Talanta 116 (2013) 376–381

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Dispersive liquid—liquid microextraction for the determination of three cytokinin compounds in fruits and vegetables by liquid chromatography with time-of-flight mass spectrometry

N. Campillo, P. Viñas, G. Férez-Melgarejo, M. Hernández-Córdoba*

Department of Analytical Chemistry, Faculty of Chemistry, Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, E-30100 Murcia, Spain

ARTICLE INFO

Article history:
Received 21 February 2013
Received in revised form
23 May 2013
Accepted 28 May 2013
Available online 5 June 2013

Keywords:
Forchlorfenuron
Thidiazuron
1,3-Diphenylurea
Dispersive liquid—liquid microextraction (DLLME)
Liquid chromatography with time-of-flight mass spectrometry (LC—TOFMS)
Fruits

ABSTRACT

The paper presents a novel approach for the determination of three cytokinin compounds, thidiazuron (TDZ), 1,3-diphenylurea (1,3-DPU) and forchlorfenuron (CPPU), in fruit and vegetables samples using liquid chromatography with electrospray ionization and time-of-flight mass spectrometry (LC—ESI—-TOFMS). Analytes were extracted from the sample matrix with ethanol, and the extract, after dilution with water, was submitted to dispersive liquid—liquid microextraction (DLLME). Once acetonitrile and 1,2-dichloroethane had been selected as extraction and disperser solvents, respectively, the influence of the following experimental parameters was studied using a Plackett—Burman design: volume of extraction and disperser solvents, sample mass and time and speed of centrifugation. The best analytical conditions were 250 μL 1,2-dichloroethane, 1.5 mL acetonitrile, 5 g sample mass, and centrifugation at 3000 rpm for 3 min. The optimized method provided DLs in the range 0.02—0.05 ng g^{-1}, depending on the compound. Satisfactory recovery values between 89 and 106% were obtained for spiked samples (kiwifruit, watermelon, grape and tomato) in the 0.2—1.0 ng g^{-1} concentration range, depending on the compound. None of the target analytes was detected in any of the samples analyzed.

1. Introduction

Plant growth regulators (PGRs) are classified into five main classes: cytokinins (CKs), auxins, gibberellins, ethylene and abscisic acid [1]. Most of these compounds are naturally produced in plants, where they regulate the growth or destroy undesired parts of plants. Synthetic PGRs are widely sold for use in agriculture because of they are cheaper and show greater stability than the natural homologs [2], although their use in the European Union is governed by the Council Directive 91/414/EEC [3]. CKs, which were discovered during the 1950s, play an important role in several phases of plant development and growth, and are used to enhance fruit set, fruit size and the yield of different crops. The functions of CK receptors in plants have been reviewed recently [4]. The CK group comprises two types of compound: adenine and phenylurea derivatives [5]. The phenylureas comprise a group of synthetic compounds, the first one identified being 1,3-diphenylurea (1,3-DPU), whereas forchlorfenuron (CPPU) and thidiazuron (TDZ) were synthetized later and show higher activity than other natural PGRs, such as the adenine-type cytokinin zeatin [5].

The main effect of CPPU is to increase fruit size (e.g. kiwifruits, grapes and apples among others), and to promote the development of the ovary into fruit without fertilization or seed formation. CPPU acts synergistically with auxins. The correct application of this compound does not involve human risk and, moreover, CPPU has been classified as not likely to be a human carcinogen or endocrine disrupter [6]. In 2006, CPPU was included in Annex I of Directive 91/414/EEC of the European Union as an authorized PGR for kiwifruit, with a maximum residue limit of 50 ng g^{-1} [7]. TDZ, also classified as not likely to be a human carcinogen, has been used for chemical defoliation before the mechanical harvesting of cotton and for promoting plant growth [8,9]. The effect of 1,3-DPU on callus induction on the surface of certain plants has been described [10], as well as its stimulatory effect on plants [11], although its cytokinin activity is 10,000 times lower than that of CPPU.

