
En el artículo se presenta el desarrollo, optimización y validación de un método analítico para la determinación simultánea de 10 plaguicidas en lechuga.

1. Escriba el nombre de cada plaguicida
2. Según la introducción del artículo, para el análisis de plaguicidas se usa cromatografía de gases con detector de fósforo y nitrógeno, detector de captura de electrones y detector de masa. Explique en qué situaciones se usa cada uno de ellos y diga cuál es el mejor de todos en función de especificidad y sensibilidad.
3. El método de extracción de los plaguicidas es por SPME, describa el fundamento
4. El uso de la SPME acoplada a GC se ha usado ampliamente para el análisis de algunos plaguicidas, explique por qué hoy en día también se usa la SPME acoplada a HPLC?
5. En el desarrollo del método SPME-HPLC-DAD. Diga qué tipo de detector es el DAD y para qué muestras es adecuado.
6. En este trabajo se propone por primera vez el uso del sistema SPME-HPLC-DAD, en el desarrollo del método diga cuáles son las variables identificadas inicialmente por los autores para establecer las condiciones óptimas?
7. Describa brevemente el sistema cromatográfico usado para el análisis de los plaguicidas (tipo de columna, fase móvil, flujo, longitud de onda del detector, temperatura) y clasifiquelo de acuerdo al mecanismo de separación
8. El diseño experimental para la optimización de la SPME ¿Cuáles son las variables independientes y cuál es la respuesta medida?
9. El diseño usado fue el diseño compacto central completamente factorial, indique como es este diseño e interprete la tabla 1, (a que se refieren los 20 experimentos)
10. Enliste las determinaciones efectuadas para la validación del método de extracción
11. El modelo cuadrático encontrado fue

\[ Y = 4937828 + 616242X_1 + 128691X_2 + 2150239X_3 + 336454X_1X_3 + 240209X_1X_2 + 756312X_2X_3 + 418302X_2^2 + 607327X_3^2 + 60133X_2^2 \]

Esta ecuación se usó para predecir las respuestas en la tabla 1
En la evaluación del modelo, indique para que se usó esta predicción.
12. ¿Cuáles fueron los términos significativos de la ecuación del modelo y como se supo esto? Recuerde que estos términos finalmente determinarán las variables importantes a ser tomadas en la optimización del método de extracción.
13. Según la metodología de la superficie de respuesta, que conclusiones se deducen de los efectos interactivos de los dos factores más importantes
4. Los 10 plaguicidas son:

1. Acetamiprid
2. Azoxystrobina
3. Cyprodinil
4. Fenhexamid
5. Fluothionil
6. Jolpet
7. Iprodione
8. Metafloryx
9. Pirimicarb
10. Tolylfluanid

3. El método de extracción de los plaguicidas por SPME. Describa fundamentalmente:

Es una técnica que se basa en la partición del analito entre la matriz de la muestra y la fase estacionaria.

Donde al equilibrio es llevado a cabo entre la concentración del analito en la muestra y la cantidad de analito internamente de la fibra, dependiendo del coeficiente de distribución.

4. ¿Por qué se indicaría también usar la SPME acoplada a HPLC?

Porque sirve para el análisis de compuestos no-semi volátiles o térmicamente inestables, y recientemente para el análisis de pesticidas debido al interés en la seguridad de alimentos, en este caso para alimentos que se consumen frescos y que tienen alta demanda, los cuales tienen residuos de pesticidas.
En el desarrollo del método SPME-HPLC-DAD, diga qué tipo de detector es el DAD y para qué muestra es adecuado.

- Detección de arreglo de diodos.
- Se usa para muestras polares, con volatilidad baja, termolábiles que no son directamente determinadas por GC.
- Puede ser usado para residuos de pesticidas.

¿Cuáles son las variables identificadas inicialmente por los autores para establecer las condiciones óptimas para el sistema SPME-HPLC-DAD?

Son:
- Tipo de fibra
- Desorption time
- Tiempo de extracción
- Temperatura de extracción
- Desorption solvent
- pH de solución de muestra
- Soaking time
- Iónico strength

¿El diseño experimental para la optimización de la SPME, ¿cuáles son las variables independientes y cuál es la variable medida?

- \( X_1 = \text{pH} \)
- \( X_2 = \% \text{ NaCl} \)
- \( X_3 = \text{Tiempo de extracción} \)

- La respuesta medida: \( Y = \text{Síntesis de área de pico de todos los pesticidas} \)

¿Indique cómo se establece e interpreta tabla 1, a que se refiere número de experimentos.

El diseño consiste de un diseño factorial de segundo grado. Existe el punto central \( (n_0) \) donde \( n_0 \leq 4 \).

