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# Determination of triazine herbicides in seaweeds: Development of a sample preparation method based on Matrix Solid Phase Dispersion and Solid Phase Extraction Clean-up

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ABSTRACT

A method using dual process columns of Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) has been developed for extracting and cleaning-up of nine triazine herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) in seaweed samples. Under optimized conditions, samples were blended with 2 g of octasilyl-derivatized silica ($C_8$) and transferred into an SPE cartridge containing ENVI$^{TM}$-Carb II/PSA (0.5/0.5 g) as a clean up co-sorbent. Then the dispersed sample was washed with 10 mL of *n*-hexane and triazines were eluted with 20 mL ethyl acetate and 5 mL acetonitrile. Finally the extract was concentrated to dryness, re-constituted with 1 mL methanol:water (1:1) and injected into the HPLC-DAD system. The linearity of the calibration curves was excellent in matrix matched standards, and yielded the coefficients of determination $\geq$ 0.995 for all the target analytes. The recoveries ranged from 75% to 100% with relative standard deviations lower than 7%. The achieved LOQs ($< 10\ \mu g\ kg^{-1}$) for all triazines under study permits to ensure proper determination at the maximum allowed residue levels set in the European Union Legislation. Samples of three seaweeds were subjected to the procedure proving the suitability of MSPD method for the analysis of triazines in different seaweeds samples.



## 1. Introduction

Seaweeds have been used since ancient times as food, fodder, fertilizer and as a source of medication. Nowadays, seaweeds are the raw material for industrial production of agar, carrageenan and alginates; however they still remain to be widely consumed as a source of food in Asian countries. Although in North America and Europe their use as food is more restricted, in recent years seaweeds have been increasingly recognized as healthy and attractive foods [1]. Edible seaweeds contain dietary fiber, high concentration of minerals, vitamins, proteins, polyunsaturated fatty acids and have a low content in saturated fats. On the other hand, seaweeds have also shown biological properties such as antibacterial, antiviral, antioxidant and antifungal [2,3]. Moreover, it has been reported that the chemical composition of seaweeds varies with species, habitats, maturity and environmental conditions [4].

Triazines, well-known herbicides, are applied to soil for the control of weeds in many agricultural crops, as well as railways, roadside and golf courses. The marine environment receives fluxes of these compounds mainly from agricultural origin. Their mechanism of action is via photosynthetic inhibition, and for this reason, they are only toxic for plants; however these compounds can affect the human health through the dietary intake. These compounds are highly persistent and can survive for many years in soil, water and organisms. Therefore, they are considered as an important class of chemical pollutants and atrazine and simazine have been included in the group of endocrine-disruptors by the Environmental Protection Agency of US [5]. As a result, the European Parliament and Council [6] concerning the residue levels in food and feed of plant and animal origins established the maximum permitted concentration in seaweed 0.01 and 0.05 mg $kg^{-1}$ for simazine and terbuthylazine respectively. Moreover, a limit is not yet established for atrazine in seaweed, but its maximum permitted limit in edible vegetables is 0.05 mg $kg^{-1}$. For this reason, analytical methods for a rapid and sensitive determination of these compounds are required. However, seaweed is a complex matrix with different types of interfering compounds which make pesticide analysis difficult; in fact, studies of pesticides in seaweeds are limited and recent [7–9] and to the best of our knowledge there is only one reference in the literature devoted to the determination of triazines in seaweeds [10].

The most frequently used methodologies for the analysis of triazines in samples of vegetable and animal origins employ

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solvent extraction procedures such as soxhlet [11,12], shake flask [13,14], sonication [10,13,15], microwave-assisted extraction (MAE) [11,12,16,17], and pressurized liquid extraction (PLE) [18,19]. Nevertheless, they generally need to add a clean-up step to decrease the presence of interferents in the final extract to reduce the detection limits of the methods and to avoid inaccurate results in the chromatographic determination, which is time consuming; many times it is expensive and simultaneously decreases the precision of the methodologies involved. Over the last years, different innovative procedures have been developed and applied for the determination of pollutants in complex matrices with improved capabilities, reduced clean up and concentration steps, the avoidance of toxic solvents and improved limits of detection. In this context, sorptive extraction techniques such as solid-phase extraction (SPE), dispersive solid phase extraction (dSPE), matrix solid-phase dispersion (MSPD), solid-phase micro-extraction (SPME) and stir bar sorptive extraction (SBSE) appear to be appropriate and they have been applied for analysis of triazines in different kinds of vegetation samples [20,21].