CPPU in fruit and vegetables can be determined by immunoassay procedures [12,13], using liquid chromatography (LC) with UV detection [14—16], tandem mass spectrometry (MS/MS) [17—21] and time-of-flight mass spectrometry (TOFMS) [22,23]. However, the literature only shows two analytical methods for the determination of TDZ in fruit and vegetables: LC—UV detection [24] and LC—MS/MS [17], the latter also including the determination of CPPU. On the other hand, TDZ has been quantified in waters

* Corresponding author. Fax: +34 868887682.
E-mail address: hcordoba@um.es (M. Hernández-Córdoba).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.05.063

[8,25,26] and fertilizers [2]. Sample treatments in the cited references include extraction of the analytes into an organic solvent, and clean-up by liquid—liquid extraction (LLE) [14], solid-phase extraction (SPE) [15,16,21,22] and dispersive solid-phase extraction (DSPE) [20,23]. The simultaneous extraction and clean-up using QuEChERS methodology has also proved satisfactory [17,19,22]. In this study, we propose the simultaneous determination of CPPU, TDZ and 1,3-DPU in fruit and vegetables by solvent extraction of the analytes, with low consumption of the organic solvent and preconcentration of the extract by means of a miniaturized technique whose main advantages are its rapidity, efficacy, low cost and lack of memory effects: dispersive liquid—liquid microextraction (DLLME) [27—30]. DLLME has only been applied for the determination of TDZ in water samples [26]. To the best of our knowledge this is the first time that the simultaneous determination of the three phenylurea cytokinin compounds has been approached. Moreover, in this case, the same friendly sample treatment (DLLME) is proposed for the analysis of fruit and vegetables by LC—ESI—TOFMS.

2. Experimental

2.1. Instrumentation

The LC system consisted of an Agilent G1312A (Agilent, Waldbronn, Germany) binary pump operating at a flow-rate of 0.8 mL min^{-1}. The solvents were degassed using an on-line membrane system (Agilent G1379B). The column was maintained at ambient temperature in a thermostated compartment (Agilent G1316A). Separation was performed on a Tracer Extrasil ODS2 column (Teknokroma) (150 mm × 4 mm, 5 μm), while injection (20 μL) was performed using an autosampler (Agilent G1367A). Autosampler vials of 2-mL capacity provided with 250 μL microinserts with polymeric feet were used. The LC system was coupled to a time-of-flight mass spectrometry device (Agilent G6220A), equipped with an electrospray ionization source (ESI) operating in positive mode, using the following operation parameters: capillary voltage, 3000 V; nebulizer gas pressure, 60 psi; drying gas flow, 11 L min^{-1}; drying gas temperature, 350 °C; fragmentor voltage, 150 V; skimmer voltage, 65 V; octapole RF, 250 V. LC—TOFMS accurate mass spectra were recorded considering a mass range of 100—1000*m/z*. Applied Biosystems/MDS-SCIEX Analyst QS software (Frankfurt, Germany) was used for data processing. Accurate mass measurements of each peak from the total ion chromatograms were obtained by means of an automated calibrant delivery system which to provide the correction of the masses. The TOF mass spectrometer carried out the internal mass calibration automatically, using a dual-nebulizer electrospray source with an automated calibrant delivery system, which introduces the flow from the outlet of the chromatograph together with small amounts (about 5 μL min^{-1}) of a calibrating solution, ES-TOF tuning mix reference (Agilent), in positive ESI mode. Ten reference masses were used between 118.0862 and 2721.8948*m/z*. Mass Hunter software, version B-02-00, was used for autocalibrating and continuously recording the results of internal reference masses and the raw data.

Analyses were carried out using the extracted ion chromatogram of the protonated molecule of each analyte: 221.0492, 213.1028 and 248.0585*m/z* for TDZ ($C_9H_8N_4SO$+H), 1,3-DPU ($C_{13}H_{12}N_2O$+H) and CPPU ($C_{12}H_{10}ClN_3O$+H), respectively, with a 20 ppm window. The exact theoretical masses based on the formula were calculated using the molecular weight calculator tools of Mass Hunter software. The accurate mass spectrum of the analytes was obtained by subtracting the background of the extracted ion chromatogram. The accurate mass of the protonated molecule was used for both quantitation and confirmation purposes. The peak areas of the extracted ions were used as analytical signals for quantitation.

An IKA A11 grinder (IKA, Staufen, Germany) and an EBA 20 (Hettich, Tuttlingen, Germany) centrifuge were used for treating the samples. Hydrophilic membrane filters, mixed cellulose esters, of 8.0 μm were used to filter the ethanolic sample extracts.

2.2. Chemicals and reagents

Analytical-reagent grade ethanol, methanol, acetonitrile (ACN), acetone, dichloromethane, chloroform, carbon tetrachloride, 1,2-dichloroethane and 1,1,2,2-tetrachloroethane were obtained from Sigma (St. Louis, MO, USA). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Sodium chloride and ammonium sulfate were obtained from Fluka (Buchs, Switzerland).

