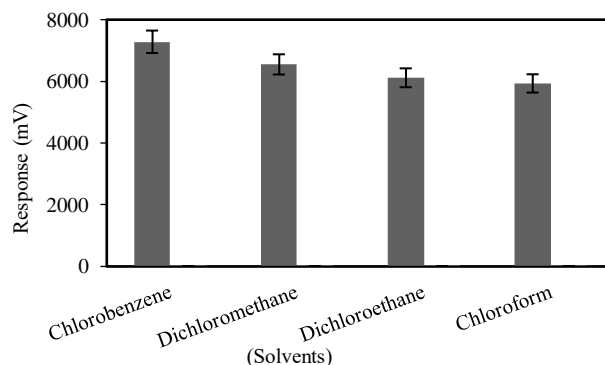


Fig. 1: Effect of extraction solvent type on the efficiency of extraction of DPA. (Conditions: 5 mL aqueous solution spiked with 500 µg/L of DPA; extraction and disperser solvents volumes, 100 µL and 1.0 mL; centrifugation time and rate, 5 min and 5000 rpm)

some organic solvents were tested as extraction solvent, including chlorobenzene, dichloromethane, dichloroethane and chloroform. Among them, chlorobenzene, demonstrated the maximum enrichment factor, which is likely due to the presence of phenyl rings in both chlorobenzene and DPA. Hence, it was selected as the extraction solvent (Fig. 1). Organic phase could easily be removed by a microsyringe and introduced into the injection port of IMS.

Various volumes of chlorobenzene (40–140 µL) as extraction solvent and a constant volume of disperser solvent (1 mL of methanol) were subjected to the mentioned micro-extraction method to evaluate the effect of the volume of the extraction solvent on the extraction efficiency. As the volume of chlorobenzene increased (up to 100 µL) more DPA was extracted, after which due to the increase in the volume of the organic phase, the concentration of the extracted DPA decreased (dilution effect) (Fig. 2). Based on these observations, 100 µL of chlorobenzene was used as the optimum volume of the extraction solvent.

Influence of type and volume of disperser solvent

The main factor in selection of disperser solvent is its miscibility in both organic (extraction solvent) and aqueous phases. Therefore; it can disperse extraction solvent into very fine droplets in aqueous phase. In the IMS instrument, due to the transfer of protons between the analyte and the reactant ions, a protonated ion species will be formed. Thus, when we have a mixture of chemical compounds with different proton affinities, competition in the ionization process is expected. Some solvents, such as acetone and acetonitrile being commonly used

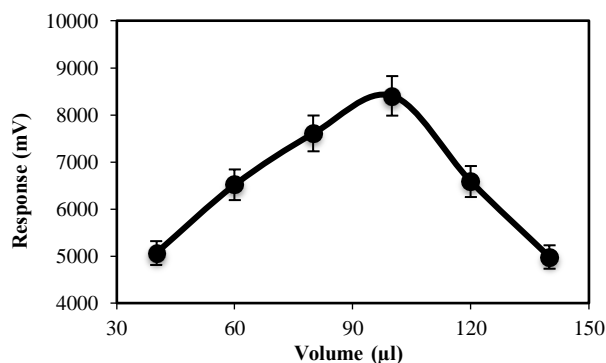


Fig. 2: The effect of extraction solvent volume on the extraction efficiency of DPA. (Conditions: 5 mL aqueous solution spiked with 500 µg/L of DPA; extraction and disperser solvents volumes, 100 µL and 1.0 mL; centrifugation time and rate, 5 min and 5000 rpm)

disperser solvents affect and reduce the intensity of the analyte signal (due to ionization and signal generation). Therefore, methanol was selected as the disperser solvent in all of the subsequent experiments [48].