- 2 puntos axiales sobre el eje de cada variable de diseño medio distancia de \( a = 1.632 \) del diseño central.

- Se usa un número total de puntos de diseño \( N = 2^k + 2k + 1 \).
Continuación de la 9:

En la tabla N=01 nos indica lo siguiente:

- El objetivo fue evaluar lo requiapto: la suma de pico de pico de todos los particulas.
- Se muestran los efectos combinados de las variables y, (pH), $Y_1 \times 10^1$, $Y_2 \times 10^1$, $Y_3$ (tiempo de extracción).

- De los experimentos, 6 de ellos (del 15 al 20) fueron replicados en el punto central para encontrar algún cambio significativo lo que nos daría la característica de precisión.

10.3 Entrela determinación para la validación del método extracción

Son los siguientes:

- Límite de cuantificación
- Exactitud
- Precisión
- Límite de detección
- Robustez
- Sinalidad

11.3 ¿Para qué se hará esta predicción?

Se usó para optimizar las variables que afectan el proceso de micromanipulación. De allí los experimentos serán concluidos con los parámetros preestados por el modelo.

12. ¿Cuáles son los términos significativos de la ecuación modelo? y cómo se interpreta esto?

Los términos significativos son:

$Y_1 = \text{pH con un valor óptimo de } 8$

$Y_2 = \% \text{NaCl con un valor óptimo de } 17.6\%$
Continuación de 13:

La función de extracción con un valor de 30 min. Se supone esto, a través del valor de $R^2 = 0.994$ y $R^2_{adj} = 0.894$ (el grado de linealidad) y otros valores como: probabilidad de error $p < 0.01$ valor $F$, los cuales nos muestran el análisis de varianza (ANOVA) de la Tabla N° 02.

13.2 Conclusión: Se deduce de los efectos interacción de la

21 importantly?

- **Fig. a.**
  - Con respecto al efecto combinado de pH y NaCl, la máxima respuesta obtenida es a pH = 8 y entre 15% - 20% de NaCl.

- **Fig. b.**
  - Con respecto al efecto combinado de pH y extracción, la máxima respuesta obtenida de suero de oveja de pico ocurri en bajo el tiempo máximo de extracción a pH 8.

- **Fig. c.**
  - El efecto combinado de % NaCl y tiempo de extracción (Fig. 1c), muestra: Para un tiempo de extracción de 30 min. el más alto respuesta obtenida de suero de oveja de pico, es entre 15% y 20% de NaCl y para 90 min de tiempo de extracción, el más alto respuesta se obtenida con 30% de NaCl.
7. Describa brevemente el sistema cromatográfico usado para el análisis de los plaguicidas.

- Tipo de columna: C18 de medidas 5 μm, 250 mm y 4,6 mm. Ultracarb® DDS (Phenomenex, Milford, MA, USA)
- Flujo: 0,75 ml/min
- Longitud de onda del detector: 224 nm
- Temperatura: 40°C
- Seguín Mecanismo de Separación es: Fase reversa
- Fase móvil: Mzcla de 2 solventes
  - Solvente A = 0,1%. Ácido Trifluoracético en Agua
  - Solvente B = Metanol

02: En qué situación su uso detector de NyP, de captura de e, y de masa. ¿Cuál es el mejor en función de especificidad y sensibilidad?

- El detector de NyP y captura de electrones y detector de masa se usan para el análisis de pesticidas.
- El mejor según especificidad y sensibilidad es el detector de masa.
- El detector de captura de también es usado para componentes orgaño metálicos.

Scribe.
Analytical Methods

Optimisation of a solid-phase microextraction/HPLC/Diode Array method for multiple pesticide screening in lettuce

Armando Melo, Ana Aguiar, Catarina Mansilha, Olívia Pinho, Isabel M.P.L.V.O. Ferreira

A new method was developed for the determination of 10 pesticides widely used in lettuce production (ametryn, chlorpyrifos, cyanazine, diclofop, folicidol, fenhexamid, fluazifop, glufosinate, metolachlor, pirimicarb, and tolylfluralin) using solid-phase microextraction (SPME) and liquid chromatography (HPLC) with diode-array detection (DAD). The extraction performance of four different SPME coatings, polydimethylsiloxane (PDMS), PDMS/dimethylvinylbenzene (PDMS/DVB), carbonaceous/silanised resin (C18/TPR), and polyacrylate (PA) was evaluated using an inline SPME-HPLC/CW/FTRP fibre was selected as the most appropriate for the extraction of the majority of these pesticides. Three variables (pH, NaCl, and extraction time) were considered key factors in the optimisation process. Interactions between these analytical factors and their optimal levels were investigated by response surface methodology based on central composite design. The method allowed the determination of acetamiprid, cyanazine, fenhexamid, fluazifop, glufosinate, metolachlor, and tolylfluralin in lettuce at concentrations between 0.8 and 25.6 mg/kg, i.e., below the maximum residues levels allowed for these compounds in lettuce. Lettuce samples that suffered pesticide treatments with fenhexamid and tolylfluralin were analysed during days to harvest to study the dissipation behaviour of the pesticides used. Concentration of fenhexamid was 92.3; 53.4; 22.1; 17.9; 7.45; 1.85 mg/kg and concentration of fenhexamid was 56.8; 31.1; 17.3; 7.24; 0.87 mg/kg. Respective for 0, 3, 6, 10, 15, 20 days, and not detected at 25, 30, and 35 days for the two pesticides.

