TRACE ANALYSIS OF FLUBENDIAMIDE IN BEE POLLEN USING ENHANCED MATRIX REMOVAL-LIPID SORBENT CLEAN-UP AND LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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Abstract

In this work, a new method has been proposed with the aim of determining flubendiamide, a recently commercialized insecticide, in bee pollen by using liquid chromatography coupled to electrospray ionization mass spectrometry. For this purpose, a novel sample treatment has been proposed that has proven efficient in terms of recovery (average analyte recoveries were between 90% and 102%) and absence of matrix effect, and one which is effective, fast and selective. This involved a solvent extraction using an acetonitrile and water mixture, and a clean-up stage where, in addition to freezing, an enhanced matrix removal-lipid sorbent was successfully used for the first time with this matrix and analyte. The chromatographic conditions were also optimized, by selecting a C_{18} based column (Gemini® C_{18}) and acetic acid (1 mM) in water and methanol as mobile phase components, allowing elution of flubendiamide in less than 4 minutes, with a total analysis time of 14 min. Validation was carried out, with the result that all the parameters studied complied with existing European legislation. It should be noted that the sensitivity of the method was excellent, with a quantification limit (4 µg/kg) well below the maximum residue level established for this insecticide in bee products (50 µg/kg). Finally, several bee pollen samples were analyzed, and flubendiamide residues were not found in any of the cases.

Keywords: Bee pollen; Electrospray ionization mass spectrometry; EMR-Lipid sorbent; Flubendiamide; Insecticides; Liquid chromatography.

1. Introduction

For centuries, bee pollen has been included in the human diet due to its nutritional and health-promoting properties related to the presence of several bioactive compounds (amino acids, vitamins, proteins and lipids, by way of example). Currently it is one of the most common food supplements [1,2]. However, in recent years, several studies have detected contaminants such as pesticides, heavy metals or antibiotics in bee products, for instance, pollen, which can compromise the quality of these products and affect the consumer's health; this has, therefore, led to a deterioration of their healthy image [3,6]. In the present study, attention is focused on the insecticide flubendiamide (see Supplementary Material, Figure 1S), which is a relatively new type of systemic insecticide belonging to the chemical class of benzenedicarboxamides or diamides of phthalic acid [7]. It is a compound which is extremely effective, especially against lepidopteron pests [8], and relatively safe for non-target organisms [9]. The use of this insecticide is currently registered for more than 200 different crops worldwide [10]. In the European Union its use was approved in 2014 for a period of 10 years, and it is currently authorized in Cyprus, Denmark and the Netherlands [11]. At present, however, some countries like China [5] and the United States [12] are reviewing their registration or cancelling the commercialization of flubendiamide because it represents a potential risk for aquatic invertebrates. It is clear that the monitoring of flubendiamide residues in foods, and in bee pollen in particular, has become a matter of public and sanitary interest in terms of ascertaining its presence, which could be of special concern in those countries that have cancelled its registration. In addition, the maximum residue level (MRL) established for this substance in bee products by the European legislation, (50 μg/kg; [13]) requires selective and sensitive analytical methodologies to be developed.

As far as we know, the determination of flubendiamide in bee pollen has been only once previously carried out [14]. In this work, 253 pesticides were examined using a as sample preparation a method based on a quick, easy, cheap, effective, rugged and safe (QuEChERS) procedure; liquid (LC) and gas chromatography (GC) with tandem mode mass detection (MS/MS) were used in both cases. Moreover, there is only one study in which this insecticide was determined in other apicultural products [3]. In the present paper, flubendiamide was analyzed in honey using solid phase extraction with C_{18} cartridges and LC-MS/MS. As it may be appreciated, an analysis of flubendiamide in apicultural matrices has been the object of scant attention, although it has been studied more frequently in other food matrices, mainly vegetables (for instance, cabbage, tomato or cucumber) and fruits [10,15-23]. A sample treatment previously described by Battu et al. [15], which mainly consisted of an extraction with acetonitrile, a partition with chloroform; and a treatment with active carbon, was the basis of several of those publications [10,15,19,22,23]. However, in recent years the QuEChERS method, which fulfils several of the requirements of the green analytical chemistry (simplicity, reduced cost, time and number/amount of reagents; [24]), has been also widely used to determine insecticides, including flubendiamide, in foods [14,16-18,20,21]. In view of the small number of publications relating to apicultural products in the literature, and our recent experience in analyzing pesticides in beeswax [25], it was deemed appropriate to consider as an alternative to the only method proposed (QuEChERS; [14]) an enhanced matrix removal-lipid (EMR-lipid) sorbent for an effective clean-up of the bee pollen extracts obtained following solvent extraction. It was composed of C_{18} and some special kinds of polymers, although specific details of the structure of EMR-Lipid are still a trade secret [25,26]. This sorbent electively interacts with the

unbranched hydrocarbon chains of lipids, one of the main constituents of bee pollen (~ 25%; [1]), without unwanted analyte loss, providing high matrix cleanup with high analytes recovery and precision [27,28].

Moreover, the related bibliography suggests that the most common technique for determining flubendiamide is LC [3,14,16-18,20,21]. Separations, meanwhile, were carried out mainly using C_{18}-based analytical columns and mixtures of water and acetonitrile in different proportions as mobile phases, although in certain cases C_8 fillings, and acetonitrile and formic acid [24] or ammonium acetate with methanol [18] mobile phases were also used. Regarding the detection systems employed, numerous articles report the use of UV-Vis or diode array detectors [10,15,16,19-23], although nowadays, due to requirements regarding sensitivity and selectivity proposed by international regulatory agencies, mass spectrometry (MS) detectors are gaining attention [3,16,14,17,18,20]. In our case, C_{18}-based columns and a LC-MS system were used in all experiments.

