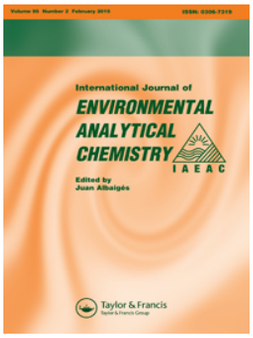

International Journal of Environmental Analytical Chemistry

ISSN: 0306-7319 (Print) 1029-0397 (Online) Journal homepage: https://www.tandfonline.com/loi/geac20

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To cite this article: Ioanna P. Kaveli, Vasilios A. Sakkas & Triantafyllos A. Albanis (2015) Development of ultrasound-assisted emulsification microextraction for the determination of seven pesticides in apple juice by gas chromatography–mass spectrometry, International Journal of Environmental Analytical Chemistry, 95:2, 145-157, DOI: 10.1080/03067319.2014.994618

To link to this article: https://doi.org/10.1080/03067319.2014.994618

Intern. J. Environ. Anal. Chem., 2015
Vol. 95, No. 2, 145–157, http://dx.doi.org/10.1080/03067319.2014.994618

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Development of ultrasound-assisted emulsification microextraction for the determination of seven pesticides in apple juice by gas chromatography–mass spectrometry

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(*Received 30 July 2014; final version accepted 28 November 2014*)

A simple, relatively rapid, sensitive and cost-effective method based on ultrasound-assisted emulsification microextraction (USAEME) followed by gas chromatography coupled with mass spectrometry has been developed for the determination of seven endocrine disruptor pesticides (chlorpyrifos, deltamethrin, dimethoate, fenitrothion, malathion, pendimethalin and procymidone) in apple juice. This approach is based on the emulsification of organic extraction solvent in a diluted apple juice sample by ultrasound radiation and further separation of both liquids phases by centrifugation. The influence of the different parameters affecting the procedure (extraction solvent, extraction solvent volume, ultrasound time, centrifugation time, ionic strength and pH) was evaluated in order to optimise the efficiency of the extraction process. Target analytes were extracted from a 0.5 g apple juice sample that was diluted by 10 times with aqueous buffer solution (pH 7). The optimised USAEME procedure used 100 μL of chloroform as extraction solvent, 8 min of ultrasound extraction, ionic strength (2.5% w/v) and 7.5 min of centrifugation at 3800 rpm. The optimised method presented recoveries between 70 and 113% for the target analytes. Acceptable linearity for all target analytes was recorded with correlation coefficients (r) higher than 0.992. The limits of quantification were found between 1.1 and 4.6 μg kg^{-1} ensuring compliance with the maximum residue limits established by the European Commission. The proposed method was applied for the determination of the endocrine disruptor pesticides in apple samples proving its suitability to the Commission Implementing Regulation (EU) no. 400/2014.

Keywords: ultrasound-assisted emulsification microextraction; endocrine disruptor pesticides; food; gas chromatography–mass spectrometry

1. Introduction

The US Environmental Protection Agency has released a list of chemicals including pesticide active ingredients for inclusion in the Endocrine Disruptor Screening Program [1]. An endocrine-disrupting compound (EDC) is defined as 'exogenous substance or mixture that alters the function of the endocrine system and generate noxious effects on the health of a safe body, its descendants, or its sub-population' [2–5]. EDCs are ubiquitous in the environment because of their very frequent use in residential, industrial and agricultural applications [6]. In terms of adverse health effects, there is concern that substances with endocrine-disrupting properties may be causally involved in a number of diseases and disabilities, including hormone-dependent cancer, reproductive disorders, a decline in fertility obesity, diabetes, cancer, heart disease, reproductive health problems, as well as neurodevelopmental and neurodegenerative disorders

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or conditions [7,8]. The major routes of humans' exposure to these EDCs are assumed to involve a normal dietary regimen that includes food containing added antioxidants, compounds leaking from food-wrapping materials and pesticide residues (i.e. vegetables, fruits, dairy products) [9,10]. Since traces of endocrine-disrupting pesticides are still detectable in many food products and environmental water resources, widespread application poses some risk to human health and safety. In this regard, maximum residue levels (MRLs) have been set by the European Commission in order to safeguard the health of those who consume vegetables and fruits treated with pesticides during production [11].

The globally increased concern towards EDCs induced a necessity to develop highly sensitive and specific analytical tools for their determination in food samples far below the established MRLs.

Several extraction techniques have been developed and employed for the determination of pesticides in liquid, semi-solid and solid samples, among them, liquid–liquid extraction, solid-phase extraction (SPE), accelerated solvent extraction, matrix solid-phase dispersion [12–16], liquid-phase microextraction [17] and dispersive liquid–liquid microextraction [18,19].

In 2008, a new ultrasound-assisted emulsification microextraction (USAEME) was developed [20]. USAEME is based on a heterogeneous system of two immiscible liquid phases, in which the main effects of ultrasounds are the fragmentation of one of the phases to form an emulsion of submicron droplet size that extends the contact surface between both liquids. The main advantage of the technique is the high extraction efficiency achievable in a short period of time [20–23]. A scarce number of studies have been reported using USAEME in food commodities [24–28].

The aim of the current study was to develop a simple, rapid, sensitive and cost-effective method based on USAEME, followed by gas chromatography coupled with mass spectrometry for the determination of seven endocrine disruptor pesticides in apple juice samples. The choice of the target analytes, as well as the matrix, is relevant to the Commission Implementing Regulation (EU), no. 400/2014, concerning a coordinated multiannual control programme of the Union for 2015, 2016 and 2017 to ensure compliance with MRLs of pesticides and to assess the consumer exposure to pesticide residues in and on food of plant and animal origin.