After the selection of methanol as the appropriate disperser solvent, its volume should be optimized. To obtain the optimized volume of methanol, three volumes of methanol (0.5, 1.0 and 1.5 mL) containing 100 µL of chlorobenzene were used in DLLME experiments. The results indicated that by increasing the volume of the disperser solvent from 0.5 to 1.0 mL, an increase was observed in the extraction efficiency. On the other hand, further increase in disperser solvent volume resulted in a decrease in the extraction efficiency. This could be attributed to the more solubility of DPA in the aqueous phase which led to the diminished partition of this compound into chlorobenzene droplets. As a result, 1.0 mL was chosen as the optimum volume of methanol.

Effect of centrifugation time

Mass transfer is a time-dependent process. In DLLME technique, the extraction time is defined as the time interval between the addition of the mixture of dispersive solvent (methanol) and extraction solvent (chlorobenzene) to the sample before centrifugation. The effect of extraction time was evaluated in the time range between 1 to 5 min but no differences in sensitivity were obtained. It shows one of the most important advantages of DLLME, it is practically time-independent, due to the large surface area between the extraction solvent and aqueous sample phase. So, the mixture was centrifuged immediately after forming the cloudy solution. This step is the most time-consuming steps.

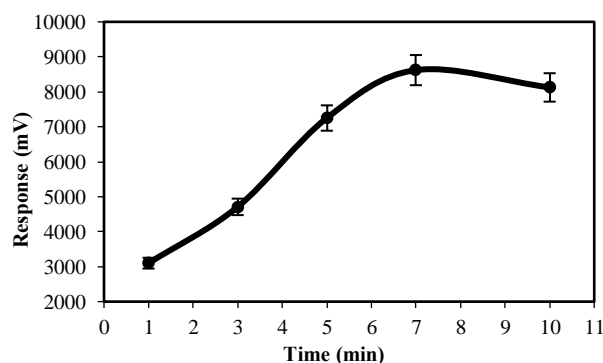


Fig. 3: The effect of centrifuging time on the extraction recovery of DPA. (Conditions: 5 mL aqueous solution spiked with 500 µg/L of DPA; extraction and disperser solvents volumes, 100 µL and 1.0 mL; centrifugation rate 5000 rpm)

The centrifugation time was studied in the range of 1 to 10 min; the best result was attained at 7 min. Figure 3 illustrates the effect of centrifugation time on the extraction efficiency.

Effect of solution pH

One of the important factors influencing the Extraction Recovery (ER) is pH of the samples. It plays a significant role in the extraction because the pH can determine the state of the analyte in the sample solution. Hence, in order to investigate the extractability of the analyte the pH of the sample solution is a crucial parameter to be considered and optimized. The effect of this parameter was studied in the pH range of 2–12 by using NaOH and H₃PO₄ (0.1 mol/L) (Fig. 4). The best result was achieved at pH value of 6.0. Therefore, it was selected for the rest of the studies.

Effect of salt concentration

The effect of increasing ionic strength was evaluated by adding NaCl (0–10%, w/v) in the sample spiked with DPA at a level of 250 µg/L. Due to the salt effect, it is expected that with increasing the percentage of salt, because of the decrease in the solubility of the extraction solvent the volume of the organic phase is increases. Results showed that salt addition had no significant effect on the extraction recovery, but due to the increase in the volume of organic phase, the enrichment factor decreased. Therefore, the extractions were performed without salt addition.

Quantitation and method validation

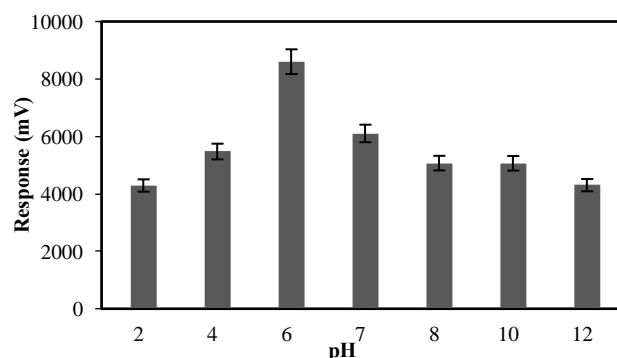
The DLLME–IMS spectra of DPA aqueous solutions at low concentration levels (50 and 250 µg/L) is illustrated in Fig. 5.

