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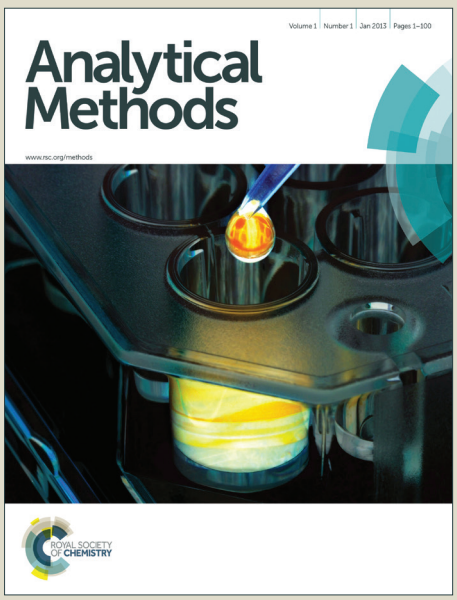

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PAPER

Analysis of pesticide residues in tea using accelerated solvent extraction with in-cell cleanup and gas chromatography tandem mass spectrometry

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A fast, simple and easily automated method was developed for the simultaneous determination of pesticide residues in tea using accelerated solvent extraction (ASE) with in-cell cleanup and gas chromatography-tandem mass spectrometry (GC-MS/MS). The method integrates extraction and cleanup processes into a single step, by adding a clean-up sorbents along with the sample into the extraction cell. The efficiency of this method was characterized in terms of its recovery (with values ranging from 90 to 98%), repeatability along with intermediate precision (showing relative standard deviations less than 15%), and sensitivity (providing detection limits between 0.001 and 0.007 µg g^{-1}). The concentration range of the pesticide residues found in the sample is from 0.008 to 0.161 µg g^{-1}. The relative expanded uncertainty achieved for this method ranged from 24% to 34%. The results indicate that the proposed method is easy and reliable for the determination of pesticide residues in tea, and it is suitable for use in routine analysis.

1 Introduction

Tea, one of the oldest and popular beverages in the world for its specific aroma and flavour as well as its health promoting properties, is obtained from the tender leaves of the plant *Camellia sinensis*.[1] The use of pesticides is rising for modern agriculture to protect and produce the high quantity and quality of tea in order to meet the demand of society. Insecticides from the pesticide groups of organochlorine pesticides (OCPs), organophosphorous pesticides (OPPs) and pyrethroids are widely used during the cultivation of tea to prevent and control mites, leafhoppers, plant bugs and aphids.[2] The current trend in pesticide residue analysis is the development of a multi-residual method that not only provide the simultaneous determination of multiple pesticides but is also applicable to a large number of samples of different origins. Traditional sample preparation methods such as liquid-liquid extraction, Soxhlet extraction, and the Luke method are laborious, time consuming, expensive, require large amounts of organic solvents and usually involve many steps leading to loss of some quantity of the analyte. As a result, modern sample preparation procedures such as accelerated solvent extraction (ASE),[3] supercritical fluid extraction (SFE),[4] microwave assisted extraction (MAE),[5] solid phase extraction (SPE),[3] solid phase microextraction (SPME),[6] matrix solid phase dispersion (MSPD)[7] extraction and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) have been developed to overcome the drawbacks in the traditional approaches.[8]

An efficient and rugged extraction method is important for the determination of trace levels of pesticides in tea. Accelerated solvent extraction (ASE), also known as pressurized liquid extraction (PLE) is an instrumental extraction technique that uses small amount of solvents to perform extraction at elevated temperature and pressure.[9] Applications of ASE resulting in better extraction efficiencies and short analysis times for the simultaneous extraction of multiple pesticides in tea have been reported in the literature.[10-11] Recent advances in these automated system with an in-cell cleanup have demonstrated the selective removal of interferences for matrices such as fish and fish oil, soil and mushroom.[12-14] This technique, which does not involves a manual transfer of sample has resulted in high sample extraction productivity and reduced the laboratory error.[15] The addition of dispersive SPE adsorbents at the outlet end and the sample on top of adsorbent provides a simultaneous extraction and clean-up process in the cell. This way, the unwanted compounds are retained in the cell by the adsorbents, while the analytes are eluted with the solvents during the extraction. This

streamlined sample preparation eliminate the manual transfer of sample for cleanup procedure using gel permeation chromatography (GPC) and/or solid phase extraction (SPE) or any other clean up procedures.