Matrix solid-phase dispersion is a sorptive extraction technique which involves the dispersion of the sample in a solid sorbent and subsequent elution with a relatively low solvent volume, allowing the simultaneous extraction and clean-up of analytes from solid samples [22]. If an additional clean up step is necessary, it is possible to use the MSPD column with another sorbent at its bottom. This technique shows a high flexibility and selectivity due to the variety of possible combinations of both sorbents and elution solvents [23]. Due to its simplicity and high throughput, MSPD methods have been developed for the extraction of different pesticides residues from different plants and plant materials [24]; however references for the determination of triazines by MSPD are still scarce and furthermore in most cases few triazines are included in these studies [25–28].

The aim of this work was the development and validation of an effective and simple method based on Matrix Solid Phase Dispersion (MSPD) and Solid Phase Extraction (SPE) Clean-up followed by High Performance Liquid Chromatography (HPLC) coupled to Diode Array Detection (DAD) for the simultaneous determination of nine triazine herbicides in seaweeds in order to be able to quantitate residues of these compounds in the range of the European maximum residue levels. Samples of three edible seaweeds were selected to illustrate the applicability of this method. To the best of our knowledge, no studies using MSPD have been done to extract these chemicals residues from seaweeds.

## 2. Experimental

### 2.1. Samples

*Dried edible seaweeds*. Sea lettuce (*Ulva Lactuca*), Wakame (*Undaria pinnatifida*), and Nori (*Porphyra umbilicalis*), from aquaculture production, were purchased from a local market in A Coruña city, NW, Spain. Samples were homogenized grounding them to a fine powder by an electric mill and stored in glass bottles out of light exposure until analysis.

### 2.2. Chemicals

(a) *Herbicide standards*. Herbicides (ametryn, atrazine, cyanazine, prometryn, propazine, simazine, simetryn, terbuthylazine and terbutryn) analytical standards were supplied by Sigma-Aldrich Inc. (St. Louis, MO, USA). The individual stock standard solutions of 1000 mg $L^{-1}$ were prepared in methanol by exact weighing of high-purity substances and stored at −18 °C in dark. Then a mixture of all these compounds was prepared in methanol containing 10 mg $L^{-1}$ each of individual triazine and stored at −18 °C. All working solutions were daily prepared by appropriate dilution of the 10 mg $L^{-1}$ standard solutions with methanol:water (1:1) ratio.

(b) *Solvents*. *n*-hexane 95% and methanol were superpurity solvents obtained from Romil (Cambridge, UK). Acetonitrile (HPLC grade) and ethyl acetate (PAR, solvents for analysis of pesticide residues by GC) for instrumental analysis were obtained from Panreac (Barcelona, Spain). Milli-Q water was obtained from a purification system from Millipore (Billerica, MA).

(c) *Sorbents*. LC-8 Bulk packing and Supelclean™ ENVI-Carb II/PSA SPE Tube 6 ml (500 mg/500 mg) were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

(d) *Filters*. Polytetrafluoroethylene (PTFE) filters of 0.45 μm were obtained from Lida Manufacturing (Kenosha, WI, USA).

### 2.3. Materials and apparatus

A Visiprep® vacuum distribution manifold from Supelco (Bellefonte, PA, USA) was employed in the purification step. A Büchi R-3000 rotary evaporator (Büchi Labortechnic AG, Flawil, Switzerland) was used in the evaporation step.

Chromatographic analyses were carried out in a High Performance Liquid Chromatography-Diode Array Detector (HPLC–DAD). The system consisted of a 2695 pump with a 996 Diode Array Detector from Waters (Milford, MA, USA) and a computer running Empower 2 data processor. The column was a stainless steel column (150 mm × 4.6 mm ID, particle size 5 μm) packed with Hypersil GOLD $C_{18}$ chemical bonded phase from Thermo Scientific (Austin, TX, USA).