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Analysis of trace levels of pesticides in foodstuff frequently requires extensive extraction and purification procedures to remove high molecular weight interfering compounds such as lipids (Ridway, Laljiie, & Smith, 2007). The extraction process often involves sample preparation such as, defatting and maceration followed by solvent extraction. Nowadays, extraction methods include solid-phase extraction (Stajnhaber & Zupancic-Kralj, 2003), supracritical fluid extraction (Kainz et al., 2004), solid-phase microextraction (SPME) (Aslaksh, Mallik, Kaur, & Schmitt-Kopplin, 2005; Hu et al., 2008), stir bar sorptive extraction (Sandra, Tienpont, & Deval, 2003) and more recently the ‘quick, easy, cheap, effective, rugged, and safe’ (QEEShES) method (Gamba, Lehayey, Mastoroska, Fernandez, & Oliveira, 2007) and matrix solid-phase dispersion (Covaci et al., 2010).

Solid-phase microextraction has introduced and developed by Pawliszyn (Arthur & Pawliszyn, 1980; Pawliszyn, 1997). The technique is based on the partition of the analyte between the sample matrix and a stationary phase that is a fibre coated with an extracting liquid (polymer) or solid (ceramic) phase. Equilibrium is reached between the concentration of the analyte in sample and the amount of analyte sorbed on the fiber, depending on the distribution coefficient. Owing to its convenience, solvent-free operation and low cost, it has gained wide applicability as an analytical technique. To date, SPME coupled with GC has been widely investigated for the analysis of volatile organic compounds including some pesticides (Rodrigues, Reyes, Rehder, & Raths, 2005; Sanchez-Palomo, Diaz-Maroto, & Perez-Coello, 2005). Moreover, SPME coupled with HPLC is receiving increased attention concerning the analysis of non-volatile, or thermally unstable compounds. Recently, the analysis of pesticides by SPME-HPLC has been reviewed by Aslaksh et al. (2005). However, in general, the optimized methodologies are applied only to water and biological samples; analyses of pesticide residues in vegetables and fruits are still scarce (Aslaksh et al., 2005; Falquies, Cao, Ung, Unry, Poveda, & Monroy, 2001; Wang, Hennion, Ung, & Monroy, 2000). Pesticides use and its residues on lettuce are of particular food safety interest. Lettuce is consumed fresh, so residues that may remain on the harvested product are not removed by processing. Pesticide residues are detected in lettuce and other leafy vegetables more often than in other fresh vegetables. Moreover, lettuce is a major fresh-market vegetable crop (Vandermeer, Stank, Chudan, & Varanada, 1992). The attractiveness of the lettuce in European fresh markets is produced in greenhouses where the conditions are favourable to plant growth. However greenhouse mild temperatures and high humidity also promote fungal and insect development. Key pests in lettuce are the diseases downy mildew and Botrytis grey mould and the insects' aphids. Growers prevent these pests using authorised pesticides. In EU the most used pesticides in lettuce are azoxystrobin, acetoxypropin, cyproconazole, fenhexamid, fludioxonil, folpet, iprodione, metalaxyl, pirimicarb, and tolyfluanid so they were used in this conduct experiment. To our knowledge, no study has been presented describing the use of SPME and HPLC-DAD for the simultaneous analysis of these pesticides (both fungicides and insecticides) in vegetables.

The development of a SPME-HPLC method requires selection of many variables (fibre type, extraction time, desorption solvent, soaking time, desorption time, extraction temperature, pH of sample solution, and ionic strength) in order to establish optimum conditions. The conventional approach for the optimisation of a multivariable system is usually one-variable-at-a-time. This can be very time consuming and, where interactions exist between the variables, it is unlikely to find the true optimum. Response surface methodology (RSM) is a very useful tool for this purpose as it provides statistical models, which help in understanding the interactions among the parameters that should be optimised (Lorevi, 2009). RSM is a collection of mathematical and statistical techniques for modelling and analysis of problems in which a response of interest is influenced by several variables (Ferreira et al., 2007). The main objective of RSM is to determine the optimum operational conditions for the system or to determine a region that satisfies the operating specifications (Ferreira et al., 2007). RSM can be a useful tool for optimisation in analytical chemistry (Bezerra, Santelli, Oliveira, Villar, & Escalante, 2008), namely applied to the optimisation of pesticides by SPME (Perrin, Borrill, & Marcé, 1999).