The presence of pigments, lipids and alkaloids in tea which are co-extracted with the pesticides may interfere with the analysis.[16] The combination of dispersive SPE clean up method utilising primary secondary amines (PSA) and octadecyl (C_{18}) adsorbents could solve the purification problems and provide high recovery of the analyte.[17-20]

Gas chromatography-mass spectrometry (GC-MS) has the advantages of a high separation power and identification capability and it has been widely applied in the analysis of pesticides in various food samples. Another advantage of MS/MS is that it can be operated in the selected reaction monitoring (SRM), which is beneficial for the accurate quantification of the analyte. It eliminates the confusion with similar compounds and thus obtain reliable identification and confirmation of the pesticide residues in samples.[17]

To the best of our knowledge, no procedures have been reported on the use of ASE with in-cell cleanup for the purpose of simultaneous extraction of multiple classes of pesticide residues in tea. In this study, five analytes which include organochlorine, pyrethroid or organophosphate pesticides, in 10 commercial tea samples were extracted using ASE with in-cell cleanup and analysed by GC-MS/MS.

2 Experimental

2.1 Reagents and chemicals

HPLC-grade acetonitrile, acetone and hexane were obtained from MERCK (Darmstadt, Germany). All of the pesticide standards used were more than 95% pure. The purity was taken into account in the calculation of the actual concentration of each standard solution. The pure pesticide endosulfan (containing alpha-endosulfan and beta-endosulfan) was obtained from Sigma Aldrich (Steinheim, Germany) whereas bifenthrin, chlorpyrifos, dieldrin, lindane and triphenyl phosphate were purchased from Dr. Ehrenstorfer (Augsburg, Germany). The primary secondary amine (PSA) and octadecyl (C_{18}) was obtained from Varian (Harbor City, USA). Cellulose filters (20 mm diameter) were purchased from Restek (Bellefonte, PA, USA) and hydromatrix was obtained from Agilent Technologies (Santa Clara, CA, USA).

2.2 Preparation of the standard solutions

Since weights can be measured with greater accuracy, the preparation of standard solution was carried out gravimetrically whereby the determination of weights is used as a means of quantifying an analyte concentration in mass/mass ratio. This way, an accurate concentration was obtained besides, error and preparation time of standard solution can be minimised.[21] Weighing was made using a four decimal analytical balance. The individual pesticide stock standard solutions (endosulfan, bifenthrin, chlorpyrifos, dieldrin and lindane) were prepared in acetonitrile by dissolving approximately 10 mg of the pure reference material in appropriate mass of acetonitrile (ρ=0.786 g mL^{-1}) to give a final mass fraction of 1000 µg g^{-1}. A stock solution of triphenylphosphate in acetonitrile at a concentration 130 µg g^{-1} was used as internal standard. The intermediate pesticide standard mixture was prepared by pooling aliquots of the individual pesticide stock standard solutions and then diluting the pooled standards with acetonitrile to produce a concentration of 100 µg g^{-1} of each sample.

2.3 Matrix-matched calibration standards

For the calibration of the GC-MS/MS, matrix-matched calibration standards were freshly prepared by combining the blank extract with the desired amount of the intermediate standard solution and triphenylphosphate (TPP) to produce five different concentration levels (0.04, 0.80, 1.2, 2.0 and 3.5 µg g^{-1}). Each concentration were prepared in duplicate and analysed ten times.

2.4 Extraction by ASE with in-cell cleanup

The accelerated solvent extraction was performed using an ASE 300 accelerated solvent extractor (Dionex, Sunnyvale, CA, USA) equipped with 33 mL stainless steel cells. The cell loading was performed in the following sequence. First, the cellulose filter was placed at the bottom of the cell. Then, the pre-weighed adsorbents (0.3 g of PSA and 0.15 g of C_{18}) was added and topped by the cellulose filter. The sample was spiked with 50 µL of TPP at a concentration of 130 µg g^{-1}, placed in the cell and then topped with a cellulose filter. Finally, the cell was filled to the top with hydromatrix to fill the vacant volume. The cell was tightly closed and inserted into the cell tray for the extraction.