### 2.4. Extraction procedure

The optimization study was carried out using a pesticide free sea lettuce seaweed sample spiked at the 1 mg $kg^{-1}$ level. 1.0000 g of dried seaweed sample was homogenized with 2.00 g of LC-8 in a glass mortar with a pestle for 5 min. The final mixture was transferred into a 12-mL SPE cartridge containing a dual sorbent layer of 1 g Supelclean™ ENVI-Carb II/PSA (500/500 mg). Once packed, MSPD/SPE columns were connected to a Visiprep® vacuum distribution manifold and were washed with 10 mL of hexane. Elution was performed with 20 mL of ethyl acetate and 5 mL of acetonitrile (80:20) and the obtained eluate was evaporated to a drop in a rotary-evaporator and brought to dryness by a gentle nitrogen stream. The residue was reconstituted in 1 mL methanol:water (1:1) ratio and the solution was filtered through a 0.45 μm PTFE syringe filter.

### 2.5. HPLC-DAD conditions

The chromatographic analysis was carried out using the following ACN:$H_2O$ gradient elution: ACN initial percentage of 30% (8 min) increased linearly to 40% in 5 min, increased to 50% in 5 min, after which the percentage was returned to the initial conditions in 9 min. A constant mobile phase flow rate of 1 mL $min^{-1}$ and 20 μL of sample volume were used.

The absorbance was measured continuously in the 200–400 nm range and peak areas quantification was carried out at 222.7 nm in order to achieve maximum sensitivity. All triazine herbicides were identified initially by retention time and then by applying spectral contrast techniques (incorporated in Millenium$^{32}$ software) the homogeneity of the spectral peak was confirmed. Finally a spectral identification was carried out contrasting the spectrum with a standard library created in the wavelength interval of 200–400 nm.

## 3. Results and discussion

### 3.1. MSPD optimization

In MSPD the analyte interacts with the solid support, the bonded phase and the dispersed matrix. For blending the sample, a glass mortar was used in order to avoid analyte losses that could occur with the use of materials such as porcelain [23]. In this technique the polarity of the sorbent and elution solvent are the key factors to achieve effectiveness of the extraction and purity of the final extract. Most methods reported have used reversed-phase materials bonded silica as the solid support. Theoretically, silica particles facilitate disruption of biological samples whereas the bonded alkyl chains contribute to dispersion and retention of lipophilic compounds [29]. $C_8$ and $C_{18}$ are by far the most popular sorbents for analyte extraction from plant tissues; in this study $C_8$ was chosen as dispersant because it has been observed that $C_8$-bonded silica provided a more selective extraction with less co-elution compounds [30].

Although in some cases the MSPD extracts are clean enough to be directly subjected to instrumental analysis, a clean up step is often desirable. For this purpose an on-line clean-up step can be integrated into the sample preparation process by placing a layer of co-sorbent, obviously with different sorption behavior, at the bottom of MSPD cartridge. Based on our experience on clean up of animal feed extracts [31], solid phase extraction was carried out employing a dual-layer tube containing carbon/primary secondary amine (ENVI™-Carb II/PSA) sorbents separated by PE frit. ENVI-Carb II is a graphitized non-porous carbon that has a strong affinity towards planar molecules and can remove pigments (e.g., chlorophyll and carotenoids), and sterols. PSA is a polymerically bonded ethylenediamine-N-propyl phase that contains both primary and secondary amines, which retains fatty acids, organic acids, sugars and some polar pigments [32].

Selection of elution solvent is a function of analyte polarity, since the target analytes should be efficiently desorbed while the remaining components should be retained in the column. The generally employed solvents in the literature for elution of triazines in vegetable extracts from MSPD–SPE systems are acetonitrile [25], dichloromethane [27] and specially ethyl acetate [26,28,33]. In this study ethyl acetate and acetonitrile were tested; dichloromethane was avoided, according to principles of green chemistry, for being a chlorinated solvent.