2. Experimental

2.1 *Chemicals and materials*

All the chemicals and reagents used were of analytical grade. Chlorpyrifos, deltamethrin, dimethoate, fenitrothion, malathion, pendimethalin, procymidone, potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). A mixture containing the investigated compounds at 1000 μg L^{-1} in methanol was prepared and used as working solution. All solutions were refrigerated at 4°C for storage. Extraction solvents used for the development of USAEME such as chlorobenzene, carbon tetrachloride and chloroform ($CHCl_3$) were purchased from Merck (Darmstadt, Germany). Methanol (99.9%) was purchased from Labscan (Dublin, Ireland). Sodium chloride (NaCl) was obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA). The pH value of the solution was adjusted by dissolving proper amount of KH_2PO_4 in water (0.1 M) and addition of 0.1 M of NaOH.

2.2 *Apparatus*

Chromatographic analysis of EDCs was performed using a Trace GC Ultra instrument (Thermo Scientific, Waltham, MA, USA) coupled to an ISQ mass spectrometer controlled by a computer

Table 1. Target analytes, relevant physicochemical characteristics, retention times, as well as diagnostic ions for their analysis in GC/MS.

Analytes	pK_a[a]	$logK_{ow}$[b]	t_R (min)	Quantification ion (*m/z*)	Identification ions (*m/z*)
Dimethoate	nd[c]	0.76	18.7	93	125–229
Fenitrothion	nd	3.32	25.7	277	125–260
Malathion	nd	2.89	26.4	173	127–158
Chlorpyrifos	nd	4.70	26.9	197	286–314
Pendimethalin	10.52	5.18	29.6	252	162–281
Procymidone	nd	2.53	30.9	96	255–283
Deltamethrin	10.62	6.10	56.5	181	209–253

Notes: [a]Obtained from http://www.chemicalize.org
[b]Obtained from http://www.syrres.com
[c]nd, not dissociated

running X-Calibur software. Aliquots of 1 µL were injected using an AI/AS 3000 auto sampler (Thermo Scientific). In splitless mode, the injector temperature was maintained at 250°C. The separation was performed using a DB-5 capillary column 30 m × 0.25 mm i.d., with film thickness of 0.25 µm (Supelco, Bellefonte, PA, USA). Helium was the carrier gas at a constant inlet flow rate of 1.0 mL min^{-1}. The GC oven temperature was programmed at an initial temperature of 60°C (held for 1.0 min), then ramped at 15°C min^{-1} to 160°C (held for 4 min), then to 230°C at 2.2°C min^{-1}, followed by a 5°C min^{-1} ramp to 290°C (held for 5 min). The ion source and transfer line temperature were kept at 250 and 280°C, respectively. Electron ionisation mass spectra at *m/z* of 50–500 were recorded at 70 eV. In the selected ion monitoring mode acquisition, target ions were monitored at different time windows defined by the corresponding retention times. Characteristic ions (Table 1) were chosen for each analyte, according to their mass spectra obtained in the full-scan mode as well as by comparison with NIST library. Identification of analytes and confirmation of results was followed according to SANCO/12571/2013 guidelines [29].

USAEME extraction process was performed on a model S30H (*Elmasonic*) ultrasonic cleaner at 280 W, 50–60 kHz with digital timer, temperature controller. A centrifuge model Jouan B 4i was used to perform the centrifuge process (220 V, 500 W, 50/60 Hz, Herblain-France).

2.3 *Sample collection and preparation*

Control (blank) apple samples were purchased on local market (labelled as organic) and were checked for absence of target pesticides before use in the validation studies. All samples were kept stored at –15°C. Before analysis, samples were left at room temperature in the absence of light, were manually cut into small pieces and homogenised using a food blender. Samples were centrifuged for 10 min at 3800 rpm and the supernatant (apple juice) was collected and subjected to the USAEME procedure.

2.4 *USAEME procedure*

An accurately weighted portion of 0.5000 g of the apple juice was diluted by 10 times after the addition of aqueous phosphate buffer solution (4.5000 g, pH 7). Then the solution was placed in a 10-mL plastic centrifuge tube with a cover. Afterwards, 2.5% w/v NaCl and 100 µL $CHCl_3$ (extraction solvent) were added and the tube was immersed horizontally in an ultrasonic bath at 60 kHz frequency at 25°C for 8 min. As a result, an emulsion of $CHCl_3$ in aqueous sample was

formed. The emulsion was disrupted by centrifugation at 3800 rpm for 7.5 min. Accordingly, the organic phase was sedimented in the bottom of the plastic tube and was removed by 100-μL syringe. The extract was evaporated by a gentle stream of nitrogen, reconstituted in $CHCl_3$ (20 μL) and transferred to a chromatography vial before GC/MS analysis.