Therefore, the main goal was to propose and validate a specific method for determining flubendiamide residues in bee pollen using LC-MS. Extraction, clean-up and determination procedures have been proposed and optimized in terms of specificity and efficiency, with the further aim of reducing the matrix effect as much as possible and avoiding potential stability problems observed during sample extraction when applying a multi-residue approach [29]. As far as we know, this has not been previously done for flubendiamide in bee pollen. In addition, there has been no report to date of the use of EMR-lipid sorbent with this insecticide or in bee pollen. Further goals of this work focused on studying the most relevant validation parameters in accordance with current

European legislation [30], as well as checking the potential presence of flubendiamide in several bee pollen samples.

2. Materials and methods

2.1. Chemicals

Flubendiamide (Det. Purity 98.2%) was obtained from Sigma-Aldrich (Madrid, Spain). Acetonitrile and methanol (LC grade) were purchased from Panreac (Barcelona, Spain). Ammonium formate, ammonium hydroxide, sodium chloride, formic acid and acetic acid were supplied by Sigma Aldrich Chemie Gbmh (Steinheim, Germany); meanwhile, ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA, USA).

2.2. Standards

Standard stock (1000 mg/L) and working solutions were prepared in methanol. Bee pollen samples (0.5 g), in which the absence of flubendiamide was previously confirmed using LC-MS, were spiked with variable amounts of flubendiamide before (BF samples) or after (AF samples) sample treatment. Those samples were used for validation studies and to prepare the quality control (QC) samples. Each QC sample was prepared with 0.5 g of bee pollen spiked with three different concentrations of flubendiamide within the linear range: low concentration- 4 µg/kg; medium concentration- 40 µg/kg; high concentration- 400 µg/kg. The stock solution was stored in glass containers in darkness at - 20ºC; working and matrix-matched solutions were stored in glass containers and kept in the dark at 4ºC. All solutions remained stable under these conditions for over two weeks. The stability of those standards was checked by preparing a new stock standard and comparing the detector responses. The means

from six replicates for each of two solutions (old and new) differed less than 10% (data not shown) as specified by the European Commission [30].

2.3. Sample treatment

Commercial bee pollen (n = 8), which were from Spanish regions where flubendiamide has been used in some cultivars, were obtained from local markets (Valladolid, Spain). Those samples were mixed and dried at 45ºC in an oven, ground and pooled for optimum sample homogeneity, and subsequently stored in darkness at 4ºC. After the above-mentioned procedure, a subsample (0.5 g) was weighed in a 50 mL centrifuge tube, and then 15 mL of an acetonitrile and water mixture (70:30, v/v) was added, the mixture was mechanically shaken for 5 min at 960 oscillations per min in a vibromatic mechanical shaker (J.P. Selecta S.A., Barcelona, Spain). Then, 1.0 g of sodium chloride was added to the centrifuge tube and the content was centrifuged for 5 min at 5°C and 10000 rpm (5810R Eppendorf centrifuge, Hamburg, Germany). Following this, the centrifugation tubes were left to cool in a polystyrene box filled with dry ice for 4 min. The upper phase was then collected and transferred to a different 50 mL centrifuge tube in which the content of the EMR-Lipid tube (15 mL, 1 g; Agilent Technologies, Palo Alto, CA, USA) had been previously added. It was shaken for 30 s in a vortex device (Heidolph, Schwabach, Germany) and centrifuged (5 min; 5°C; 10000 rpm). Finally, the supernatant was transferred to a 25 mL conical flask and evaporated to dryness in a rotary evaporator (R-210/215 Buchi, Flawil, Switzerland) at 60°C. The dry extract was reconstituted with 2 mL of methanol and the resulting solution was passed through a 0.45 μm nylon filter (Nalgene, Rochester, NY, USA), before injection into the LC–MS system (see scheme in Figure 1).

2.4. LC-MS conditions

It was used an Agilent Technologies 1100 LC coupled to a MS detector (single quadrupole) equipped with an electrospray ionization (ESI) source. A Gemini® C_{18} (3 µm; 110 Å, 30 × 4.6 mm; Phenomenex, Torrance, CA, USA) analytical and guard column, which was protected by a guard column (Gemini® C_{18}, Phenomenex), was used for separation at 30ºC. The mobile phase, which was applied in gradient elution mode, consists of acetic acid (1 mM) in water and methanol at a flow rate of 0.5 mL/min (see Table 1). Injection volume was set at 2 µL. Regarding selection of the MS conditions, ESI in negative mode was chosen according to the existing literature [3,16,14,17,18,20]. Several experiments (flow injection analysis) were performed with the aim of selecting the optimum MS parameters by analyzing solvent based and matrix-matched standards. The studied ranges and optimal values for each parameter of ESI are summarized in Table 2. Full-scan spectra were obtained by scanning from m/z 100 to 800, and flubendiamide showed an intense ion (*m/z* 254.1), which was used for quantifying with the selected ion monitoring mode (SIM) mode; meanwhile, two other ions with the highest signals (*m/z* 681.2 $[M-H]^-$; 274.2) were used for confirmation (see Figure 2). These ions have been usually employed in the above-mentioned studies.