The optimization of the MSPD–SPE procedure was carried out by spiking the sorbents with 1 mL of a standard solution containing 1 mg $L^{-1}$ of each triazine. As ethyl acetate is the most common solvent for elution of triazines in MSPD procedures, the time consumed in the evaporation step is much lower with ethylacetate than with acetonitrile; hence preliminary assays were carried out with ethyl acetate. In order to determine solvent volume required for the complete elution of the target analytes, eluates of 5 mL were collected and the obtained results showed that at least 15 mL of ethyl acetate was necessary for recovering all compounds from the MSPD–SPE system. Recoveries obtained were higher than 90% for all compounds except for simetryn (70%), which although had a lower value than for the rest of triazines, it is acceptable in trace analysis. However, with a view for improving the recovery of simetryn, the sequence of 10 mL of ethyl acetate and 5 mL of acetonitrile was assayed and recoveries obtained were similar to those achieved with ethyl acetate for most of compounds but recovery of simetryn increased until 86%. Therefore the combination of 10 mL ethyl acetate + 5 mL acetonitrile was selected as elution solvent.

The selected conditions were applied to samples of sea lettuce, which did not contain triazine residues at detectable concentrations. For this purpose, 1.0000 g of dried seaweed was spiked at the 1 mg $kg^{-1}$ level (equivalent to 0.08 mg $kg^{-1}$ of fresh tissue) and subjected to the procedure previously optimized with standards. Recoveries obtained for spiked seaweed were between 50% and 65% for all analytes which implies a strong interaction of all studied compounds with the matrix. Therefore additional volumes of ethyl acetate and/or acetonitrile were collected but very unsatisfactory recoveries were obtained in all cases, and only the tandem of 20 mL ethyl acetate and 5 mL acetonitrile achieved recoveries higher than 60% for all compounds. Several authors have pointed out that washing the column with a solvent, such as hexane or water, prior to elution of the target analytes can have a huge influence on the performance of the MSPD method [29,30]. Therefore an experiment on rinsing the sample with 10 mL of hexane, prior to analytes elution with 20 mL ethyl acetate and 5 mL acetonitrile, was carried out. With this procedure, schematized in Fig. 1, satisfactory recoveries were obtained for all triazines (up to 80%). As an illustration of the results obtained, Fig. 2 shows the chromatograms corresponding to unspiked and spiked sea lettuce extracted and purified under the procedure considered.