3. Results and discussion

3.1 *Preliminary experiments*

Despite the advanced nature of the GC/MS tools, one of the most common problems that occur is its susceptibility to matrix effects, which adversely affect quantification when analysing complex matrices such as food samples. The percentage of matrix effect (%ME) was obtained for each analyte from the slopes of the calibration curves and was determined by comparing solvent and matrix-matched slopes by using the formula: $\%ME = [1 - (slope_{SOLVENT}/slope_{MATRIX})] \times 100\%$. Signal enhancement would occur if the percentage of the difference was positive. If the result is negative, then this is indicative of signal suppression. To evaluate the presence and extension of this effect, standards of different concentrations were analysed in pure solvent and apple juice matrix. The combination of a dilution factor of 1–10, as well as the use of matrix-matched standards (calibration with extracts of free-analyte apple juice spiked after the USAEME), compensated the suppression signal effects, achieving an accurate quantification (a percentage between −20% and +20% was considered as no matrix effect because this variation would be close to the repeatability values) (Table 2).

Concerning the USAEME procedure, initially the effect of horizontal and vertical sonication was investigated at 25°C. Conical tubes located inside the sonicator received horizontal (with the water level at about 5 cm) and vertical ultrasonication (with the water level at about 13 cm). The results showed that in the latter configuration the solvents used, aggregated in the bottom of the conical tubes, while during horizontally positioning, the extraction solvents formed fine droplets in the sample solution. For the establishment of an efficient USAEME procedure, several other parameters were explored, such as extraction solvent, extraction solvent volume, ultrasound extraction time, centrifugation time, ionic strength and pH. The experiments were performed by modifying one parameter at a time while keeping the remaining parameters constant, at a concentration level of 75 μg kg^{-1}. Each optimisation experiment was replicated for three times.

Table 2. Validation parameters obtained for target analytes after USAEME and GC/MS.

Compounds	Correlation coefficient (r)[a]	Intra-day RSDr (%)	Inter-day RSD_{WR} (%)	Rec[b] (%)	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	MRL (μg kg^{-1})	Matrix effect[c] (%)
Dimethoate	0.999	8	10	113	1	4	20	+10
Fenitrothion	0.992	8	11	86	0.7	2	10	−8
Malathion	0.997	12	13	98	0.3	1	20	−19
Chlorpyrifos	0.995	11	14	87	0.4	1	500	+18
Pendimethalin	0.9999	9	11	83	0.9	3	50	+8
Procymidone	0.995	7	9	70	1	5	10	+14
Deltamethrin	0.995	4	6	94	1	4	200	+8

Notes: [a]Method linearity was evaluated by performing matrix-matched calibration curves (calibration with free-analyte apple juice samples spiked before the USAEME) at a concentration range starting from LOQ for each analyte to 500 μg kg^{-1}.
[b]Spiking blank apple juice samples (treated with the procedure described in Section 2.3) at concentration level, corresponding to 5xLOQ for each analyte.
[c]Positive sign indicates signal enhancement and negative sign signal suppression.

3.2 *Selection of extraction solvent*

The selection of extraction solvent is critical for developing an efficient USAEME procedure since the physicochemical properties of the solvent govern the emulsification phenomenon and, consequently, the extraction efficiency. The extraction solvent should have a higher density than water, a low solubility, a high extraction capability for the target analytes, as well as a relatively low surface tension, so that it can disperse in the sample and form a stable emulsion after ultrasound radiation. Furthermore, the solvent must be compatible with the GC injection requirements and separation technique. In this study, three organic solvents, namely chlorobenzene, $CHCl_3$ and carbon tetrachloride, at a volume of 80 µL were investigated. Figure 1 illustrates the effect of the extraction solvents on the extraction efficiency. The results showed that $CHCl_3$ has the highest extraction efficiency for all the target analytes. As a result, $CHCl_3$ was chosen as the best extraction solvent for subsequent studies.

3.3 *Effect of extraction solvent volume*

In order to study the effect of extraction solvent volume on the extraction efficiency of the target analytes, the volume of $CHCl_3$ was varied in the range from 80 to 120 µL (considering the fact that the sediment volume of $CHCl_3$ after USAEME ranged between 30 and 40 µL). As it can be seen (Figure 2), peak areas increase with the increment of extraction