The extraction was performed using the following ASE parameters as described previously[11]; extraction temperature, 120 °C; extraction pressure, 1500 psi; heating time, 5 min; static time, 10 min; purge time, 60 s; extraction solvent, acetone-hexane (2:1, v/v); flush volume, 60% and static cycles, 2. The extracts were collected in the collection vessel, concentrated to 1 mL with a gentle stream of nitrogen at 40 °C, and transferred into a vial for the GC-MS/MS analysis.

2.5 Recovery assay and method validation

The accuracy and precision of the method was assessed from the recoveries of three different spiked concentrations (0.04, 2.0 and 3.5 µg g^{-1}) which covered the low, medium and high regions of each compound. Solutions at each spiked level were prepared in triplicate and were injected 10 times. Spiked samples were left to stand for at least 1 hour to allow pesticide absorption onto the sample. They were then extracted according to the extraction procedures described above.

The limit of detection (LOD) and quantification (LOQ) was determined from the analytical curve where the analytical curves for each analyte at a level approximating the LOD and LOQ were constructed using spiked sample at four concentration levels (0.005, 0.01, 0.08 and 0.15 μg g^{-1}). The LOQ obtained subsequently validated by the independent analysis of spiked samples prepared at the quantification limit.

2.6 Analysis of real samples

Ten processed black tea samples from various tea brands were randomly selected from the local supermarket for the study. A 200 g of each tea sample was blended using a food processor to produce a fine powdery material, sieved and then stored in the container at 4 °C. The samples were mixed by a shaker before analysis to ensure that the samples were fully mixed and homogenized. Approximately 1 g of each sample was taken for analysis and prepared in duplicate. Samples were mixed with TPP and were subjected to the extraction process, which is described in the "extraction by ASE with in-cell cleanup" section. Each replicate samples was then measured 10 times with GC-MS/MS.

2.7 GC-MS/MS analysis

The GC-MS/MS system consisted of a ThermoFinnigan Gas Chromatography, an AS 200 autosampler and a Polaris Q ion trap mass spectrometer (San Jose, CA). The data acquisition and processing were performed using X-calibur software. The pesticides were separated on a DB-5MS (30 m x 0.25 mm i.d., 0.25 μm film) capillary column from Agilent. The splitless mode was used for the injection. The oven temperature was held at 80 °C for 1 min, heated to 280 °C at a heating rate of 20 °C min^{-1} and then kept at 280 °C for 8 min. Helium was used as the carrier gas at a constant flow rate of 1.5 mL min^{-1}. The injection port temperature and transfer line temperature were maintained at 260 °C and 280 °C, respectively. The ion source temperature was set at 250 °C and the injection volume was 1 μL. The solvent delay was set for 4 minutes. The total run time for GC-MS/MS was 17 minutes. The mass spectrometer was operated in the electron ionization (EI) mode at 70 eV. The MS/MS detection method was first performed with individual injections of the pesticides and TPP in full scan mode at 1.2 μg g^{-1} to obtain their retention times and select their parent ions.

2.8 Estimation of measurement uncertainty

The uncertainty on pesticides measurement using this ASE with in-cell cleanup method was evaluated based on top down approach according to Eurachem/CITAC Guidelines.[22] The uncertainties of the gravimetric measurements, as well as the standard purity, were estimated and integrated in the calculation of the total combined uncertainty. The contributions of uncertainty were obtained from the statistical analysis of repeated measurements and some sources were obtained from calibration certificates. Uncertainty was further divided into recovery, precision and analytical curve. After the estimation of all sources of uncertainty, they were combined according to the law of propagation of uncertainties, obtaining the combined standard uncertainty, u_c. The expanded uncertainty, U is obtained by multiplying the u_c by a coverage factor k, assuming a normal distribution of the measurand.