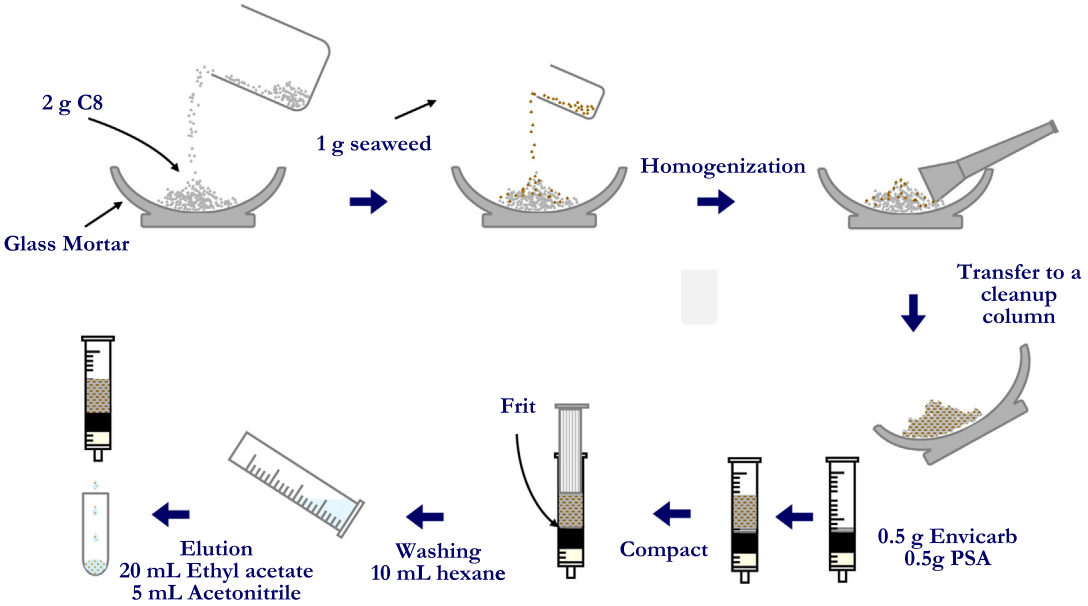


**Fig. 1.** Scheme of the final conditions of MSPD–SPE procedure.

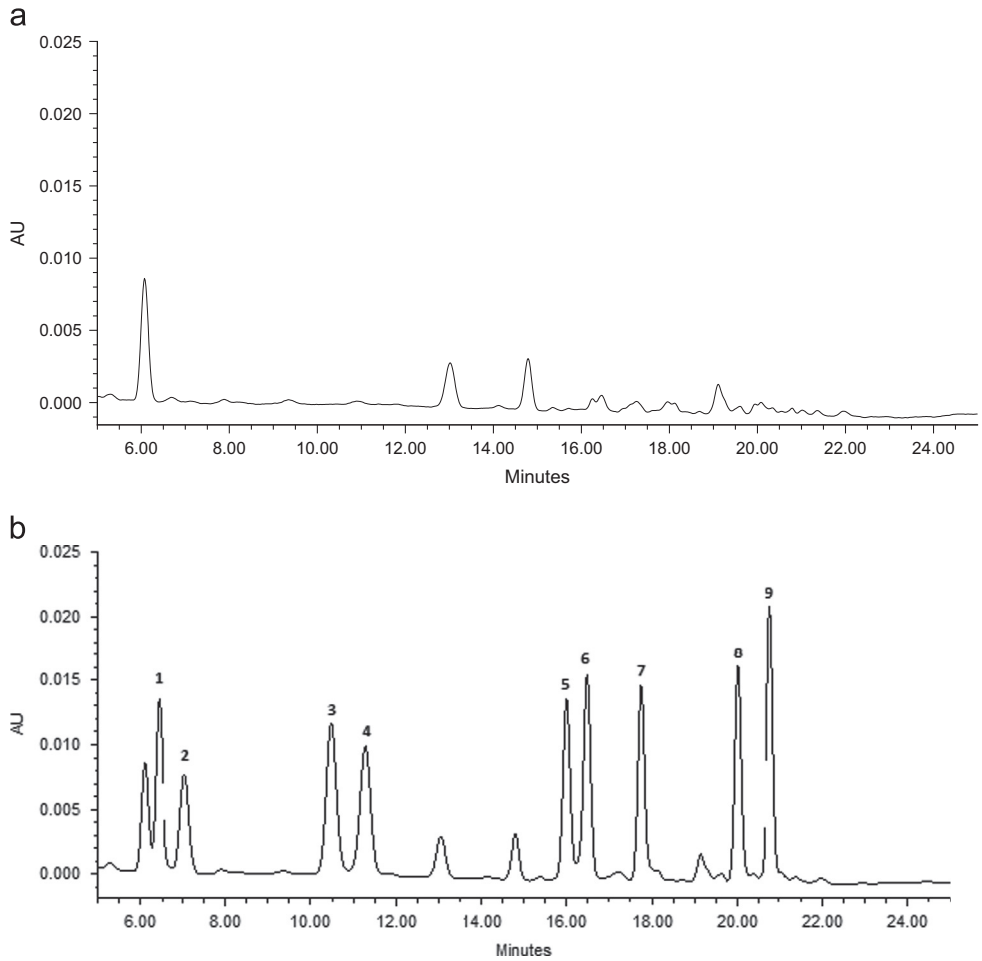


**Fig. 2.** (a) MSPD sea lettuce extract chromatogram and (b) MSPD spiked sea lettuce extract chromatogram. Target compounds are numbered as follows: (1) simazine, (2) cyanazine, (3) simetryn, (4) atrazine, (5) ametryn, (6) propazine, (7) terbuthylazine, (8) prometryn, and (9) terbutryn.