3. Results and discussion

3.1. Sample treatment

One of the main goals was to propose an alternative procedure to the only previous publication [14] for an efficient and simple extraction of flubendiamide from bee pollen. Firstly, then, in terms of selecting the extraction solvent, it can be seen in the related literature that acetonitrile and acetonitrile and water mixtures have mainly been chosen to extract flubendiamide (see Introduction). Consequently, after testing some acetonitrile and

water (100:0; 70:30; 50:50, 30: 70, v/v) mixtures, a 70:30, v/v mix (see Figure 3) was selected as, in the other cases, recoveries were lower than 70%. Once the extractant mixture was selected, optimal conditions were ensured by sequentially testing the amounts of bee pollen (0.1-1.0 g) and sodium chloride (0.5-1.5 g), volume (5-20 mL), extraction and centrifugation times (1-10 min). Sodium chloride (salting-out) was used to facilitate the passing of flubendiamide to the organic phase as it provided good results according to a recent publication [31]. The amount of bee pollen (0.5 g) selected as the optimal value was the result of sensitivity and extraction efficiency (data not shown). The best rates of extraction efficiency for 0.5 g of bee pollen (recovery percentages > 90%; see Figure 3) were achieved when using the conditions summarized in Figure 1. However, a strong matrix effect was also observed (> 40% of signal suppression) that affected analyte ionization, which could be due to the presence of bee pollen constituents, especially the principal ones (proteins and lipids; [1]). A freezing step was then introduced in order to precipitate some of the bee pollen components [14,25], but this was not enough for removing some matrix components reducing flubendiamide ionization, such as lipids; this resulted in a significant signal suppression, (> 30%). Therefore, a recently commercialized sorbent (EMR-lipid), which was successfully employed for removing lipids without losing analytes in other bee matrices like honey bees and beeswax [25,32] was tested for cleaning-up the sample with the aim of reducing as much as possible the signal suppression. In this case, due to the amount of extractant being greater than the capacity of the tube, the EMR-lipid sorbent was transferred from the original to the centrifuge tubes. Thus, the organic phase was collected after the cooling step, transferred to the 50 mL centrifuge tube, containing 1 g of EMR-lipid sorbent, and centrifuged. The extraction efficiency continued to be good (recoveries > 90%), but more importantly, the matrix effect was drastically reduced (< 10% in all cases), demonstrating the suitability of EMR-

lipid sorbent for the clean-up of complex samples. After evaporation of the supernatant, different amounts of methanol (1.0-3.0 mL), which was selected on the basis of a previous study [3], were tested in order to obtain the best means of reconstituting the dry extract. According to the recovery rates (data not shown), 2 mL was considered appropriate.

Finally, the overall performance of the proposed sample treatment has demonstrated that it can be considered an efficient and simpler alternative to the QuEChERS proposal, as recoveries (average values were between 90%-102%; see Table 3), matrix effect, overall procedure time (< 30 minutes), and number of steps and chemicals required are similar to or even better than the reported values (80%-84%; < 30 min; the requirement of solid-phase extraction and matrix-matched calibration curves; [14]). Indeed, the absence of a significant matrix effect is a relevant finding and an important advantage in comparison with the only previous study [14], as it implies that reference standard in solvents could be used for quantifying flubendiamide instead of matrix-matched samples. This observation could be directly related to the pioneer use of EMR-Lipid sorbents with this matrix or compound. It can also be remarked that the selected sample treatment can also be considered as environment-friendly in relation to the organic solvent consumption. Although the amount is higher in the present study (12.5 mL) than in the previous research (4 mL; [14]), it is much lower than the amounts required when solvent extraction is employed to determine flubendiamide in other food matrices (> 30 mL), and comparable to that of other QuEChERS studies [10,15-23].

3.2. Chromatographic conditions

A more economical detector (single quadrupole) instead of the one used previously (quadrupole-time of flight; [3]) was used in this research in order to make it possible for

the method to be widely employed, as MS/MS detectors are not easily affordable. It was, therefore, necessary to modify the chromatographic conditions. Firstly, tests were made of two columns with the same stationary phase (Gemini® C_{18}) as in our previous study but different in terms of size (length and internal diameter: 50 x 2.0; 30 x 2.0 mm). An examination of the chromatograms showed that in both cases analysis times were relatively short (< 5 minutes), although slightly longer for the 50 mm column; meanwhile, when the 30 mm column was used peak areas were 20% larger, peak height values were almost double, and peak shapes were also enhanced. Therefore, the latter column was considered appropriate for continuing the optimization procedure. As regards the mobile phase components, acetonitrile and water have generally been employed when determining flubendiamide in food matrices, but in most cases, this has been by means of UV-Vis or diode array detectors (see Introduction). In the present study, ESI applied in negative mode was employed in the LC-MS analyses; here, the use of methanol as an organic modifier is recommended not only because previous studies have shown that this solvent is more compatible with the environment than other compounds (for instance, acetonitrile, formaldehyde or tetrahydrofuran) [33], but also due to the greater effectiveness reported when ionizing molecules if ESI is employed in negative mode [34]. In addition, the use of weak acids, and specifically acetic acid at lower concentrations (~ 1 mM), has been suggested as a mobile phase component since this acid fulfils many of the requirements of a good modifier when ESI has been operated in negative mode [35]. The fastest analysis was achieved with the conditions previously stated (see subsection 2.4.1), which demonstrated the validity of the above-mentioned claims, as the best chromatographic performance (retention time, area, peak height, symmetry; data not shown) was obtained when using methanol and acetic acid (1 mM) in water as mobile phase components. Flubendiamide eluted in less than 4 min (see Figure 4) and the overall run time was 14

minutes. To our knowledge, this is the only LC method that has been reported regarding specific analysis of flubendiamide in bee pollen, as a result of which it is not possible to compare the results; for instance, in terms of the number of compounds or the matrix having a great influence on separation. Finally, it is also interesting to mention that the proposed conditions could be used with other detectors (diode array or ultraviolet detectors) as flubendiamide was baseline separated from matrix components.