Forchlorfenuron (1-(2-chloro-4-pyridyl)-phenylurea, $C_{12}H_{10}ClN_3O$, CPPU, 99.9%), thidiazuron (1-phenyl-3-(1,2,3-thidiazol-5-yl)urea, $C_9H_8N_4SO$, TDZ, 99.9%) and 1,3-diphenylurea ($C_{13}H_{12}N_2O$, 1,3-DPU, 98%) were obtained from Fluka (Buchs, Switzerland). Standard stock solutions of each compound at 1000 mg L^{-1} were prepared in methanol and stored in darkness at 4 °C. Working standard mixture solutions were prepared daily by diluting the stock solutions with Milli-Q water.

2.3. Samples and analytical procedure

Several fresh fruit and vegetable samples (kiwifruit, melon, watermelon, grape and tomato) were purchased in a local supermarket. Kiwifruit, melon and watermelon samples were peeled before being triturated, and all analyses were carried out with the pulp. After trituration, the samples were placed in 100 mL closed polyethylene flasks before storing at 4 °C until analysis.

For analyte extraction from the sample matrices, 5 g of the homogenate were weighed into a capped 15 mL glass centrifuge tube and 3 mL of ethanol were added. The mixture was vigorously shaken for 1 min and then centrifuged at 6000 rpm for 5 min. The supernatant was recovered and filtered through a hydrophilic 8.0 μm Millipore filter and the filtrate diluted to 10 mL with water and placed in a 15 mL screw cap glass centrifuge tube with conical bottom. Next, an extraction mixture, consisting of 1.5 mL of acetonitrile (disperser solvent) and 250 μL 1,2-dichloroethane (extraction solvent) was rapidly injected into the diluted sample extract with a gas-tight syringe. This solution was manually shaken for about 20 s. The cloudy solution formed as consequence of the dispersion of the organic extraction droplets in the aqueous solution was centrifuged for 3 min at 3000 rpm, sedimenting the 1,2-dichloroethane phase at the bottom of the conical tube. The lower phase was isolated and evaporated to dryness by using a mild argon stream and the residue reconstituted in 50 μL of acetonitrile before a 20 μL-aliquot was injected in the LC system. The sample concentration in the final extract would correspond to 100 g mL^{-1}. The mobile phase used in isocratic conditions was a 40/60 (v/v) ACN/water mixture with a flow rate of 0.8 mL min^{-1} throughout the process. For the final results, three portions of each sample were analyzed separately.

2.4. Recovery assays

For recovery studies, samples were fortified by adding different volumes of a standard solution containing the analytes to 20 g of sample, leading to fortification levels in the range of 0.2—1.0 ng g^{-1}, depending on the compound. The fortified samples were left for 2 h at room temperature to equilibrate before carrying out the analysis as described above. The fortification procedure was applied to four different samples (kiwifruit, watermelon, grape and tomato) at

three concentration levels. Three aliquots of each sample at each concentration level were analyzed separately. The samples used for the recovery assays were previously checked as free of the analytes studied.

3. Results and discussion

3.1. Liquid chromatographic separation

Reversed phase chromatography was used. The optimal separation conditions were established by injecting 20 μL of an aqueous standard solution containing the analytes at a concentration level of 1 μg mL^{-1}, into the Tracer Extrasil ODS2 column. Different ACN/water mixtures were assayed as the mobile phase in isocratic mode at a 0.8 mL min^{-1} flow-rate, and the best separation was achieved with the mixture 40/60 (v/v), when the compounds were eluted with retention times of 4.06, 9.46 and 11.25 min for TDZ, 1,3-DPU and CPPU, respectively.

3.2. Sample preparation

The solid sample was treated using a suitable solvent, capable of extracting the analytes quantitatively from the matrices. Three different solvents (methanol, ethanol and acetonitrile) were assayed. Preliminary experiments were carried out using 5 g of a fortified kiwifruit at a concentration level of 15 ng g^{-1} of each compound and 2 mL of the organic solvent, submitting the mixture to ultrasounds by means of a probe directly immersed. Even when low ultrasound power was applied, the vegetal tissues were extremely disrupted and the organic extracts were filtered with high difficulty. Consequently, analyte extraction from the matrices was accomplished by manual shaking. Ethanol provided the best extraction efficiencies and after centrifugation this ethanolic phase was made up to 10 mL with water to be preconcentrated.

3.3. Optimization of the DLLME conditions

The first step in the DLLME optimization procedure was to select the most appropriate dispersant and extractant solvents. For this purpose, 10 mL of water containing the ethanolic sample extract (obtained from a kiwifruit sample fortified at 3 ng g^{-1} for TDZ and 0.3 ng g^{-1} for 1,3-DPU and CPPU) were used and 20 μL of the settled phase was injected into the LC.