3 Results and discussion

3.1 Gas chromatographic determination

The analysis was performed in the selected reaction monitoring (SRM) mode based on the use of one target and two qualifier ions. Pesticides were identified according to retention times as well as their target and qualifier ions. The quantitation was based on the peak area ratio of the target ion divided by the internal standard. Table 1 summarizes the observed ions used in SRM mode.

Table 1 Quantitation parameters for pesticides in tea analysed by GC-MS/MS

Compound	Parent ion (m/z)	Product ion (m/z)	Retention time (min)
Lindane	181	183, 182	11.45
Chlorpyrifos	258	194, 240	12.89
Dieldrin	263	193, 228	14.39
Endosulfan	241	170, 206	14.02, 14.80
Triphenylphosphate (TPP)	325	227, 231	15.51
Bifenthrin	181	153, 166	15.89

The selectivity of the extraction method in this study was determined by comparing the chromatograms of blank matrix with those of spiked extracts. Figures 1 and 2 show the full scan chromatogram obtained from a spiked sample (1.2 μg g^{-1}) and blank sample, respectively. There are no interfering compounds except caffeine, which was detected in the chromatogram. The results suggested that, the combination of sorbent PSA and C_{18} was able to remove those interferences. This is in agreement with previous study and that makes sorbent primary secondary amines (PSA) and C_{18} widely used to clean the tea extracts. It was reported that PSA helps to remove acidic components, certain pigments and some sugar whereas the C_{18} was shown to be effective to retain the chlorophyll and do not cause pesticides losses.[23-24] However, there is no application have been reported using the PSA and C18 for ASE with in-cell cleanup.

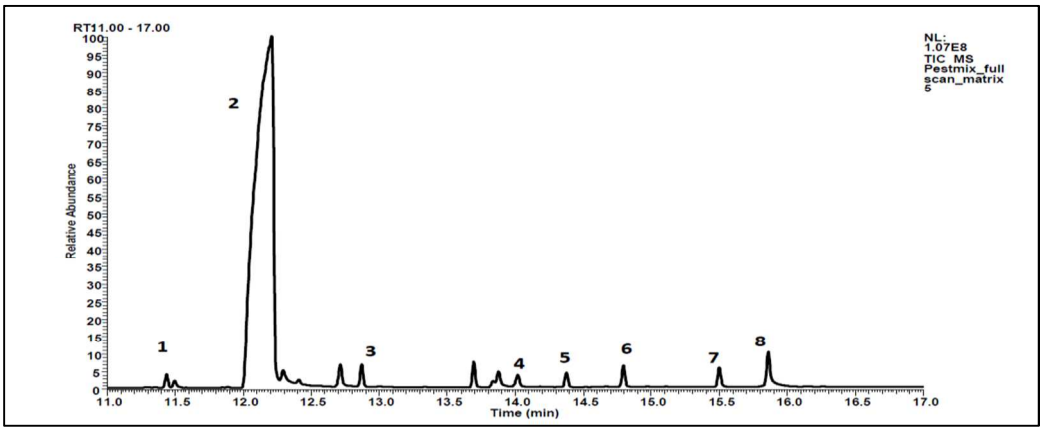

Figure 1 Full scan total ion chromatogram (TIC) obtained from a spiked sample at concentration of 1.2 µg g^{-1} (expanded time 11-17 min) 1. Lindane, 11.45 min; 2. Caffeine, 12.03 min; 3. Chlorpyrifos, 12.87 min; 4. Alpha-Endosulfan, 14.02 min; 5. Dieldrin, 14.38 min; 6. Beta-Endosulfan, 14.80 min; 7. Triphenylphosphate, 15.51 min; 8. Bifenthrin, 15.87 min.