3.3. Method validation

The validation study was performed based on the current European legislation (SANTE guidelines; [30]). The criteria include selectivity, limits of detection (LODs) and quantification (LOQs), matrix effect, linearity, precision and trueness. It should be commented that some of the main elements of uncertainty (sample amount, recovery and precision; [36]) were considered.

3.3.1. Selectivity

It was initially assessed by comparing the chromatograms of non-spiked samples (n=6) with those obtained for spiked samples. The lack of co-eluting peaks at the analyte retention time (3.5 min; see Figure 4) or in the close vicinity indicated that no interfering compounds with significant influence were present. In addition, it was observed a high similarity between the flubendiamide mass spectra in solvent based and matrix-matched standards (see Figure 2), especially for the tree ions selected for quantification and confirmation purposes as recommended by the European legislation [30].

3.3.2. Limits of detection and quantification

The LOD (1 µg/kg) and LOQ (4 µg/kg) were experimentally estimated to be three and ten times the signal-to-noise ratio, respectively. The LOQ value of the method is more than ten times lower than the established MRL value (50 µg/kg; [13]), and also slightly

better than LOQs previously reported using LC-MS/MS (5 μg/kg; [14]). This is a significant result and a potential advantage, as the LOQ was obtained with a MS detector (single quadrupole) and not with a MS/MS detector, which unfortunately are not affordable in all analytical laboratories.

3.3.3. Matrix effect

The responses (flubendiamide peak areas) obtained in solvent based standard solutions and QC samples, which were spiked after treatment (AF samples) were compared with the aim of evaluating the potential influence of the matrix on flubenidamide signal. The responses were comprised between 94% and 105% in all cases (see Table 3), which fulfilled the criteria of the European Commission (± 20% of the response from standard solutions; [30]), and implies that the matrix did not significantly affect the ESI-MS signal as it was previously discussed in subsection 3.1.

3.3.4. Linearity

As it has been above-mentioned, reference standard in solvents can be used to quantify flubendiamide due to the lack of a significant matrix effect. This was also confirmed by the overlapping at the confidence intervals of the slopes from the solvent and matrix-matched standard calibration curves (762808 ± 46202, solvent; 739860 ± 61965, matrix-matched). The linearity of the instrument response was achieved by evaluating the coefficient of determination (R^2) and the residuals of the matrix-matched calibration curves established by seven concentration levels (LOQ, 5, 10, 25, 50, 75, 100 μg/L) that correspond to LOQ and 400 μg/kg (LOQ, 20, 40, 100, 200, 300, 400 μg/kg) in samples, in line with the proposed sample treatment and unit conversion. The coefficient of the determination values (R^2) was above 0.99 in all cases (0.997, solvent; 0.994, matrix-matched). Moreover, the relative standard deviation (%RSD) of the residuals was lower than 7% in all cases (data not shown). This was also verified with the deviation of back-

calculation concentration from true concentration, which was lower than 20% (data not shown) as specified by the European Commission [30].

3.3.5. Precision

Precision, which was expressed as relative standard deviation (%RSD), was evaluated through repeated sample analysis using blank bee pollen samples spiked before sample treatment (BF samples) at three different concentration levels (QC samples), either on the same day of (n=6) (intra-day precision experiments), or over three consecutive days (n=6) (inter-day precision). The obtained %RSD values were below 8% in all cases (see Table 3), which are lower than the values reported in the only study in which flubendiamide was investigated in bee pollen (< 14%; [14]). Finally, it must be remarked that those values are consistent with the current European legislation (%RSD ≤ 20; [30]).

3.3.6. Trueness

This was evaluated by the mean recoveries (as a measure of trueness), calculated by comparing the results (analyte peak area) obtained from the different QC samples (AF or BF). Recoveries ranged from 90% to 102% with %RSD values lower than 8% in all cases (see Table 3). These values fulfilled the requirements established by the European Commission [30] (recovery percentages between 70% and 120%; %RSD ≤ 20), and are better than the recoveries obtained in previous work (< 85%; [14]).

3.4. Application of the method

Eight bee pollen samples (see Subsection 2.3) were analyzed, and flubendiamide residues were not found in any of them, as was the case in the previous study in which flubendiamide was investigated in this matrix [14]. Perhaps this absence of residues could be explained by reasons such as a potential lack of stability in bee pollen or the

physical-chemical characteristics of flubendiamide, especially its solubility and mobility (see Supplementary Material, Figure 1S). As regards of the stability issue, despite the fact that we searched for the ions of its metabolite (desiodo-flubendiamide; 555, 274, 254) that were listed in a previous publication [16], none were detected. Neither was it possible to obtain the metabolite standard after contacting several manufacturers and other researchers, and for that reason the method was only developed and optimized for flubendiamide.

4. Conclusions

A new LC-MS based method for specifically determining flubendiamide in bee pollen has been developed, optimized and validated. A sample treatment has been proposed that has proven to be fast, efficient and one that involves a relatively low consumption of organic solvents (12.5 mL), as recommended by green analytical chemistry. Moreover, the performance of this treatment has been compared with the only existing proposal where flubendiamide was determined in pollen by means of a multi-residue approach. As a result, it can be concluded that although the consumption of solvents is higher in the present study, it is simpler (few steps), requires a smaller number of reagents, and there is an absence of matrix effect; this implies that flubendiamide may be quantified with solvent-based standards. This finding could be directly linked to the pioneer use with this insecticide or matrix of EMR-Lipid sorbents. LC analysis was performed with a C_{18} column (Gemini®), the dimensions of which were selected after two different columns were studied. Meanwhile, the mobile phase composition, which consisted of methanol and acetic acid applied in gradient elution mode, was also optimized in order to obtain the fastest chromatographic run while avoiding coelution with matrix components and maximum effectiveness when ionizing flubendiamide. The

proposed method was validated according to current European legislation, and has been shown to be selective, precise and to allow for a wide range of linearity. In addition, the LOQ obtained is much lower than the established MRLs and slightly better than the only LOQ value previously reported, using a cheaper and simpler MS detector. Eight samples from different Spanish regions were analyzed with the proposed methodology and flubenidamide residues were not detected in any of them. To conclude, this method demonstrates the suitability of EMR-lipid sorbents for performing an effective clean-up in a complex matrix (bee pollen). Finally, our study has shown some of the potential advantages of developing specific approaches instead of multi-residue methods, such as the absence of matrix effect or greater precision and extraction efficiency.