Table 3. Analytical results for determination of DPA by DLLME-IMS.

Analyte	DLR ($\mu\text{g/L}$)	R^2	LOD		RSD (%)		EF	ER (%)
			in standard ($\mu\text{g/L}$)	in sample ($\mu\text{g/kg}$)	Intraday (n=5)	Interday (n=3)		
Diphenylamine	25-550	0.997 $y = 11.574x + 2315.7$	7.5	17	6.0	7.8	36.0	46.85

Table 4. Determination of DPA in fruit samples using DLLME-IMS

Sample preparation method	Added ($\mu\text{g/kg}$)	Found ($\mu\text{g/kg}$)	Recovery %	RSD % (n=3)
Apple	0	ND ^a	-	-
	50	48.80	97.61	5.0
	70	66.26	94.66	4.9
	90	87.95	97.72	3.1
	110	106.50	96.81	2.1
Pear	0	ND	-	-
	50	46.12	92.24	7.3
	70	67.19	95.99	4.2
	90	86.92	96.58	2.3
	110	106.10	96.46	2.1

^a Not detectedFig. 4: The effect of pH on the extraction recovery of DPA. (Conditions: 5 mL aqueous solution spiked with 500 $\mu\text{g/L}$ of DPA; extraction and disperser solvents volumes, 100 μL and 1.0 mL; centrifugation time and rate, 5 min and 5000 rpm)

Analysis of real samples

In order to assess the applicability of the present method it was applied for the extraction and determination of DPA in the fruit samples including apple and pear under optimal conditions. The contents of DPA were determined by IMS, the samples were free of the analyte. An added-recovery method was used to explore the method's accuracy and matrix effect in different samples.

Under the optimal conditions, the figures of merit of the method such as linear range, relative standard deviation, enrichment factor, and limit of detection were evaluated. The obtained results are summarized in Table 3.

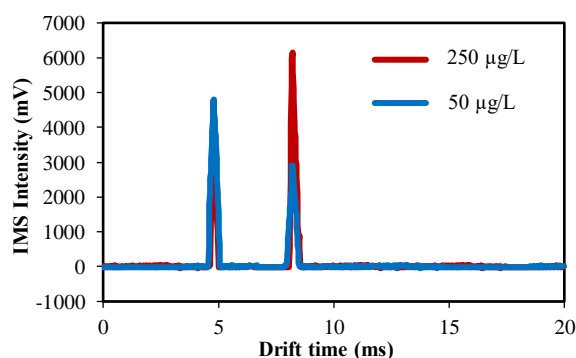


Fig. 5: The ion mobility spectra of DPA.

In the range of 25-550 $\mu\text{g/L}$ the calibration graph showed good linearity. The LODs calculated based on S/N of 3 were obtained as 7.5 $\mu\text{g/L}$ in solution and 17 $\mu\text{g/kg}$ in solid samples, respectively. The repeatability and reproducibility (RSD) were calculated by analyzing samples spiked with 150 $\mu\text{g/L}$, which were 6.1 and 7.5%, respectively. The LOQ (not included in Table 3) was obtained as 25 $\mu\text{g/L}$.

The samples were spiked at four concentration levels (50, 70, 90 and 110 $\mu\text{g/kg}$) of DPA, and the proposed procedure was carried out in order to calculate relative recoveries in the samples. The results given in Table 4 depict the effectiveness of the method (mean relative recoveries in the range of 92.2–97.6%), i.e., the matrices of the samples had no significant effect on the proposed method. Therefore, the proposed method could be used

Table 5. Comparison of DLLME-IMS with other analytical techniques for determination of DPA.