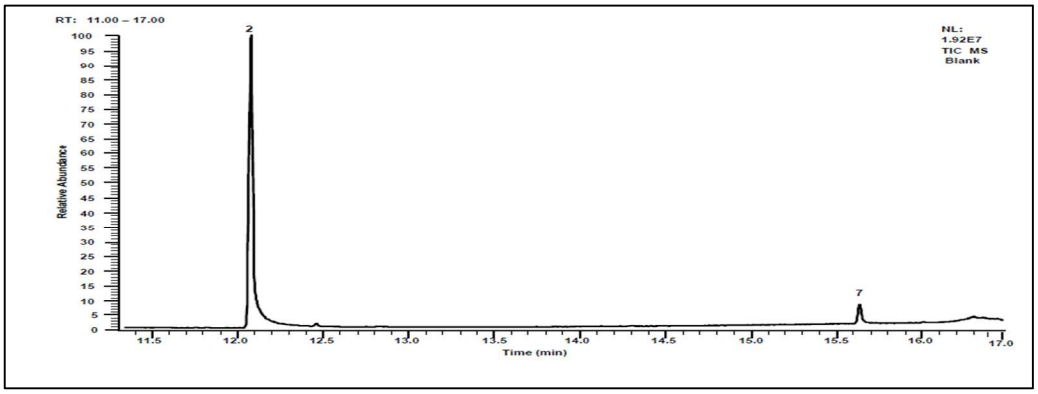

Figure 2 Full scan total ion chromatogram (TIC) of blank sample (expanded time 11-17min). 2. Caffein, 7. Triphenylphosphate

3.2 Validation of the method

Quantitative analysis was carried out using an internal calibration method. The analytical curves for each compound using five different concentration levels of matrix-matched calibration standards were generated by plotting the peak area ratio (peak area of the analyte over peak area of internal standard) versus the concentration ratio (concentration of the analyte over concentration of internal standard). The use of matrix-matched standards is important to eliminate matrix effects in quantitation of pesticide residues.[25] The mass spectrometer detector response was found to have good linearity for all of the pesticides with determination coefficients (r^2) were greater than 0.995. The calibration range was linear from 0.04 µg g^{-1} to 3.5 µg g^{-1}.

The limits of detection (LOD) and quantification (LOQ) were determined based on the analytical curves. LOD was calculated as LOD=3.3σ/S and LOQ=10σ/S where σ is the standard deviation of the response and S is the slope of the analytical curve. The σ was measured as the standard error of the analytical curve or the standard deviation of the y-intercept.

Table 2 shows the LOD and LOQ obtained for all of the investigated pesticides. The LOD and LOQ of each pesticides are well below the maximum residual limits (MRLs) allowed by the European Community. Table 3 shows the accuracy and precision of the LOQ.

Table 2 LODs, LOQs and MRLs of the pesticides

Compounds	Pesticide level, µg g^{-1}		
	LOD	LOQ	MRLs[26]
Endosulfan[a]	0.007	0.021	30.0
Bifenthrin	0.005	0.015	5.00
Chlorpyrifos	0.006	0.018	0.10
Dieldrin	0.001	0.003	0.02
Lindane	0.003	0.009	0.05

Table 3 Accuracy and precision of the LOQ.

Compounds	Concentration, µg g^{-1} (n=6)		
	Conc.	Recovery (%)	RSD
Endosulfan[a]	0.021	94	10.3
Bifenthrin	0.015	92	13.2
Chlorpyrifos	0.018	90	9.9
Dieldrin	0.003	89	14.5
Lindane	0.009	91	11.7

a= sum of alpha-endosulfan and beta-endosulfan

The method detection limit values were found to be between 0.001 and 0.007 and the quantification limit were found to be between 0.003 and 0.021. The recovery and precision of the spiked sample at the concentration of LOD was satisfactory with recovery ranged from 89 to 94%, with relative standard deviation of less than 15%. The LOQ and LOD values obtained in this study are lower and comparable from previous study.[11, 16-17] The differences of LOD and LOQ values from component to component originate from the noise, the response factor of instruments and matrix interference. This extraction method has good purification effect and therefore, resulting in a better detection and quantification by the GC-MS/MS.

The accuracy and precision of the method were determined by evaluating the recovery and the repeatability of the spiked samples. The analysis was carried out in two separate performance tests, where the sample was measured on the same day (intra-day) and on four different days (inter-day). The precision represents an estimate of the variability of measurements and the reproducibility of the test method, and the recovery tests for each pesticide at different fortified levels were carried out to assess the accuracy of the presented method. The mean percentage recoveries and relative standard deviations of each pesticide for triplicate spiked samples at three different concentration levels are reported in Table 4. The intra-day recoveries of the analytes varied in the range of 91 to 97%, with RSD values within the range of 6.3 to 9.9%. The inter-day recoveries varied from 90 to 98%, with RSD values ranging from 7.1 to 12.1%. All of the investigated pesticides met validation requirements to achieve 70 to 120 % recoveries.[27] Precisions of less than 15% was achieved for both intra- and inter-day analyses even at low concentrations (0.04 µg g^{-1}).