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Conflict of interest statement

The authors of this manuscript declare no conflict of interest.

Abbreviations:

AF, samples spiked after sample treatment; **BF**, samples spiked before sample treatment; **ESI,** electrospray ionization ion; **EMR**, enhanced matrix removal-lipid; **MRL,** maximum residue level; **QC,** quality control; **QuEChERS**, quick, easy, cheap, effective, rugged and safe; **RSD**, relative standard deviation; **SIM,** selected ion monitoring.

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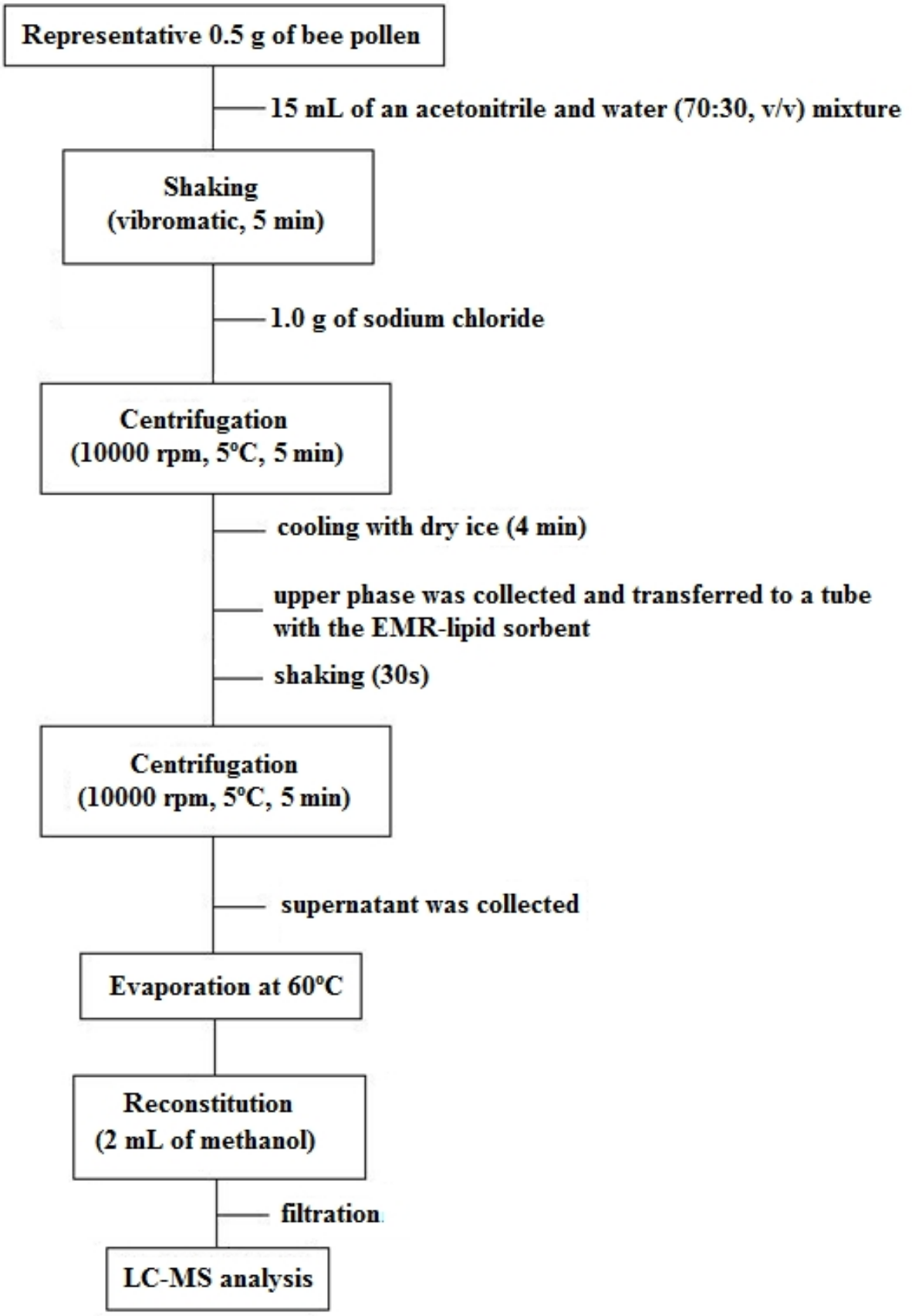
Figure captions