The purpose of this study was the development and validation of a SPME/HPLC-DAD method for multiple pesticide screening in lettuce (Lactuca sativa). RSM based on central composite design was used to optimise some variables in SPME. The method was applied to the monitoring of pesticides in greenhouse-grown lettuce samples to study the dissipation behaviour of some of these pesticides during the days to harvest, aiming to evaluate the use of good agricultural practices.

2. Experimental

2.1. Chemicals and reagents

All analytical-grade chemicals (purity >98%) including acetamiprid, azoxystrobin, cyproconazole, fenhexamid, fludioxonil, folpet, iprodione, metalaxyl, pirimicarb, and phosmet (used as internal standard) were purchased from Sigma-Aldrich (Steinheim, Germany). All the solvents used were HPLC-grade from Merck (Darmstadt, Germany). Ultra-pure water (0.054 μS/cm) was obtained by using a Milli-Q system from Millipore (Milford, MA, USA).

Pesticides standards and calibration solutions were prepared as recommended by SANOCO/16084/2009. Stock standards of each pesticide were prepared in acetonitrile and kept from light in a freezer. Working solutions of each pesticide were mixture of appropriate concentrations prepared weekly and kept at low temperature in the dark.

Different buffers for pH (ranging between 2.64 and 9.36) were prepared to study the effect of pH on SPME pesticide extraction (Perrin & Dempsey, 1974). Buffer with pH 8.5 selected for analysis of real samples contained 50 mL 0.1 M KH₂PO₄ and 45.1 mL NaOH diluted to 100 mL (Perrin & Dempsey, 1974).

2.2. Sampling strategy

Lettuce (L. sativa L. var. capitata) were planted in a 300 m² plastic greenhouse situated in Póvoa de Varzim, in December 2008. Planting density was 12 plants/m² (30 cm x 23 cm). The culture was irrigated whenever necessary with a total of 150 L/m². Pesticides were sprayed when decided by grower according to the results of risk assessment for the main pests and diseases. Lettuce plants (1 kg or at least 10 units, depending on the growth phase) were collected before the addition of pesticides (pesticide free samples) and at 0, 1, 3, 6, 7, 9, and 14 days after pesticide addition, respectively. Six (s₁, s₂, s₃, s₄, s₅, and s₆) to study the dissipation behaviour of some of these pesticides after pre-harvest interval in accordance with Directive 2002/69/EC and SANOCO/16084/2003. Lettuce samples were immediately transported to the laboratory, freeze for 2 h and mashed with a blender.

2.3. HPLC-DAD conditions

The chromatographic analysis was carried out in an analytical HPLC unit (Jasco, Tokyo, Japan) equipped with Jasco PU-1580 HPLC pumps, a Column Heater (Model 7581); Jones Chromatography, Hengoed, UK, an MD-510 Plus multichannel detector. The
SPME-HPLC interface with Rheodyne valve (Supelco, USA) was used to perform the injection in HPLC system. The column was a reversed-phase Ultracarb ODS (30 μm, 250 mm × 4.6 mm) (Phenomenex, Milford, MA, USA). The Varian PDA Controller Software (JMS Developments, Le Fontaine, France) was also used. The HPLC was carried out by gradient elution with a mixture of two solvents and a flow of 0.75 ml/min. Solvent A consisted of 0.1% of trifluoroacetic acid in water and solvent B consisted of methanol. The linear gradient program was 0-7.5 min, 20-100% B in A (7.5-11.5 min). 60-85% B in A 16.5-25 min, 60-85% B in A; 25-45 min, column rinse and re-equilibration. Separations were carried out at 40 °C of temperature. Diode Array detection was set at 224 nm. Peak identification in samples was carried out by comparing retention times and spectra of unknown peaks with reference standards, as well as peak purity.