The correct selection of the extraction solvent to be used must take into account several properties: higher density than water in order to achieve an ease recollection of the sedimented phase, high extraction capability, low solubility in water and good chromatographic behavior. Bearing these factors in mind, 1,1,2,2-tetrachloroethane ($C_2H_2Cl_4$), carbon tetrachloride (CCl_4), chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2) and 1,2-dichloroethane ($C_2H_4Cl_2$) were tested using 100 μL of the extractant solvent and 1 mL of ACN as dispersant solvent. The high solubility of dichloromethane in water (13 g L^{-1} at 20 °C) prevented the sedimented phase from being discernible. The use of $CHCl_3$ was also discarded because volumes lower than 20 μL were obtained for the settled phase. Comparing the analytical signals obtained with $C_2H_2Cl_4$, CCl_4 and $C_2H_4Cl_2$, the latter provided higher sensitivity for the three analytes. The main parameter considered for the selection of the disperser solvent is its miscibility in the extraction solvent and the aqueous phase. ACN, methanol and acetone have this property, and were tested in this study using 1.0 mL of each one and 100 μL of 1,2-dichloroethane as the extraction solvent. No significant differences were observed between the three disperser used, being ACN finally selected considering its close consonance with the mobile phase.

The presence of salt in the aqueous phase generally reduces the solubility of polar organic compounds and increases the partitioning coefficient between the organic solvent and water. Sodium chloride and ammonium sulfate were used to increase the ionic strength of the aqueous phase. Salt concentrations higher than 5% (w/v) prevented the sedimented phase from being discerned, and when different salt percentages lower than 5% (w/v) were assayed, the analytical signals for the studied compounds decreased slightly related to the absence of salt. Therefore, the addition of salt was discarded. The extraction time, defined as the time elapsing between the addition of dispersant and extraction solvents and the beginning of the centrifugation step, was varied between 20 s and 3 min and, as expected, no differences in sensitivity was attained for any of the studied compounds, confirming the DLLME characteristic of being time-independent, one of its most relevant advantages. Consequently, the minimum time necessary to form the cloudy solution (about 20 s) was adopted.

To evaluate the main factors affecting the efficiency of the DLLME step, a Plackett—Burman design was followed, using a statistical package to generate the experimental matrices and to evaluate the results. In Plackett—Burman designs the number of experiments is a multiple of four and exceeds by one the number of factors. In this study, the main effects of five factors [volume of extractant (1,2-dichloroethane) and dispersant solvent (ACN), sample mass, centrifugation time and centrifugation speed] were considered for the multivariate approach, each one being considered at two levels (low and high). The values corresponding to each factor level are reported in Table 1, as well as the 28 experiments carried out joined with four central points.

In order to identify the variables which significantly affected the response of the analytes, an analysis of variance at a 95% confidence level was carried out and the mean results obtained are represented by means of Pareto charts (Fig. 1), in which the length of the bars is proportional to the absolute value of the estimated effects. The dashed line represents 95% of the confidence interval and effects that cross this line are significant values with respect to the response. Each bar has a numerical value that also indicates the magnitude and whether the factor has a positive or negative effect on the response. The three analytes showed a similar behavior and Fig. 1 represents the results obtained for TDZ. A positive effect (from low to high values) was obtained for the five factors studied, the sample mass being the most significant factor for the three analytes, followed by the volume of extractant and dispersant solvents. According to the results, the time and speed of centrifugation, had no significant effect on the extraction efficiency and were consequently eliminated from further study, selecting in all cases the values corresponding to the maximum point: 3 min and 3000 rpm.

On the other hand, the volume of the extractant and dispersant solvents were significant parameters, so a more detailed study was carried out in order to achieve maximal efficiency. Thus, different volumes of 1,2-dichloroethane in the range 150—300 μL were assayed using volumes of ACN from 1 to 2.5 mL. The highest analytical signals were obtained with 1.5 mL of acetonitrile and 250 μL of extractant.

To check the performance of the procedure, sample masses of 2—7 g were submitted to the optimized extraction procedure. A sample mass of 5 g is recommended, since, as observed in Fig. 2, higher values did not lead to the corresponding increase in sensitivity. Under the conditions finally selected for analyte extraction from the sample matrices and for the DLLME preconcentration step, sedimented phases of about 200 μL were recovered. These were then evaporated and reconstituted in 50 μL of ACN, which corresponds to the minimum volume necessary to operate the autosampler using the vial microinserts.