Table 4 Results of the validation study intra- and inter-day recoveries (Rec., %) and precision (RSD, %)

Compound	Intra-day recovery and precision (n=10)						Inter-day recovery and precision (n=10)					
	0.04 µg g^{-1}		2.0 µg g^{-1}		3.5 µg g^{-1}		0.04 µg g^{-1}		2.0 µg g^{-1}		3.5 µg g^{-1}	
	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD	Rec.	RSD
Endosulfan	91	9.8	97	7.6	95	6.6	93	11.1	98	9.8	95	8.3
Bifenthrin	94	9.9	96	8.6	97	7.3	95	10.2	94	8.9	98	7.9
Chlorpyrifos	92	9.7	94	8.7	95	9.4	90	12.1	93	8.9	96	7.1
Dieldrin	92	9.9	95	8.7	96	8.3	90	10.4	95	9.2	97	8.6
Lindane	95	9.4	96	8.7	95	6.3	95	9.8	97	7.9	94	7.2

3.3 Uncertainty of results

The measurement uncertainty gives information about the range in which the measurement results can be expected. It takes into account the random and systematic errors contributed to the measurement process. In other meaning, it addresses the probabilistic estimation of the maximum error of the measurement. Uncertainty is necessary to establish the comparability of results from different measurements.[28] An adequate identification and estimation of each uncertainty sources allows the accuracy of the results to be established and balanced with the time consumption and costs.[29-30]

Uncertainty associated with precision (u_p), recovery (u_R), calibration standard solution (u_{std}) and analytical curve (u_{cal}) has been identified as the major contributor to the estimation of uncertainty for the pesticides in tea measurement of this method. The uncertainty in recovery provides information associated to the uncertainty in extraction method.

The uncertainty associated with chromatographic method arises from the measurement of precision and analytical curve. The standard uncertainty (u) of precision (u_p) was quantified by evaluating the pooled standard deviation of the spiked samples at three different concentrations (0.04, 2.0 and 3.5 µg g^{-1}). The u associated with recovery (u_R) was quantified from the recovery of spiked concentration at the LOQ value of each pesticides because this value is close with the concentration of most test samples. The u of standard solution (u_{std}) consists of the u of the purity of the pure substance and the u of the balance used in the preparation of standard solution. Table 5 shows the relative standard uncertainty and combined standard uncertainty for each pesticide.

Table 5 Relatives standard uncertainty and combined standard uncertainty (u_c) of the investigated pesticides for linear range of 0.04 to 3.5 ug g^{-1}

Pesticide	u_c	u_p/p	u_R/R	u_{std}/std	u_{cal}/cal
Endosulfan	0.0038	0.0560	0.0956	0.0002	0.0010
Bifenthrin	0.0043	0.0522	0.0931	0.0003	0.0032
Chlorpyrifos	0.0046	0.0554	0.0996	0.0001	0.0019
Dieldrin	0.0043	0.0544	0.0930	0.0004	0.0009
Lindane	0.0041	0.0483	0.0911	0.0002	0.0011

3.4 Application to real sample

Concentrations of the pesticide residues in the sample were obtained from the mean value of 20 measurements. Pesticide residues were found in most of the samples but at levels lower than the maximum residual limits. The concentration and the uncertainty values for each pesticide found in the samples are shown in Table 6. When considering the uncertainty, the result indicated that the concentration of dieldrin in samples 6 and 8 exceeded its maximum residual limits (MRLs). The relative expanded uncertainty values were achieved from 24 to 34% and were acceptable considering the complexity of the matrix, the analyte level and the complexity of the analytical procedure. Figure 3 shows the chromatogram of pesticide occurrence in tea sample.