2.4. Selection of SPME fibre and desorption conditions

The SPME device for manual extraction, consisting in a holder assembly and several replaceable fibers, was purchased from Supelco (Montigny-le-Bretonneux, France) and used without modification. The three coatings assayed in this work were: PDMS (100 μm), PDMS/divinylbenzene (PDMS/DVB, 60 μm), and PDMS/divinylbenzene/carboxylated resin (PDMS/porous carbon, 35 μm). Each fiber was conditioned in methanol with stirring for 30 min. Assays for selection of SPME fibre were performed by direct immersion of the fibre into 4.0 ml of a standard solution containing 1 μg/ml of each pesticide under study in acetonitrile-water (10:90). For preliminary experiments, extraction was carried out at room temperature (22 ± 3 °C, approx.) for 30 mins (agitation at 1000 rpm), 1200 μl of acetonitrile were added (300 μl/w). The desorption chamber of the interface SPME-HPLC (60 μl) was previously filled with a solution of methanol-water (9:1). Afterwards, and in order to select the appropriate desorption solvent and soaking time, other experiments were performed with the selected fibre using methanol and mixtures of methanol/water (9:1) and (8:2) and soakings times of 5, 10 and 15 min.

2.5. Statistical design of experiments for SPME extraction

Experiments with three independent variables pH (X1), NaCl (X2) and extraction time (X3) were conducted using the full factorial Central Composite Design (CCD). In this study, the full CCD consisted of (i) a complete 2³ factorial design, (ii) n₀ centre point (n₀ - 1) and (iii) two axial points on the axes of each design variable at a distance of γ = 1.682 from the design center. Hence a total number of design points of N = 2³ + 2n₀ points was used. The centre point was replicated six times to give five degrees of freedom for calculation of errors in the experiments. The response Y was the sum of peak area of all pesticides, since it grouped information from all compounds under study. The optimal values of response Y were obtained by solving the regression equation and by analyzing the response surface contour plots. The variables were coded according to the Eq. (1):

\[ X_i = \frac{x_i - x_{i0}}{Dx} \]  

where, \( x_i \) is the coded value of variable i, \( x_i0 \) the uncoded real value of an independent variable, \( x_{i0} \) the value of \( x_i \) at the centre point and \( Dx \) is the step change between levels -1 and 1. The behaviour of the system was explained by the following second order polynomial equation (Eq. (2)):

\[ Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=j}^{3} \beta_{ij} X_i X_j \]  

where, Y is the sum of peak area of all pesticides under study, \( \beta_0 \) the constant coefficient, \( \beta_i \) the coefficient of linear effect, \( \beta_{ii} \) the coefficient of squared effect, \( \beta_{ij} \) the coefficient of interaction effect and \( X_i \) is the coded value of variable i.

The goodness-of-fit of the regression model and the significance of the parameters estimates were determined through appropriate statistical methods. Work on experimental design, data analysis, response surfaces and contour diagrams, was performed by Design Expert trial-Version 7 (Stat-Ease Inc, Minneapolis, MN).

2.6. SPME method validation and application to real samples

The limit of quantification (LQ) is assumed to be the lowest amount of an analyte in a sample that can be quantified with acceptable precision and accuracy whereas the limit of detection (LD) is the lowest concentration of an analyte that can be reliably differentiated from the background noise but not necessarily quantified as an exact value. Both were calculated through the calibration curve parameters, using the intercept and the residual standard deviation obtained for the calibration line (Perfetti et al., 1999; Miller & Miller, 2005).

Eight-point calibration curves were constructed for each pesticide using lettuce, cucumber and tomatoes fortified within the range of concentrations of 0.8-2.5 mg/kg. Phosmet was chosen as internal standard (IS) because its use is forbidden in European Union lettuce crops.

Analyses of real samples were performed as follows: before SPME extraction, 2.5 g of lettuce, cucumber or tomatoes were mixed with 10 μl of a 200 μg/ml acetonitrile solution (10 min at 30 °C with 1 ml of acetonitrile in a ultrasonic bath, followed by centrifugation (10 min, 11.5 g).

Optimal SPME conditions for the extraction of the pesticides were: 2 ml aliquot of lettuce extract, 0.7 g of NaCl (185 μl/w) and 2 ml of buffer solution (pH = 8) were transferred to a 4-ml Teflon-lined septum cap vial equipped with a magnetic bar. The CW/TPR SPME fibre was immersed directly into the sample solution, and the extraction took place at room temperature (22 ± 3 °C, approx) for 30 mins with continuous stirring at 1000 rpm. After extraction, the fibre was withdrawn into the needle, the needle was removed from the septum and was inserted in the desorption chamber of the interface SPME-HPLC (with Rheodyne valve) that is off-line under ambient pressure when the injection valve is in load position. The chamber (60 μl) was previously filled with a solution of methanol-water (9:1) and the fibre was soaked for 10 min (soaking time). Then, the valve was switched to inject position and the analytes were delivered to the column. The valve was returned to load position after 1 min (desorption time). Between extractions the fibre was cleaned with methanol and ultrapure water for 10 min.