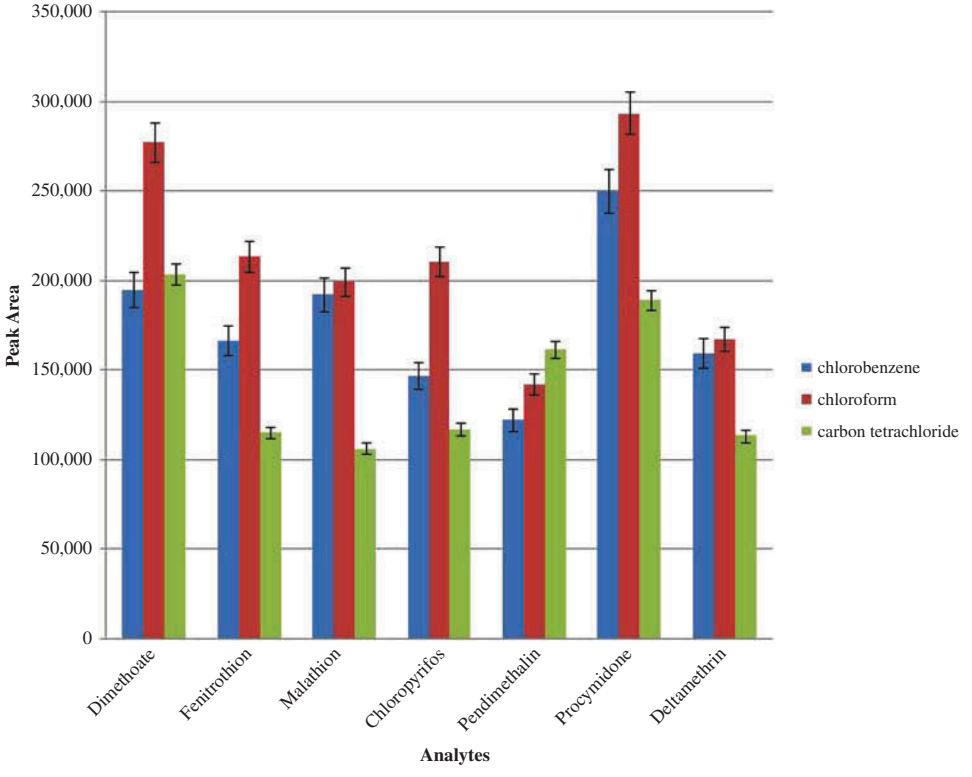

Figure 1. Effect of different extraction solvents on the extraction efficiency of target analytes obtained by USAEME (USAEME conditions: extraction solvent volume 80 µL, sonication time 5 min, temperature of ultrasonic water bath 25°C, centrifugation time 10 min).

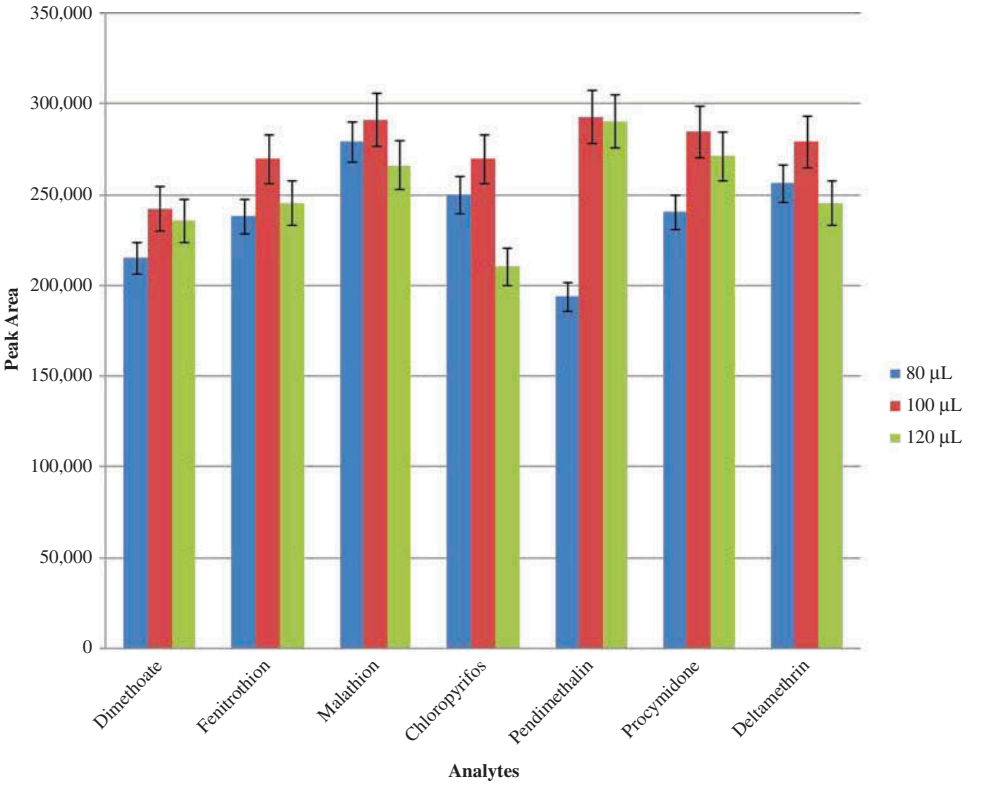

Figure 2. Influence of different extraction solvent volumes on the extraction efficiency of target analytes obtained by USAEME (USAEME conditions: extraction solvent $CHCl_3$, sonication time 5 min, temperature of ultrasonic water bath 25°C, centrifugation time 10 min).

solvent volume. However, after 100 µL, the analytical signals decreased. This was attributed either to the dilution of the final extract since a higher solvent volume was recovered or to inefficient dispersion of $CHCl_3$ [30]. Therefore, 100 µL of $CHCl_3$ was chosen as the optimal volume for further studies.

3.4 *Effect of ultrasound extraction time*

The application of ultrasonic irradiation accelerates the mass-transfer process between two immiscible phases, which together with the large surface of contact between both phases leads to an increment in the extraction efficiency in a minimum time. This step is critical since $CHCl_3$ was broken up into small microdrops and dispersed to the diluted apple juice sample. The effect of extraction time was examined in the range of 2–14 min with constant experimental conditions. The results (Figure 3) demonstrated that the highest responses were obtained after ultrasonication of 8 min, while at longer extraction time (11 min) there were no significant changes in signal intensities ensuring that equilibrium was reached. At 14 min, the extraction efficiency was slightly decreased due to the instability of the emulsion that delaminates (divides into layers). Therefore, a 8 min sonication time was used in the following studies.