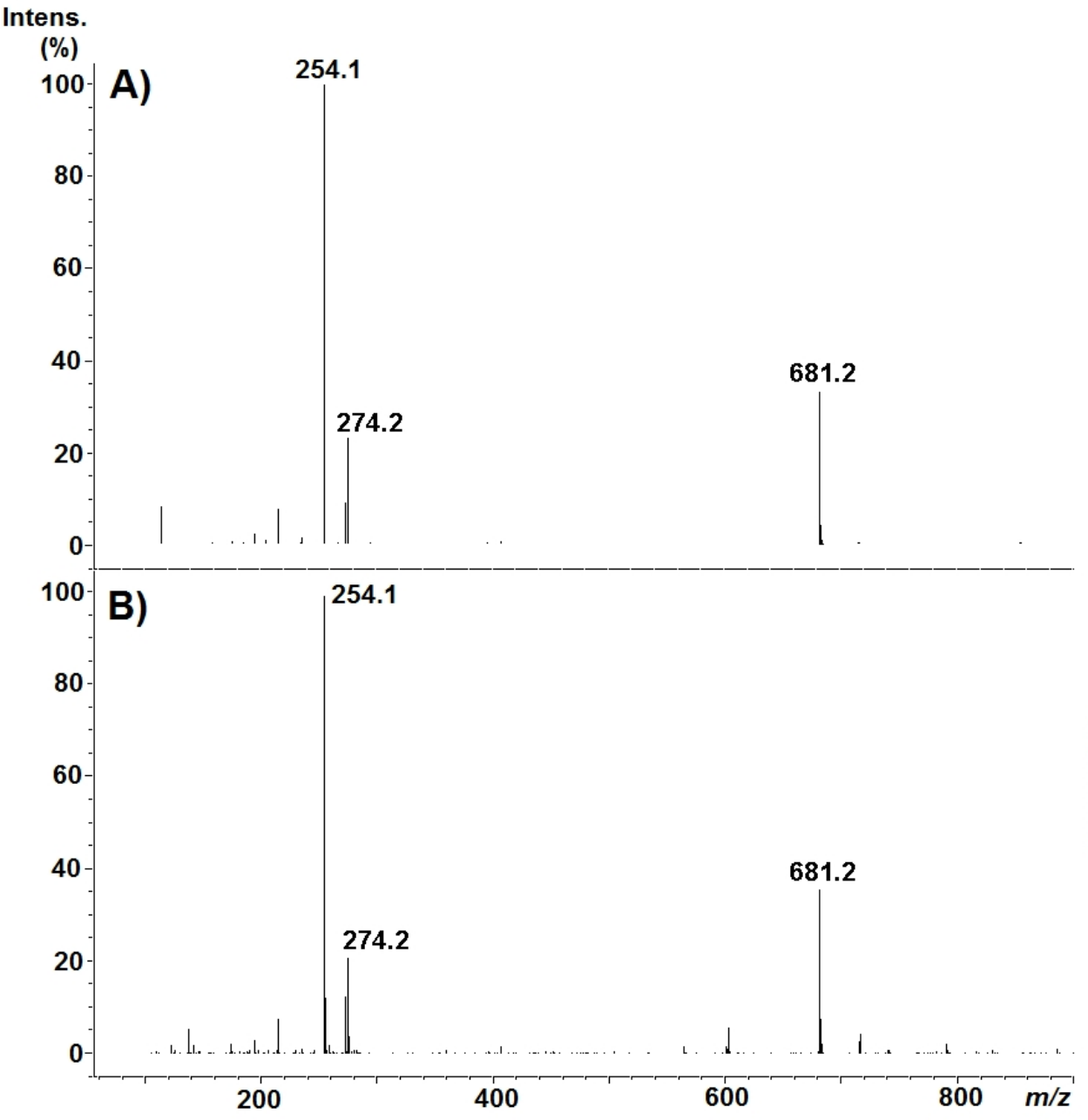
Figure 1.- Scheme of the proposed sample treatment.

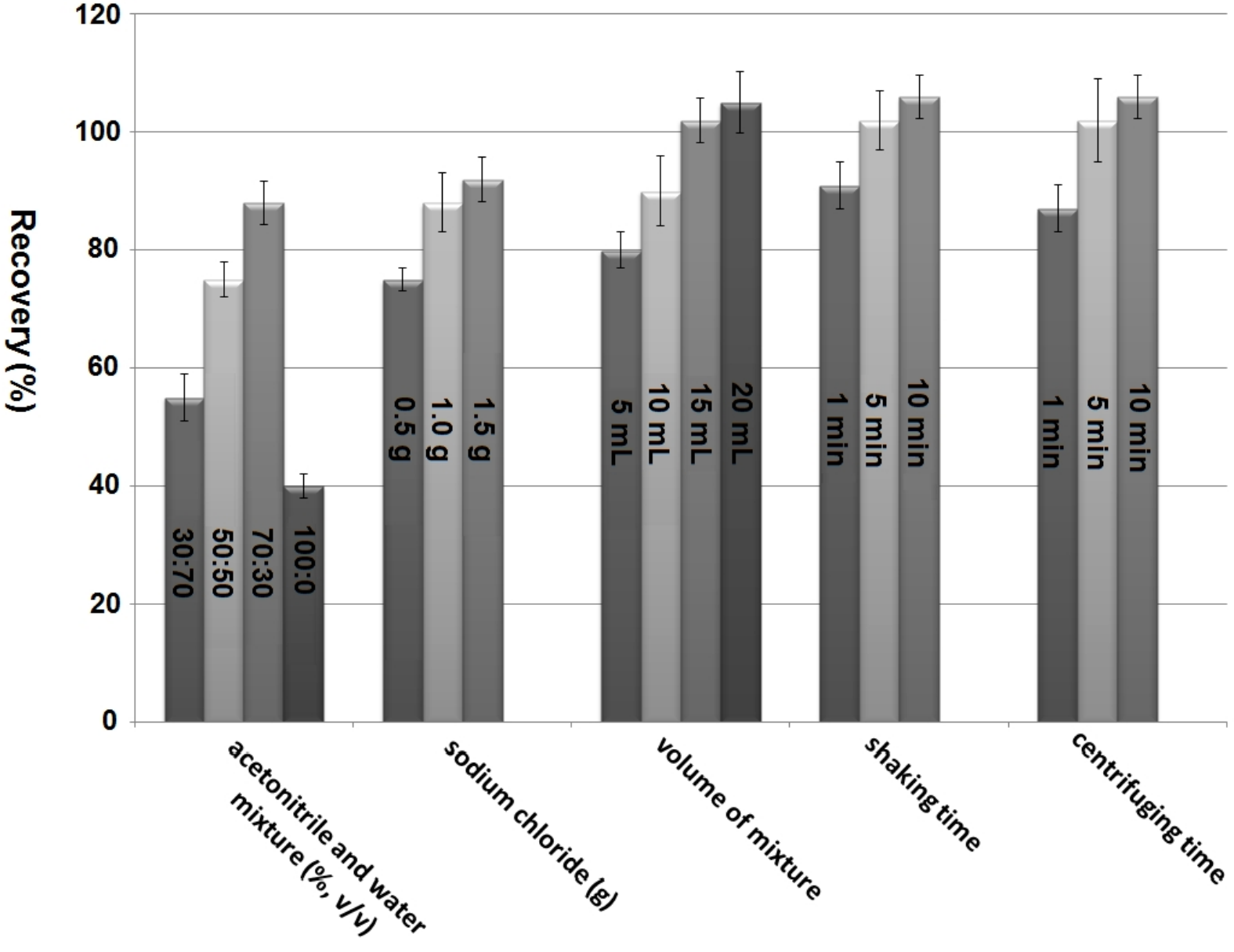
Figure 2.- Full scan ESI-MS spectra of flubendiamide in (**A**) solvent standard solution (100 μg/L) and (**B**) spiked (400 μg/kg) bee pollen sample.