3. Results and discussion

3.1. HPLC-DAD conditions

The mobile phase containing 0.1% trifluoroacetic acid in water and methanol was selected since it is not very expensive and keeps a constant acid pH that is important to improve peak resolution. Thus, after optimisation of gradient the chromatographic separation of 11 pesticides (10 pesticides under study and IS) was carried
out with a mixture of these two solvents using a flow of 0.75 mL/min. The gradient conditions described in (Section 2.3) were adapted from Otero, Grande, and Gandara (2003) for the separation of all target compounds. The advantages of the programmed gradient conditions are shorter running time and the use of more economical solvents.

3.2. Selection of SPME fibre and desorption conditions

PDMS, PDMS/DVB, PA, and CW/TPR fibres were tested using the conditions described in Section 2. Among the four fibres tested, CW/TPR showed the best extraction performance for most of the pesticides, which is in agreement with results reported by other authors (Sagatini et al., 2007). However, acetylamid was not extracted, owing to its very low value of log P (0.80), additionally, princaicarb and metanexizol presented low peak areas as also expected, since these two pesticides have low octanol/water partition coefficients as well (log P = 1.70 and 1.85, respectively) and thus they present a low affinity for the SPME coating (Redway et al., 2007). All other pesticides under study present log P > 2 and high affinity for the SPME coating. As a result, CW-TPR fibre was selected for subsequent experiments. Afterwards, and in order to select the appropriate desorption solvent and soaking time preliminary experiments were developed with the CW-TPR fibre using methanol, and mixtures of methanol/water (9:1) and (8:2) and soaking times of 5, 10, and 15 min. The best desorption was obtained using methanol/water (9:1) and 10 min of soaking (data not shown).

3.3. Statistical design of experiments for SPME extraction

To evaluate the sum of peak area of all pesticides (Y) 20 experiments were conducted according to the CCD method. The design of this experiment is given in Table 1, together with the experimental results and predicted values. To study the combined effects of pH (X1), NAC (X2) and extraction time (X3), experiments were performed at different combinations of these parameters using statistically designed experiments. Six experiments (runs 15–20) were replicated at the centre point to verify any change in the estimation procedure as a measure of the precision property.

Regression analysis was performed to fit the response function (Y). The model expressed by Eq. (2), where the variables take their coded values, represents the sum of peak area of all pesticides (Y) as a function of X1, X2 and X3. The final empirical model in terms of coded factors for the sum of peak area (Y) is given by Eq. (3):

\[ Y = 4937828 + 616242X_1 + 12809X_2 + 21503X_3 
+ 33645AX_1X_2 + 240290X_1X_3 + 756312X_2X_3 
+ 418302X_1^2 - 687527X_2^2 + 60133X_3^2 \] (3)

This equation was used to predict the sum of peak area in Table 1. Apart from explaining the linear effects of pH, NAC and extraction time on the sum of peak area, the CCD approach described the quadratic and interaction effects of the parameters too. The relationship between observed and predicted sum of peak area showed that the plotted points cluster around a diagonal line, indicating good fitness of the model, since the values of R^2 and R^2_adj were found to be 0.944 and 0.864, respectively.

ANOVA is important in determining the adequacy and significance of the quadratic model. The analyses were done by means of Fisher’s F-test. Generally the model F-value of 18.69 with a low probability P-value indicates high significance of the regression model (Miller & Miller, 2005). From the ANOVA summary (Table 2), the model was found to be statistically significant (P < 0.01) at the 99% confidence level. X1, X2, X3, X1X2, X1X3, X2X3 are significant model terms. The lack-of-fit term is non-significant as it is desired. The non-significant value of lack-of-fit (more than 0.05) showed that the quadratic model was valid for the present

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<td>Significant</td>
</tr>
<tr>
<td>X2X3</td>
<td>2.46 12</td>
<td>1</td>
<td>0.8605</td>
<td>2.03877</td>
<td>0.0004</td>
<td>Significant</td>
</tr>
<tr>
<td>X1^2</td>
<td>5.15 12</td>
<td>1</td>
<td>0.5156</td>
<td>1.94518</td>
<td>0.0000</td>
<td>Significant</td>
</tr>
<tr>
<td>X2^2</td>
<td>0.70 10</td>
<td>1</td>
<td>0.0710</td>
<td>0.33046</td>
<td>0.1281</td>
<td>Significant</td>
</tr>
<tr>
<td>Residual</td>
<td>4.93 12</td>
<td>10</td>
<td>0.8670</td>
<td>1.21547</td>
<td>0.0292</td>
<td>Not significant</td>
</tr>
</tbody>
</table>
study [Montgomery, 2001]). Thus, the sum of peak area was adequately explained by the model equation (Eq. (5)).