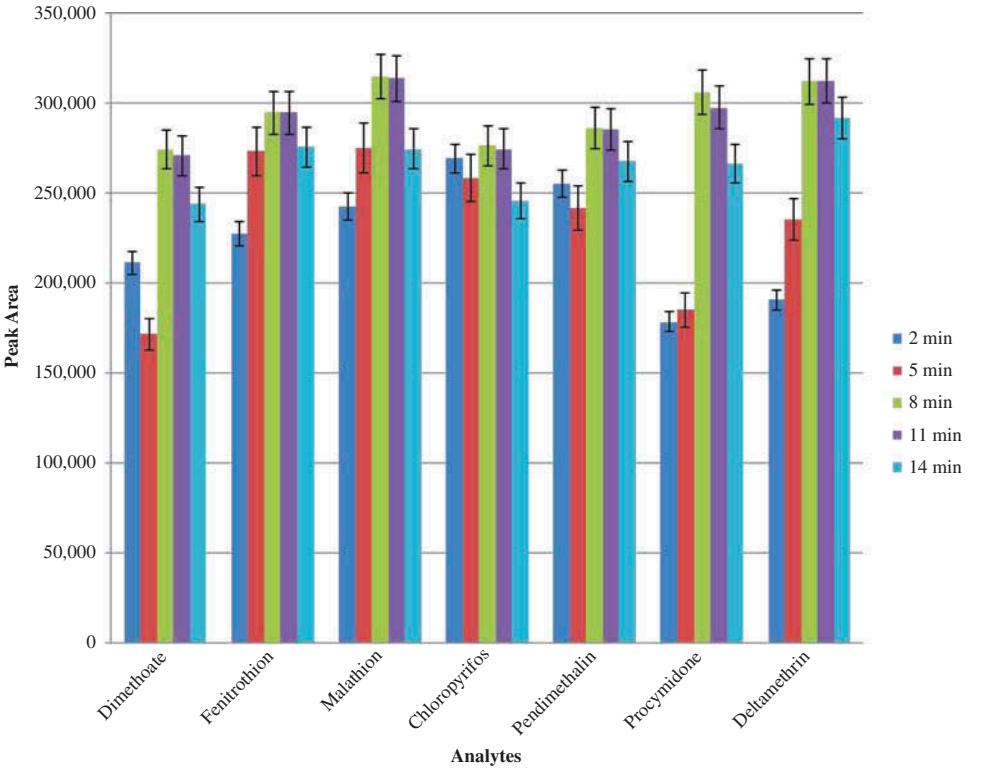

Figure 3. Effect of ultrasound extraction time on the extraction efficiency of the target analytes (USAEME conditions: $CHCl_3$ 100 μL, temperature of ultrasonic water bath 25°C, centrifugation time 10 min).

3.5 *Effect of centrifugation time*

After USAEME extraction, the emulsion was disrupted by centrifugation to enable phase separation and adequate recovery of the $CHCl_3$ droplets. Centrifugation times at 3800 rpm were examined in the range of 2.5–10 min (Figure 4). Based on our findings, 7.5 min was sufficient for complete separation of phases of the investigated compounds since an increasing centrifugation time does not influence the extraction efficiency. Thus, 7.5 min was selected as the centrifugation time to get a satisfactory biphasic system.

3.6 *Effect of ionic strength*

For many equilibrium-based extraction approaches, salting out, that is, increasing the ionic strength has been commonly used technique to promote analyte transfer [31]. To investigate the influence of ionic strength on the extraction efficiency, experiments were carried out with the addition of NaCl (0–5% w/v) into the diluted apple juice sample. The results (Figure 5) revealed that the extraction efficiency was increased with increasing NaCl concentration up to 2.5% (w/v). At higher concentration, a decrease on the extraction efficiency occurred. The salting-out effect can decrease the solubility of analytes in the aqueous phase and promote the transfer of the analytes towards the organic phase, thus improving the extraction efficiency. On the other hand, as the ionic strength of the medium increases, both the viscosity and density of the