Figure 3.- Evaluation of the extraction efficiency (recoveries) obtained after testing different solvent mixtures, amounts of sodium chloride, volumes of mixture, shaking and centrifuging times with spiked blank bee pollen samples at medium concentration (40 μg/kg). Data represent the mean of three replicates ± the relative standard deviation of the mean (narrow bars).

Figure 4.- Representative LC-MS chromatograms (SIM in negative mode using the quantification ion; see Subsection 2.4) obtained from: (**A**) a solvent standard solution (10 μg/L) of flubendiamide; (**B**) a blank bee pollen sample; a spiked (40 μg/kg) bee pollen sample.


Representative 0.5 g of bee pollen
15 mL of an acetonitrile and water (70:30, v/v) mixture
Shaking
(vibromatic, 5 min)
1.0 g of sodium chloride
Centrifugation
(10000 rpm, 5°C, 5 min)
cooling with dry ice (4 min)
upper phase was collected and transferred to a tube with the EMR-lipid sorbent
shaking (30s)
Centrifugation
(10000 rpm, 5°C, 5 min)
supernatant was collected
Evaporation at 60°C
Reconstitution
(2 mL of methanol)
filtration
LC-MS analysis


Intens.
(%)
A)
254.1
274.2
681.2
B)
254.1
274.2
681.2
100
80
60
40
20
0
200
400
600
800
m/z


Recovery (%)
120
100
80
60
40
20
0
30:70
50:50
70:30
100:0
0.5 g
1.0 g
1.5 g
5 mL
10 mL
15 mL
20 mL
1 min
5 min
10 min
1 min
5 min
10 min
acetonitrile and water mixture (%, v/v)
sodium chloride (g)
volume of mixture
shaking time
centrifuging time