Table 6 The pesticide level (µg g^{-1}) found in the samples and its uncertainty

Sample	Endosulfan	Bifenthrin	Chlorpyrifos	Dieldrin	Lindane
			Concentration µg g^{-1}		
S1	ND	0.038±0.010	0.026±0.005	ND	0.019±0.005
S2	<LOQ	0.152±0.038	0.020±0.005	0.011±0.003	0.013±0.003
S3	ND	0.161±0.041	<LOQ	0.009±0.001	0.012±0.003
S4	ND	0.050±0.013	0.022±0.004	0.008±0.002	0.015±0.004
S5	ND	0.048±0.012	0.028±0.008	0.010±0.003	0.025±0.007
S6	0.021±0.006	0.017±0.004	0.020±0.005	0.018±0.005	0.013±0.003
S7	ND	0.027±0.004	0.026±0.005	ND	0.018±0.005
S8	0.035±0.009	0.016±0.004	0.036±0.004	0.017±0.005	0.026±0.007
S9	ND	0.021±0.005	0.022±0.006	0.008±0.002	<LOQ
S10	0.055±0.014	0.057±0.014	0.046±0.010	0.014±0.004	0.018±0.005
MRLs[26]	**30**	**5**	**0.1**	**0.02**	**0.05**

ND = Not detected
MRL = Maximum residual limits

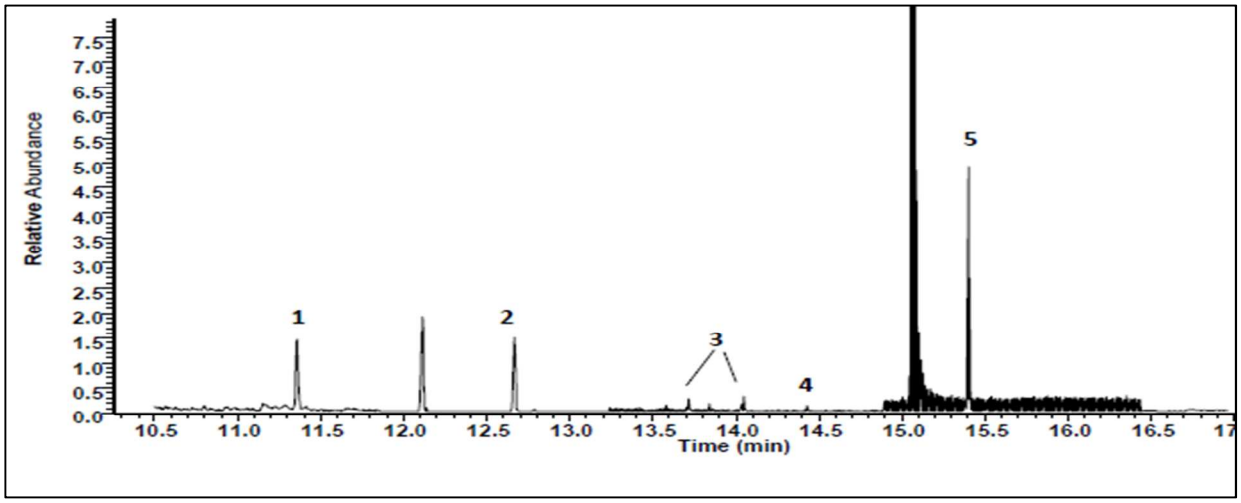

Figure 3 Chromatogram of pesticides occurrence in tea sample; 1. Lindane; 2. Chlorpyrifos; 3. Endosulfan; 4. Dieldrin; 5. Bifenthrin

4 Conclusions

In this study, multiple classes of pesticide residues in tea were simultaneously extracted by accelerated solvent extraction with in-cell cleanup and determined by gas chromatography tandem mass spectrometry. The usefulness of the proposed approach has provided remarkable analytical features, which allow the proposed methodology to be applied as a routine analysis for the monitoring of pesticide residues. Moreover, accelerated solvent extraction with in-cell clean up can eliminate the need for the use of GPC or SPE clean up procedures. In addition, a combination of the cleanup sorbents PSA and C_{18} provides a good recovery, good precision and low detection limit for all of the investigated pesticides, and it also involves both less solvent consumption and waste generation.

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