Table 1
Factors, codes, levels in the Plackett—Burman design matrix.

Factors			Levels		
			Low (−1)		High (+1)
(F_1) Volume of 1,2-dichloroethane (μL)			150		250
(F_2) Volume of acetonitrile (mL)			1.0		2.0
(F_3) Centrifugation time (min)			1.0		3.0
(F_4) Centrifugation speed (rpm)			1000		3000
(F_5) Sample mass (g)			3		7
Runs	F_1	F_2	F_3	F_4	F_5
1	+1	+1	−1	+1	−1
2	0	0	0	0	0
3	−1	+1	−1	−1	−1
4	−1	−1	−1	−1	−1
5	−1	+1	+1	+1	−1
6	+1	+1	−1	+1	+1
7	+1	−1	+1	−1	−1
8	−1	−1	−1	+1	+1
9	−1	+1	+1	+1	−1
10	+1	+1	−1	+1	−1
11	0	0	0	0	0
12	0	0	0	0	0
13	−1	−1	−1	+1	+1
14	−1	−1	+1	+1	+1
15	−1	+1	−1	−1	−1
16	0	0	0	0	0
17	+1	−1	+1	−1	−1
18	−1	+1	+1	−1	+1
19	−1	+1	+1	−1	+1
20	−1	−1	−1	−1	−1
21	+1	−1	+1	+1	−1
22	+1	+1	+1	−1	+1
23	+1	+1	−1	+1	+1
24	−1	−1	+1	+1	+1
25	+1	−1	+1	+1	−1
26	+1	−1	−1	−1	+1
27	+1	−1	−1	−1	+1
28	+1	+1	+1	−1	+1

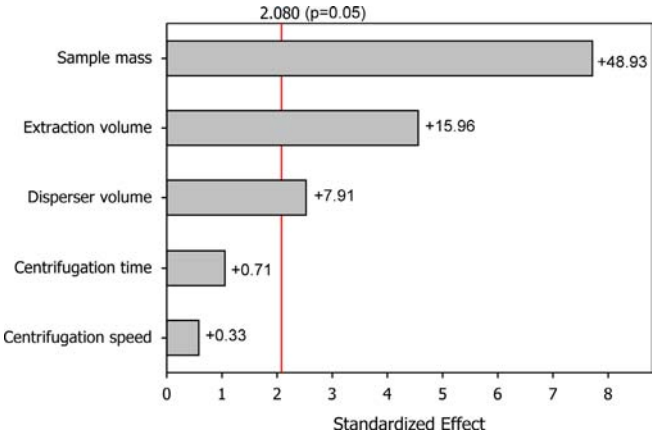

Fig. 1. Standarized main effect Pareto chart for the Plackett—Burman design. Vertical line in the chart defines 95% confidence level.

On the other hand, when the sample extraction and DLLME preconcentration step had been optimized, the sensitivities achieved for the studied compounds using TOFMS and an ion trap analyzers (IT-MS) were compared. Higher sensitivity was obtained for all the compounds using TOFMS both for aqueous standard solutions and a watermelon sample. The slopes were 10—27 fold and 30—45 fold higher for aqueous standard calibration graphs and standard additions, respectively, when using TOFMS compared with IT-MS. Therefore, TOFMS was selected.

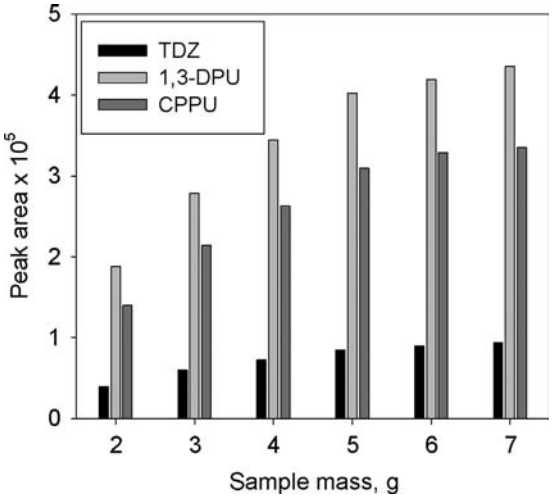

Fig. 2. Influence of sample mass on the sensitivity of the procedure.

Table 2
Slopes[a] of standard additions calibration graphs (mL n g−[1]).