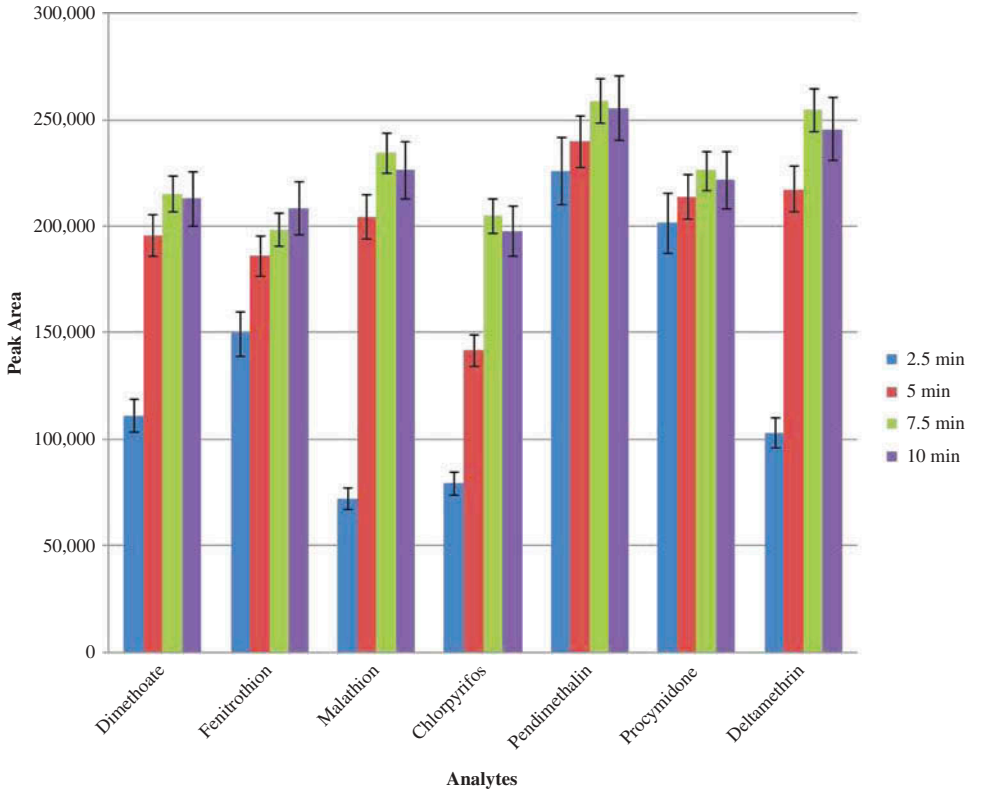

Figure 4. Effect of centrifugation time on the extraction efficiency of target analytes (USAEME conditions: $CHCl_3$ 100 μL, sonication time 8 min, temperature of ultrasonic water bath 25°C).

aqueous solution are enhanced, leading to a reduction of the efficiency of the mass transfer process and thus the extraction efficiency of the procedure [32]. Based on the experimental results, 2.5% w/v NaCl was added in the sample solution.

3.7 *Effect of pH*

It is obvious that ionised species have a great tendency for remaining in aqueous phase and it is necessary to convert them to molecular form before $CHCl_3$-based extraction. As can be observed (Figure 6), at neutral pH values, higher responses were obtained for the target analytes compared with acidic or basic conditions. From the target analytes, only pendimethalin (pKa = 10.52) and deltamethrin (pKa = 10.62) behave as weak acids (Table 1) and therefore if the solution pH is lower than 8.6 (pKa 2), they mainly exist in their molecular undissociated state and could be efficiently extracted with $CHCl_3$. On the basis of these findings, pH was adjusted to 7 with aqueous phosphate buffer solution for further studies.

3.8 *Method validation*

Performance characteristics of the optimised method were established by a validation procedure, studying trueness, precision, linear range, sensitivity and specificity. The validation scheme followed was based on the SANCO/12571/2013 European Guidelines [29].

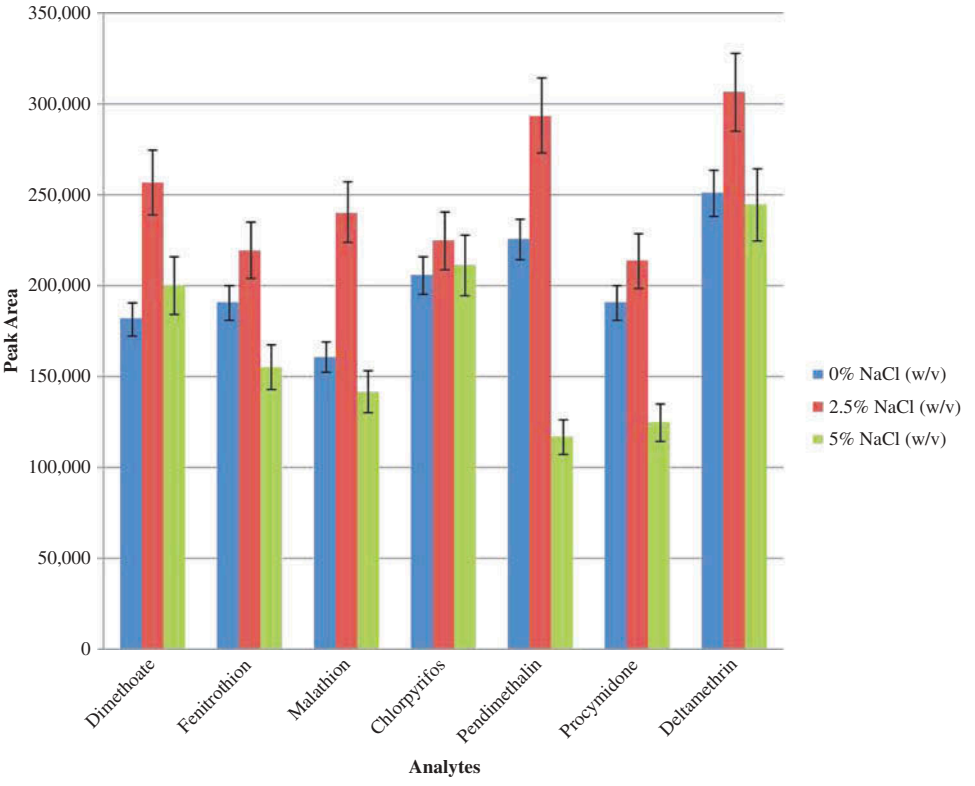

Figure 5. Effect of ionic strength on the extraction efficiency of target analytes obtained by USAEME (USAEME conditions: $CHCl_3$ 100 µL, sonication time 8 min, temperature of ultrasonic water bath 25°C, centrifugation time 7.5 min).