### 3.2. Method validation

The method was validated in terms of linearity, recoveries, precision and limits of detection and quantification. The validation data are presented in Table 1.

All quantitative results were calculated using matrix matched standards prepared by spiking the final extracts from un-spiked samples of sea lettuce with different levels of triazines as recommended by the European guidelines [34]. Good linearity of the calibration curves was obtained for all compounds over the whole range (0.1–2 mg $kg^{-1}$ dried sample) evaluated by duplicate analysis at five different concentration levels. The target compounds showed coefficients of determination ($R^2$) higher than 0.9992 for all triazines except for prometrin and terbutryn which showed only slightly lower $R^2$ ($>$ 0.995).

The matrix effect was evaluated since signal suppression or enhancement can severely compromise quantitative analysis of the compounds at trace levels, and therefore can greatly affect the method reproducibility and accuracy. The matrix effect was studied by comparison of the slopes of the calibration curves in solvent and in the extract obtained after the MSPD procedure. Both the solvent and the matrix calibration curves had good linearity, with determination coefficients higher than 0.9999 for solvent calibration curves and 0.9952 for matrix matched ones. All compounds showed a strong matrix effect since the deviation of the matrix calibration slope from the solvent calibration slope was higher than 20% (between 29% and 42%), which indicated the need of using matrix matched standards for quantification purposes.

**Table 1**
Validation data for the MSPD-HPLC-DAD method obtained employing sea lettuce seaweed.

| Compound | LOD[a] (μg $kg^{-1}$) | LOQ[a] (μg $kg^{-1}$) | Correlation coefficient ($R^2$) | Recovery ± RSD (%) ($n=5$) | |
|---|---|---|---|---|---|
| | | | | **0.1 mg $kg^{-1}$** | **1 mg $kg^{-1}$** |
| Simazine | 3.6 | 6.5 | 0.9997 | 81.2 ± 6.3 | 88.2 ± 0.9 |
| Cyanazine | 3.0 | 7.3 | 0.9998 | 80.2 ± 5.7 | 79.5 ± 0.6 |
| Simetryn | 3.8 | 6.2 | 0.9999 | 80.8 ± 5.3 | 83.0 ± 2.5 |
| Atrazine | 2.7 | 5.3 | 0.9992 | 85.9 ± 5.5 | 81.0 ± 2.8 |
| Ametryn | 2.9 | 5.9 | 0.9997 | 90.8 ± 7.3 | 83.0 ± 1.0 |
| Propazine | 3.6 | 6.0 | 0.9992 | 91.7 ± 5.1 | 91.8 ± 0.6 |
| Terbuthylazine | 1.5 | 4.3 | 0.9996 | 76.0 ± 7.4 | 84.8 ± 1.7 |
| Prometryn | 1.4 | 4.1 | 0.9973 | 82.8 ± 7.2 | 96.6 ± 2.8 |
| Terbutryn | 1.6 | 4.5 | 0.9952 | 86.1 ± 3.6 | 83.2 ± 1.1 |

[a] Results expressed in μg $kg^{-1}$ fresh sample.

The accuracy and precision of the proposed method were investigated by analysis of five replicates of uncontaminated sea lettuce samples spiked at two different concentration levels (1 and 0.1 mg $kg^{-1}$ dried sample, equivalent to 0.08 and 0.008 mg $kg^{-1}$ fresh sample respectively). The lowest spiked concentration was selected to test the method performance at the recent restrictive residue level set by European Union Legislation for simazine in edible seaweeds [35]. The obtained results demonstrated that the method achieved satisfactory recoveries in the range of 75–100% in all cases, with associate standard deviations below 7% for all compounds,

**Table 2**
Mean recoveries and RSD values of triazines in wakame and nori samples ($n=5$) spiked at two levels applying the MSPD-HPLC-DAD method.