Sample	TDZ	1,3-DPU	CPPU
Aqueous	845,805 ± 82,775	2,469,689 ± 92,239	2,563,891 ± 32,164
Kiwifruit	491,555 ± 38,834	2,249,110 ± 59,961	1,650,394 ± 48,663
Watermelon	496,936 ± 26,658	2,317,880 ± 61,529	1,761,177 ± 66,564
Melon	475,809 ± 37,210	2,297,084 ± 57,876	1,765,323 ± 86,404
Grape	505,849 ± 40,530	2,285,966 ± 36,090	1,691,227 ± 97,953
Tomato	482,877 ± 36,389	2,289,127 ± 37,978	1,677,055 ± 73,157

[a] Values are mean ± SD (n=6).

3.4. Analytical characteristics of the method

To study the relevance of any matrix effect, an ANOVA test was used to compare the slopes of aqueous standards calibration graphs and those obtained when the standard additions method was applied to the different types of sample studied (Table 2). The presence of a matrix effect was confirmed because "p" values lower than 0.05 were obtained for the three analytes in the five samples. Nevertheless, no significant differences were observed by the ANOVA test between the slopes obtained when the standard additions method was applied to different samples (p values ranging between 0.051 and 0.645) (Table 2). Consequently, matrix-matched calibration standard curves were used to quantify the target analytes, being prepared in the concentration ranges of 0.2—20 ng g^{-1} for 1,3-DPU and CPPU and 0.5—50 ng g^{-1} for TDZ. The correlation coefficients obtained under the selected conditions showed a directly proportional relationship between the amount of analyte extracted and its concentration in the sample extraction solution, being in all cases higher than 0.995.

Detection limits (DLs) and quantification limits (QLs) were calculated on the basis of three and 10 times the standard deviation of the intercept of the calibration graphs obtained by matrix-matched standards. The data obtained for DLs appear in Table 3, QLs of about 0.17, 0.07 and 0.12 ng g^{-1}, were obtained for TDZ, 1,3-DPU and CPPU, respectively. The repeatability was calculated using the relative standard deviation for 10 successive analyses of a watermelon sample fortified at a concentration level of 0.5 ng g^{-1} for all compounds (Table 3). The slopes obtained by aqueous calibration by means of DLLME—LC—TOFMS pointed to an increase in sensitivity compared with those obtained in the absence of a preconcentration step, of between 50 and 130-fold for TDZ and CPPU, respectively. Certainly, the developed procedure showed higher sensitivity than those previously reported for the

Table 3
Analytical characteristics of the proposed method.

Compound	Correlation coefficient	DL[a] (ng g^{-1})	RSD[b] (%)
TDZ	0.9920	0.050	11.5
1,3-DPU	0.9938	0.020	7.2
CPPU	0.9953	0.035	8.5

[a] Using the standard deviation of the ordinate.
[b] Calculated at a concentration level of 0.5 ng g^{-1} for each compound ($n=10$).

Table 4
Recoveries[a] from different samples.

Compound	Spike level (ng g^{-1})	Kiwifruit	Watermelon	Grape	Tomato
TDZ	0.5	94 ± 6	98 ± 5	93 ± 6	93 ± 4
	1.0	96 ± 3	105 ± 4	97 ± 4	96 ± 4
	5.0	89 ± 7	91 ± 6	101 ± 5	91 ± 6
1,3-DPU	0.2	92 ± 5	90 ± 5	95 ± 5	90 ± 5
	0.5	101 ± 4	97 ± 4	103 ± 5	92 ± 4
	5.0	96 ± 7	92 ± 5	90 ± 7	98 ± 3
CPPU	0.2	90 ± 6	92 ± 5	90 ± 6	102 ± 5
	0.5	104 ± 4	95 ± 4	105 ± 3	106 ± 3
	1.0	93 ± 5	97 ± 3	95 ± 4	98 ± 4

[a] Mean value ± standard deviation ($n=3$).