Analytical technique	Sample	DLR	LOD	RSD %	Ref
Single multicommutated fluorometric optosensor	Apple & Pear	250-5000 $\mu\text{g}/\text{kg}$	60 $\mu\text{g}/\text{kg}$	<3	[9]
LC/ESI-MS/MS	Vegetable juice	-	<5 $\mu\text{g}/\text{Kg}$	<14	[49]
LC/ESI-TOF-MS	Baby food matrix with 100% fruit content	-	1000 $\mu\text{g}/\text{Kg}$	9.5	[50]
Spectrofluorimetry	Apple, Pear & Orange	10-100 $\mu\text{g}/\text{Kg}$	4-7 $\mu\text{g}/\text{Kg}$	2-6	[3]
GC/MS	Apple, Pear & Peach	20-200 $\mu\text{g}/\text{Kg}$	-	6.4-13.7	[51]
SFE-SSME-HPLC		500-7000 $\mu\text{g}/\text{Kg}$	300 $\mu\text{g}/\text{Kg}$	<10.3	[12]
DLLME-IMS	Apple and Pear	25-550 $\mu\text{g}/\text{Kg}$	17 $\mu\text{g}/\text{Kg}$	<7.5	This work

Comparison of the present DLLME-IMS results for determination of DPA with those reported earlier reveals that the proposed method has a lower detection limit than the most other methods without any further pretreatment, the results demonstrate that that IMS and DLLME provides a simple and sensitive method for determination of DPA in water and fruits samples without any further pretreatment.

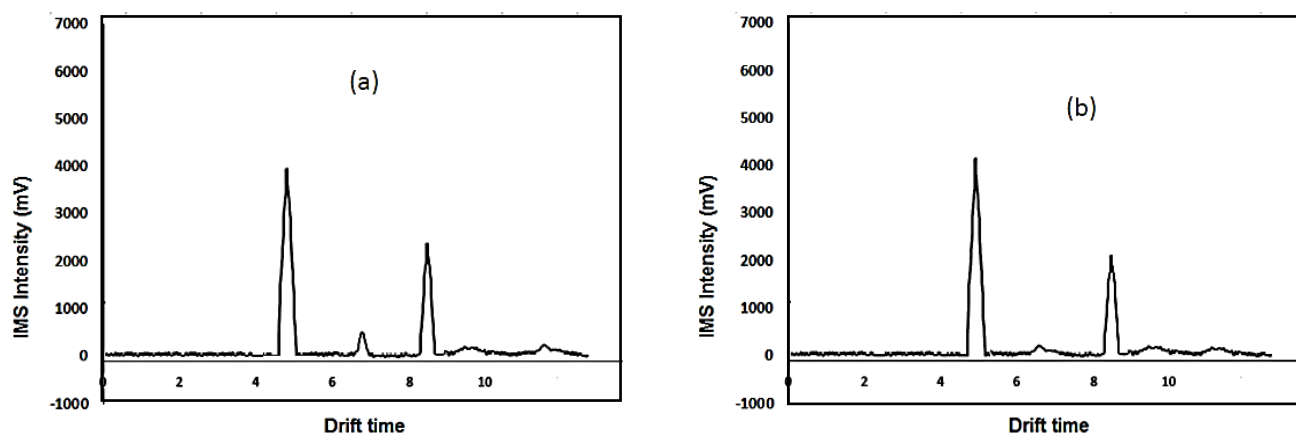


Fig. 6: The ion mobility spectra of DPA in optimization conditions for (a) apple and (b) pear samples.

as an applicable and adequate method for the analysis of DPA in the studied fruit samples. The DLLME-IMS spectra of the real sample are illustrated in Fig. 6.

Comparison with other methods

The results obtained from this study were compared with other reported methods in the literature regarding the analysis of DPA in several samples (Table 5).

CONCLUSIONS

In this study, a simple, rapid, and accurate method for the determination of DPA residues in apples and pears has been developed. Other advantages of the proposed DLLME-IMS method are a lower detection limit and the relatively wide linear range for the determination of the studied analyte. Finally, the present work was successfully used to study real fruit samples.

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