3.8.1 *Trueness and precision*

Method trueness was assessed through recovery studies, spiking blank apple juice samples (treated with the procedure described in Section 2.3) at three different concentration levels, corresponding to limits of quantification (LOQ), MRL and five times the LOQ limits for each compound. Recovery (R%) was defined according to Equation (1):

$$\mathrm{R}\% = \frac{C_{\mathrm{sed}} \times V_{\mathrm{sed}} \times 100}{C_{\mathrm{o}} \times V_{\mathrm{o}}} \tag{1}$$

were C_{sed}, V_{sed}, C_{o} and V_{o} are the concentration of analytes in the sedimented extraction solvent, volume of sedimented extraction solvent, initial concentration of analytes in apple juice sample and volume of apple juice sample, respectively. Recoveries were between 70% and 113% (Table 2), whereas intraday precision (RSD_r%), and within-lab reproducibility (RSD_{WR}%), calculated as the percentage standard deviation of five replicates, were found between 3% to 12% and 5% to 14%, respectively, complying with the requirements of SANCO document (R% 70–120%, RSDr, $RSD_{WR} \leq 20\%$). The enrichment factor (EF) was calculated using the following equation: $EF = C_{\mathrm{sed}}/C_{\mathrm{o}}$, where C_{sed} and C_{o} are the concentration of the analytes in the sedimented organic phase ($CHCl_3$) and initial concentration of the analytes in the apple juice sample, respectively. EF of analytes ranged from 18 to 28.

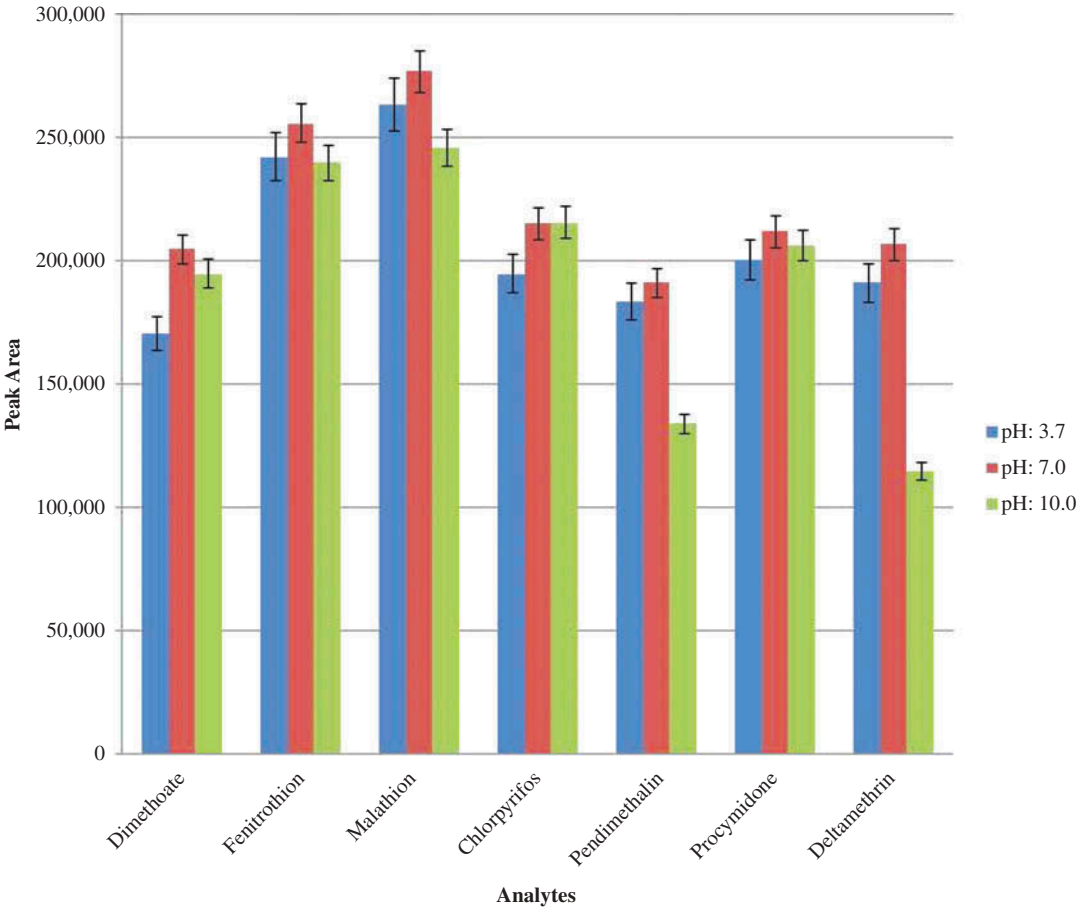

Figure 6. Influence of pH on the extraction efficiency of target analytes (USAEME conditions: $CHCl_3$ 100 μL, sonication time 8 min, temperature of ultrasonic water bath 25°C, NaCl 2.5% w/v, centrifugation time 7.5 min).

3.8.2 *Linear range*

Method linearity was evaluated by performing matrix-matched calibration curves (calibration with free-analyte apple juice samples spiked before the USAEME) at a concentration range starting from LOQ for each analyte to 500 μg kg^{-1} (seven points, each calibration point was determined in triplicate, Table S1). The obtained peak area versus concentration was linear (least-squares linear regression) in the assayed range achieving correlation coefficients between 0.992 and 0.9999 (Table 2).