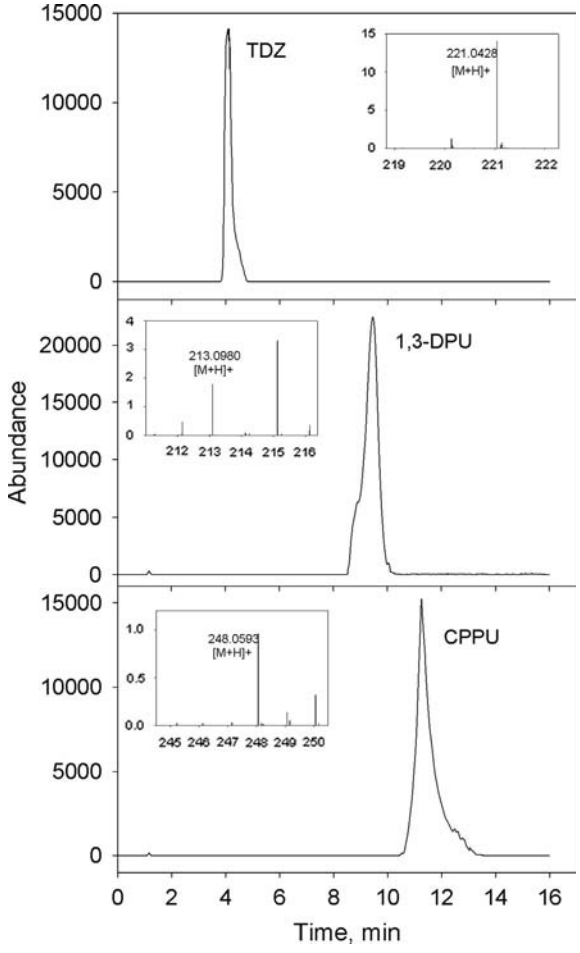

Fig. 3. Extracted ion chromatograms by DLLME—LC—ESI—TOF-MS showing the spectra of compounds obtained from a kiwifruit sample fortified with a standard mixture at a concentration level of 3.0 ng g^{-1} for TDZ and 0.3 ng g^{-1} for 1,3-DPU and CPPU.

analysis of fruit and vegetable samples using MS/MS [17—21] or TOFMS [22,23] as detection systems for LC.

3.5. Analysis of samples and validation of the method

The optimized procedure was applied to a total of 15 different samples corresponding to five different types of fruit. The analyzed samples did not contain the studied compounds, at least above their corresponding detection limits.

Recovery studies were carried out in order to verify the accuracy of the proposed method by fortifying four samples (kiwifruit, watermelon, grape and tomato) in triplicate at three concentration levels in the range 0.2—1.0 ng g^{-1}, depending on the compound. The results obtained appear in Table 4, being in accordance with the validation criteria established for pesticide residue analysis [31]. An average recovery ± standard deviation ($n=108$) of 96 ± 5 was obtained. No differences in the RSD values between the four different matrices were obtained, ranging between 1% and 8%.

The accurate masses were obtained for all the standards and spiked samples analyzed in the recovery studies. The mass error was calculated by using as reference the mass values of 220.0419, 212.0950 and 247.0512m/z for TDZ, 1,3-DPU and CPPU, respectively, which were given by the TOF software. The errors obtained were for all the standards and fortified samples between −1.8 and 0.3 ppm for TDZ, −2.8 and 2.4 ppm for 1,3-DPU, and between −4.7 and −0.1 ppm for CPPU. These values were in all cases lower than the accepted accuracy threshold of 5 ppm for confirming elemental composition [31].

Fig. 3 shows the DLLME—LC—ESI—TOFMS elution profiles as well as the mass spectra obtained from a kiwifruit sample spiked with a standard mixture of the analytes, in the selected conditions.

4. Conclusion

This is the first time that TDZ, CPPU and 1,3-DPU have been determined simultaneously. A simple extraction of the analytes from the sample matrix using a low volume of organic solvent and preconcentration of the extract by the environmentally friendly miniaturized technique DLLME in conjunction LC with the sensitive TOFMS detection system, permit quantification of the analytes with low detection limits. Moreover, the selectivity of the detection system used provided unequivocal identification of the individual analytes based on accurate mass measurements. The analytical characteristics of the proposed method make it a useful tool for the routine monitoring of the three phenylurea CKs in fruit samples

Acknowledgments

The authors acknowledge to the Comunidad Autónoma de la Región de Murcia (CARM, Fundación Séneca, Project 15217/PI/10) and the Spanish MEC (CTQ2012-34722) for financial support. G. Férez-Melgarejo acknowledges a fellowship financed by CARM.

References

[1] P.J.J. Hooykass, M.A. Hall, K.R. Libbenga, Biochemistry and Molecular Biology of Plant Hormones, Elsevier, Amsterdam, 1999.
[2] G.L. Gambino, P. Pagano, M. Scordino, L. Sabatino, E. Scollo, P. Traulo, G. Gagliano, J. AOAC Int. 91 (2008) 1245–1256.
[3] Regulation (EC) no. 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. Available from: ⟨http://europa.eu/legislation_summaries/food_safety/plant_health_checks/sa0016_en.htm⟩ (accessed 07.02.13).