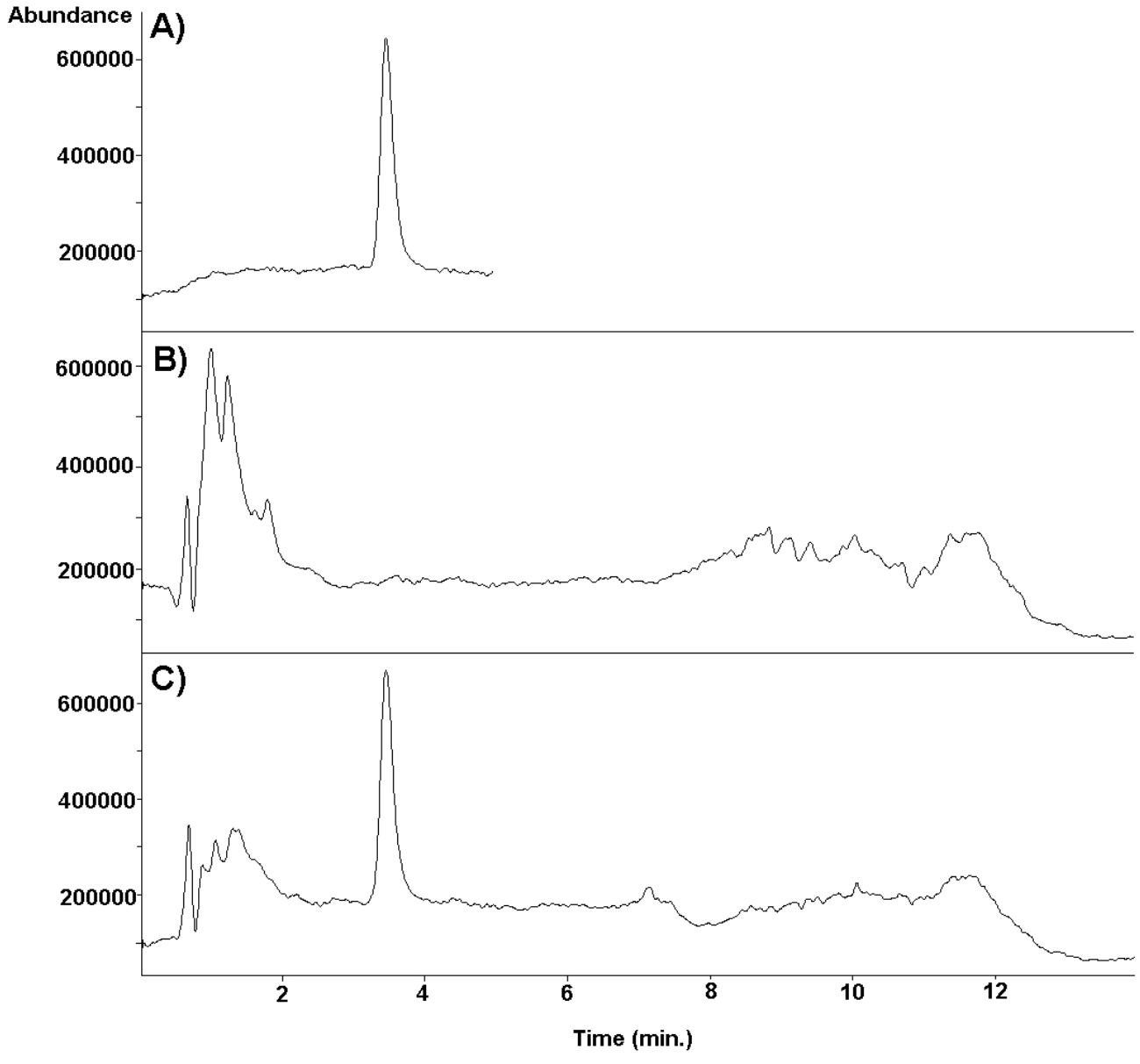

Abundance
A)
600000
400000
200000
B)
600000
400000
200000
C)
600000
400000
200000
2
4
6
8
10
12
Time (min.)

Table 1.- Gradient elution program for LC-MS analysis of flubendiamide.

Time (min)	%Acetic acid (1 mM) in water	%Methanol
0.0	32	68
5.0	32	68
6.5	0	100
8.5	0	100
10.0	32	68
14.0	32	68

Table 2.- Results obtained from the flow injection analysis tests of the ESI-MS parameters in negative mode for the selected mobile phase.

ESI-MS parameter	Studied range	Optimal value
Capillary voltage (V)	2000 - 5500	4500
Drying gas (N_2) flow (L/min)	6 -12	9
Drying gas (N_2) temperature (ºC)	100 - 350	300
Fragmentor voltage (V)	50 - 300	185
Nebulizer gas (N_2) pressure (psi)	10 – 60	40
Gain	1-15	10

Table 3.- Evaluation of the efficiency (recoveries) of the sample treatment, matrix effect (comparison of responses), and precision. Data obtained as described in subsections 3.1 and 3.3 (n = 6).

	Evaluation of the sample treatment	**Evaluation of the matrix effect**	**Precision**	
Quality control (QC) sample	**Mean (%) ± RSD (%)**	**Mean (%) ± RSD (%)**	**Intraday precision (%RSD)**	**Interday precision (%RSD)**
Low concentration	97 ± 6	105 ± 5	7	7
Medium concentration	102 ± 5	100 ± 6	5	7
High concentration	90 ± 5	94 ± 6	7	6

QC samples. -Low concentration-4 μg/kg; **Medium concentration-** 40 μg/kg; **High concentration-** 400 μg/kg.

TRACE ANALYSIS OF FLUBENDIAMIDE IN BEE POLLEN USING ENHANCED MATRIX REMOVAL-LIPID SORBENT CLEAN-UP AND LIQUID CHROMATOGRAPHY-ELECTROSPRAY IONIZATION MASS SPECTROMETRY

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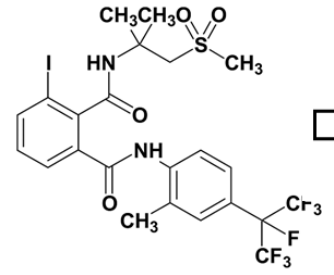
Figure 1S.- Main physical-chemical characteristics of flubendiamide.

Solubility (mg/L; 20°C)	
acetone	102000
1,2-dichloroethane	8120
ethyl acetate	294000
n-heptane	0.835
methanol	26000
p-xylene	488
water	0.029



Toxicology	
Mammals-Acute oral LD50 (mg/kg)	≥ 2000
Acceptable Daily Intake (mg/kg, bw/day)	0.017
Fish-Acute 96 h LC50 (mg/L)	0.06
Aquatic invertebrates-Acute 48 h EC50 (mg/L)	0.06
Honey bees-Oral Acute 48 h LD50 (µg/bee)	> 200

***N*²-[1,1-Dimethyl-2-(methylsulfonyl)ethyl]-3-iodo-*N*¹-{2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl)ethyl]phenyl}-1,2-benzenedicarboxamide**

Empirical formula	$C_{23}H_{22}F_7IN_2O_4S$
Molecular weight (g/mol)	682.39
Melting point (range)	217.5ºC-220.7ºC
pH (20°C)	6.05
Density (20°C)	1.659
Vapor pressure (Pa; 25°C)	10^{-4}
Log K_{OW} (pH 7; 20°C)	4.14
Aqueous photolysis DT50 (pH 7)	32.5
Soil degradation DT50 (20°C)	500
Soil adsorption and mobility (Linear K_{OC})	2197