To investigate the interactive effect of two factors on the sum of peak area, the response surface methodology was used and three dimensional and contour plots were drawn. The interferences so obtained are discussed: (i) the combined effect of pH and NaCl percentage is shown in Fig. 1a; the maximum response is obtained at pH 8 and between 15% and 20% NaCl; (ii) the combined effect of pH and extraction time is shown in Fig. 1b. It is observed that for an extraction time of 30 min the higher response is observed between 15% and 20% NaCl and for 90 min extraction time higher response is obtained for 30% NaCl.

Design Expert allows the establishment of criteria for all variables, including factors and propagation of error. Assignment of optimisation parameters was performed to maximise response and minimise extraction time keeping the other parameters in the range. Desirability indices (di) were constructed combining the individual desirability into single number and then searches the greatest overall desirability. Additionally, it was given higher relative importance to desirability of response and extraction time as a compromise between higher sensitivity with lower analysis time. The combined effect of pH and extraction time on desirability is shown in Fig. 1d. Graphs show a maximum desirability at 30 min and pH 8, keeping the percentage of NaCl at 17.6%.

Further to support the optimised data as given by numerical modelling under optimised condition, the confirmatory experiments were conducted with the parameters as suggested by the model ($X_1$, (pH) 8, $X_2$ (NaCl) 17.6% and $X_3$ (extraction time) 30 min). The response obtained ($3.19 \times 10^3$) was within 95% prediction interval (2.27E+08 – 5.9E+08) indicating a good accordance with the model given by RSM software.

3.4. Calibration and matrix effect evaluation

Lettuce has compounds that can interfere in the SPME of the selected pesticides. Thus, according to SANCO/10084/2009 the potential for matrix effects to occur should be assessed at method validation. For this purpose calibration curves were constructed using lettuce pesticide free samples (collected before the addition of any pesticide and analysed to confirm the absence of pesticides). Four different calibration curves were constructed using 6, 0.25, 1.0 and 2.5 g of lettuce, added of increasing amounts of pesticides. Calibration curves for each pesticide were prepared by plotting their area relative to that of the internal standard (propranolol) vs. the analyte concentration. Under the operational conditions detailed previously, the chromatographic separation achieved for lettuce samples proved to be lacking in interfering peaks that co-eluted with the pesticides studied (Fig. 2). Good linearity was observed for azoxystrobin, cyprodinil, fenhexamid, fludioxonil, flutriafol, iprodione, and tolylfluanid with $R^2$ greater than 0.996. Acomine/pid is not extracted using this SPME fiber; metalaxyl and pirimicarb were extracted only for concentrations above 20 mg/kg. Thus, owing to its low octanol/water partition coefficients (log P value); alternative methods should be selected for these three compounds. Phosmet was a good choice as IS since it presented chromatographic characteristics similar to that of the compounds under study and presents an intermediary value of log P.
recovery for IS was 97.1 ± 7.5%. The intra-day repeatability (RSD [%], n = 6 in the same day) was 4.1% and the inter-day repeatability (RSD [%], n = 17 in five different days) was 7.7%. These results are in the same range of those reported by Sargentini et al. (2007) for the analysis of carbamates and phenylurea pesticide residues in fruit juices by solid-phase microextraction and liquid chromatography.

A statistical comparison between the calibration curves constructed using 0.025, 1.0, and 2.2 g of lettuce was performed. Significant differences were observed between calibration curves, indicating a matrix effect on SPME extraction. Additionally, multiple linear regressions with backward stepwise method were constructed using lettuce amounts and pesticide concentrations to predict the pesticide concentration. The regressions obtained for thiourea and fipronil were the only ones without a significant effect from the lettuce amount.

Table 3 presents the linear range tested for each pesticide, linearity (peak area = k×C + a) and respective determination coefficients ($r^2$), and the statistical parameters obtained when carrying out the linear regression, the repeatability of peak areas, the LD and LQ obtained for these compounds were far below the legislated LMR. Thus, the sensitivity of the method using 2.5 g of sample is sufficient to ensure compliance with food legislation, except for metoxuron and primicarb that were extracted only for concentrations above the respective LMR (Regulation (EC) No. 396/2005).

Fig. 2. Chromatograms of lettuce samples after SPME extraction under optimum extraction conditions (pH 8; 17.6% NaCl; 30 min extraction time): uncontaminated sample spiked with pesticides. Peak identification and concentration: 1, primicarb (16 mg/kg); 2, acetamiprid (16 mg/kg); 3, cyprofloxacins (5.8 mg/kg); 4, metalaxyl (56 mg/kg); 5, phenoxam (4.4 mg/kg); 6, fenhexamid (11.2 mg/kg); 7, fludioxonil (8.4 mg/kg); 8, fenhexamid (11.2 mg/kg); 9, flupentox (8.4 mg/kg); 10, iprodione (22.4 mg/kg); and 11, tolledfluindol (22.4 mg/kg).