[4] A. Heyl, M. Riefler, G.A. Romanov, T. Schmülling, Eur. J. Cell Biol. 91 (2012) 246–256.
[5] D.W.S. Mok, M.C. Mok, Annu. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 89–118.
[6] United States Environmental Protection Agency. Available from: ⟨http://www.epa.gov/opp00001/chem_search/reg_actions/registration/fs_PC-128819_01-Sep-04.pdf⟩ (accessed 07.02.13).
[7] EU Commission Directive 2006/10/EC of 27 January 2006, amending Council Directive 91/414/EC to include forchlorfenuron and indoxacarb as active substances. Off. J. Eur. Union L25 (2010) p. 24.
[8] T.L. Potter, L. Marti, S. Belflower, C.C. Truman, J. Agric. Food Chem. 48 (2000) 4103–4108.
[9] United States Environmental Protection Agency. Available from: ⟨http://www.epa.gov/oppsrrd1/REDs/thidiazuron_factsheet.pdf⟩ (accessed 07.02.13).
[10] F. Kaczyna, R. Megnet, Hydrobiologia 268 (1993) 57–64.
[11] A. Piotrowska, R. Czerpak, J. Adamowicz, A. Biedrzycka, M. Potocka, Acta Soc. Bot. Pol. 74 (2005) 111–118.
[12] C. Suárez-Pantaleón, J.V. Mercader, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes, J. Agric. Food Chem. 58 (2010) 8502–8511.
[13] C. Suárez-Pantaleón, F.A. Esteve-Turrillas, J.V. Mercader, C. Agulló, A. Abad-Somovilla, A. Abad-Fuentes, Anal. Bioanal. Chem. 403 (2012) 2019–2026.
[14] D. Sharma, M.D. Awasthi, Chemosphere 50 (2003) 589–594.
[15] J.Y. Hu, J.Z. Li, J. AOAC Int. 89 (2006) 1635–1640.
[16] M. Kobayashi, I. Takano, Y. Tamura, S. Tomizawa, Y. Tateishi, N. Sakai, K. Kamijo, A. Ibe, T. Nagayama, Food Hyg. Soc. Jpn. 48 (2007) 148–152.
[17] X. Shi, F. Jin, Y. Huang, X. Du, C. Li, M. Wang, H. Shao, M. Jin, J. Wang, J. Agric. Food Chem. 60 (2012) 60–65.
[18] J. Wang, D. Wotherspoon, J. AOAC Int. 90 (2007) 550–567.
[19] A. Valverde, L. Piedra, A. Aguilera, M. Boulaid, F. Camacho, J. Environ. Sci. Health B 42 (2007) 801–807.
[20] K. Banerjee, D.P. Oulkar, S. Dasgupta, S.B. Patil, S.H. Patil, R. Savant, P.G. Adsule, J. Chromatogr. A 1173 (2007) 98–109.
[21] D.P. Oulkar, K. Banerjee, M.S. Ghaste, S.D. Ramteke, D.G. Naik, S.B. Patil, M.R. Jadhav, P.G. Adsule, J. AOAC Int. 94 (2011) 968–977.
[22] A. Valverde, A. Aguilera, C. Ferrer, F. Camacho, A. Cammarano, J. Agric. Food Chem. 58 (2010) 2818–2823.
[23] D.P. Oulkar, K. Banerjee, S. Kulkarni, J. AOAC Int. 94 (2011) 1715–1721.
[24] J. Hu, Y. Hu, Y. Chen, T. Yang, Bull. Environ. Contam. Toxicol. 87 (2011) 448–451.
[25] Y. Li, J.E. George, C.L. McCarty, S.C. Wendelken, J. Chromatogr. A 1134 (2006) 170–176.
[26] M. Saraji, N. Tansazan, J. Sep. Sci. 32 (2009) 4186–4192.
[27] M. Rezaee, Y. Assadi, M.R.M. Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, J. Chromatogr. A 1116 (2006) 1–9.
[28] C. Bosch Ojeda, F. Sánchez Rojas, Chromatographia 69 (2009) 1149–1159.
[29] M. Rezaee, Y. Yamini, M. Faraji, J. Chromatogr. A 1217 (2010) 2342–2357.
[30] M. Asensio-Ramos, L.M. Ravelo-Perez, M.A. González-Curbelo, J. Hernández-Borges, J. Chromatogr. A 1218 (2011) 7415–7437.
[31] European Commission, Directorate General Health and Consumer Protection, Document SANCO/10684/2009, implemented in January 2010, Method validation and quality control procedures for pesticide residue analysis in food and feed.