3.8.3 *LOD and LOQ and specificity*

The limit of detection (LODs) and LOQs for all target analytes were determined by successive analyses of chromatographic extracts of blank apple juice samples with decreasing amounts of the analytes until a signal-to-noise ratio 3:1 was reached, whereas the LOQs were determined considering a signal-to-noise ratio 10:1. The calculated LODs and LOQs (Table 2) are

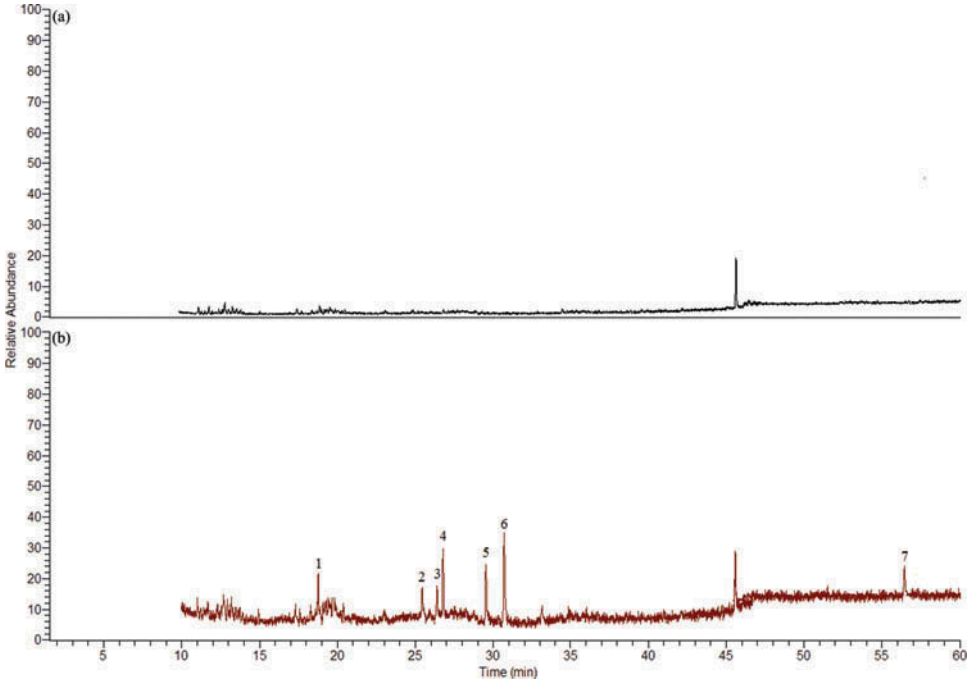

Figure 7. USAEME-GC-MS chromatograms (full-scan mode) obtain from (a) a blank sample and (b) a spiked apple juice sample following the validated USAEME procedure. 1, dimethoate; 2, fenitrothion; 3, malathion; 4, chlorpyrifos; 5, pendimethalin; 6, procymidone; 7, deltamethrin. Spiking level corresponds to 2xLOQ for each analyte.

acceptable, exhibiting compliance with the MRLs established by the European Commission for EDCs residues in apple [33].

The specificity of the method was tested by the analysis of blank samples. The absence of any chromatographic peak in the apple juice matrix, at the same retention times as the target analytes, indicated that there were no matrix compounds that might give a false-positive signal in these blank samples (Figure 7).

3.9 *Comparison with other extraction methods and application to real samples*

The analytical characteristics of the proposed method could not be directly compared with other methods since they may differ in the selection of the target analytes and/or the sample matrix itself. Considering the determination of malathion, chlorpyrifos and pendimethalin in apple juice samples, the proposed USAEME method was compared to QuEChERS, SPE and solid-phase microextraction (SPME) [34]. Among them, SPME was more sensitive allowing to reach lower LOQs; however, it was the most time-consuming procedure. On the other hand, SPE without additional clean-up seemed less adequate for the investigated matrices. Dispersive liquid–liquid microextraction and multidimensional gas chromatography–mass spectrometry was employed recently for the analysis of 24 pesticide residues in apple juices, including malathion, chlorpyrifos and procymidone. Mean recoveries of the three pesticides ranged from 66% to 104%, and the limits of quantification were between 3.2 and 4.7 μg L^{-1} [35]. In the proposed dilute, extract (USAEME) and shoot method, there is no need for an additional clean-up step or a need to use a

fibre coated with a polymeric phase (which is relatively expensive). Based on this, USAEME could be an alternative simple, relatively rapid and sensitive method, suitable for the analysis of apple juice samples. The bottleneck of the proposed method could be the use of toxic chlorinated solvent such as $CHCl_3$, in comparison with other recently developed liquid-phase microextraction methods such as USAEME [36]. The latter method provided for procymidone recoveries up to 99.5% and LOQ 2 mg L^{-1}.

To demonstrate the applicability of USAEME for routine analysis, the described method was applied to the determination of the seven endocrine disruptor pesticides in six apple juice samples obtained from local markets from the region of Epirus (N.W. Greece). The results revealed that the selected samples were free from contamination from the target analytes. To validate the results, samples spiked with known amount of EDCs were analysed (50 μg kg^{-1}). Good recoveries were obtained in the range of 72–109%.

4. Conclusions

In this study, USAEME followed by GC-MS was developed for the determination of seven endocrine disruptor pesticides in apple juice samples. Optimisation of the variables affecting the extraction efficiency of USAEME was carried out. Applicability, accuracy, precision and sensitivity of the proposed method have been demonstrated based on SANCO/12571/2013 European guidelines. The proposed method is simple, cost-effective and provides high sensitivity and reproducibility in a short analysis time, and it is suitable for its application for the determination of the selected EDCs in apple juice samples following the Commission Implementing Regulation (EU) no. 400/2014.

Funding

This research project has been co-financed by the European Union (European Regional Development Fund-ERDF) [MIS: 348148] and Greek national funds through the Operational Program 'THESSALY-MAINLAND GREECE AND EPIRUS-2007–2013' of the National Strategic Reference Framework [NSRF 2007–2013].

Supplemental data

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/03067319.2014.994618.

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