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Family</th>
<th>E.U.'s MRL (mg/kg)</th>
<th>$K_{ow}$ log $P$</th>
<th>LD</th>
<th>LQ</th>
<th>Range of concentration tested (mg/kg)</th>
<th>$R$ × (95% C$^2$)</th>
<th>$R$ × (95% C$^2$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
<td>Herbicide</td>
<td>3</td>
<td>8.80</td>
<td>0.55</td>
<td>-</td>
<td>1.0-12.8</td>
<td>0.017 ± 0.017</td>
<td>0.030 ± 0.001</td>
<td>0.596</td>
</tr>
<tr>
<td>Anatoxacin</td>
<td>Herbicide</td>
<td>3</td>
<td>2.00</td>
<td>0.48</td>
<td>1.09</td>
<td>1.6-12.8</td>
<td>0.017 ± 0.017</td>
<td>0.030 ± 0.001</td>
<td>0.596</td>
</tr>
<tr>
<td>Cyprofloxcins</td>
<td>Fungicide</td>
<td>10</td>
<td>3.90</td>
<td>0.48</td>
<td>0.91</td>
<td>1.6-6.80</td>
<td>0.017 ± 0.017</td>
<td>0.030 ± 0.001</td>
<td>0.596</td>
</tr>
<tr>
<td>Fenoxam</td>
<td>Fungicide</td>
<td>3</td>
<td>5.51</td>
<td>0.73</td>
<td>1.79</td>
<td>1.6-12.8</td>
<td>0.159 ± 0.004</td>
<td>0.124 ± 0.0001</td>
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</tr>
<tr>
<td>Fludioxonil</td>
<td>Fungicide</td>
<td>5</td>
<td>4.12</td>
<td>0.71</td>
<td>1.59</td>
<td>1.2-12.6</td>
<td>0.150 ± 0.019</td>
<td>0.192 ± 0.001</td>
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<td>Flupentox</td>
<td>Fungicide</td>
<td>2</td>
<td>3.85</td>
<td>0.47</td>
<td>1.57</td>
<td>1.2-12.6</td>
<td>0.313 ± 0.042</td>
<td>0.341 ± 0.001</td>
<td>0.596</td>
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<tr>
<td>Iprodione</td>
<td>Fungicide</td>
<td>10</td>
<td>3.90</td>
<td>1.54</td>
<td>5.14</td>
<td>3.2-25.6</td>
<td>0.263 ± 0.033</td>
<td>0.081 ± 0.001</td>
<td>0.596</td>
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<tr>
<td>Metoxuron</td>
<td>Fungicide</td>
<td>3</td>
<td>1.50</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Polycarb</td>
<td>Insecticide</td>
<td>2</td>
<td>1.70</td>
<td>-</td>
<td>-</td>
<td>1.6-12.8</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Tolledfluindol</td>
<td>Insecticide</td>
<td>3</td>
<td>3.90</td>
<td>1.53</td>
<td>5.13</td>
<td>3.2-25.6</td>
<td>0.0018 ± 0.019</td>
<td>0.048 ± 0.001</td>
<td>0.596</td>
</tr>
</tbody>
</table>

LD, limit of detection (mg/kg); LQ, limit of quantification (mg/kg); C, concentration in mg/kg; $r^2$, determination coefficients (n = 5).


3.1. Real sample analysis

The applicability of the method was evaluated by the analysis of lettuce samples from a greenhouse that were sprayed with
4. Conclusions

In this work, it has been selected the most appropriate conditions of SPEME coupled to HPLC/DAD detection for the quantification of azoxybenzene, cyanodim, fenhexamid, fipronil, iprodione, and tolyfluanid in lettuce. DAD although less powerful than other detectors has the advantage of being easier to acquire and use, enabling quantification of these pesticides below the maximum residue levels allowed for those compounds in lettuce. The use of SPEME as a purification step allows determining them quantitatively without interferences and allows the simplification of the clean-up step. The factorial designs were used to optimize variables affecting the microextraction process: Confirmatory experiments were conducted with the conditions suggested by the model $X_1$ (pH) 8, $X_2$ (NaCl) 17.6% and $X_3$ (extraction time) 30 min and the response obtained was within 95% prediction response interval.

The proposed methodology can be used for screening of pesticides in lettuce samples or to study the dissipation behaviour after pre-harvest interval. Analysis of lettuce samples sprayed with fipronil and fenhexamid indicated that after the days to harvest the concentration level (1 the uncertainty of the result) found for the pesticide residue was below the EU's MRLs, thus causing no problems in terms of food consumption.

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References