| Compound | Wakame | | Nori | |
|---|---|---|---|---|
| | 0.1 mg kg$^{-1}$ | 1 mg kg$^{-1}$ | 0.1 mg kg$^{-1}$ | 1 mg kg$^{-1}$ |
| | Recovery ± RSD (%) ($n=5$) | | | |
| Simazine | 98.7 ± 4.7 | 90.4 ± 4.1 | 83.6 ± 6.7 | 85.9 ± 1.5 |
| Cyanazine | 87.0 ± 3.4 | 85.7 ± 1.2 | 64.8 ± 8.8 | 87.5 ± 1.9 |
| Simetryn | 94.2 ± 4.5 | 84.6 ± 0.3 | 65.1 ± 3.0 | 85.5 ± 1.4 |
| Atrazine | 92.3 ± 0.5 | 89.1 ± 1.4 | 78.6 ± 1.5 | 89.1 ± 1.7 |
| Ametryn | 102.1 ± 3.9 | 89.6 ± 1.2 | 77.5 ± 2.2 | 91.0 ± 0.8 |
| Propazine | 92.9 ± 6.8 | 90.5 ± 1.5 | 64.6 ± 3.3 | 90.5 ± 1.2 |
| Terbuthylazine | 86.0 ± 1.0 | 90.8 ± 1.4 | 70.4 ± 2.4 | 86.7 ± 1.4 |
| Prometryn | 120.2 ± 3.1 | 91.0 ± 1.8 | 70.9 ± 2.8 | 86.8 ± 0.7 |
| Terbutryn | 99.1 ± 0.7 | 89.4 ± 1.8 | 64.7 ± 5.2 | 85.4 ± 1.0 |

which are in the acceptance range [34]. The obtained recoveries and relative standard deviation are comparable or even better than those provided by other authors for the determination of some of these pollutants by MSPD in fruits and vegetables [27,28,33].

The limits of detection (LODs) and limits of quantification (LOQs) were calculated as the minimum amount of target analyte that led to a chromatogram peak with a signal-to-noise ratio of 3 and 10 respectively, determined experimentally from fortified samples [34]. As can be seen in Table 1, the LODs varied from 1.4 to 3.8 μg kg$^{-1}$ and LOQs values varied from 4.1 to 7.3 μg kg$^{-1}$ in fresh sample. Therefore, the obtained LODs and LOQs were satisfactory and allowed the determination of these compounds at the levels required by the legislation of seaweed for human consumption. By comparing the LODs obtained by the proposed methodology with those reported in the literature using MSPD in horticultural matrices, better sensitivity is attained [27,28,33].

Reliability of the method was evaluated in terms of recovery by spiking two edible seaweed samples: a red one (nori) and a brown one (wakame) at a concentration level of 1 and 0.1 mg kg$^{-1}$ dried samples. The analytical recoveries, calculated using matrix matched standards, obtained for five replicates ($n=5$) of the samples spiked with the triazine herbicides are presented in Table 2. As can be seen the recovery values obtained for wakame ranged between 85% and 100% for all compounds, except for prometrin at the low level (120%), with RSD lower than 7%. In the case of nori recoveries fluctuated from 85% to 90% with RSD values below 2% at the high level whereas they were between 65% and 85% with RSD below 9% at the low level. Therefore we can conclude that this method could be established as a suitable method for routine analysis to screen trace levels in different types of seaweed in compliance with EU directives.

## 4. Conclusion

A procedure for the analysis of nine triazines from seaweed samples based on MSPD and SPE has been developed. The method uses $C_8$ as dispersant with ENVI-Carb™/PSA co-column and *n*-hexane as washing solvent followed by a combination of 20 mL ethyl acetate and 5 mL acetonitrile as elution solvent. The developed method provides satisfactory accuracy and precision for the determination of triazines in seaweed with LODs and LOQs adequate to carry out analysis of samples in the concentrations required by the European Union regulations. The method was successfully applied to the analysis of three seaweed samples (sea lettuce, wakame and nori). The main advantages of this methodology when compared with conventional methods of sample preparation to screen triazines in vegetable matrices are easy of work-up, fast, low cost, avoidance of clean-up procedure, as well as the significant reduction of organic solvents in agreement with the principles of the Green Chemistry.

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