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Screening of Contaminants of Emerging Concern in Microalgae Food Supplements

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Received: 20 April 2020; Accepted: 14 May 2020; Published: 20 May 2020

Abstract: The frenetic lifestyle in the developed countries has driven us to be deficient in some nutrients, which may be overcome by supplements. Microalgae, like spirulina (*Arthrospira platensis*) and chlorella (*Chlorella* ssp.) are widely used as supplements due to their high contents of macro- and micronutrients. Chlorella and spirulina can be grown naturally in a range of water bodies, showing their high adaptability to harsh environments. They are mainly produced in countries with poor water quality and sometimes inexistent water legislation, which can be a vector of micropollutant introduction into the food chain. Thus, a method for the simultaneous determination of 31 emerging contaminants commonly found as micropollutants in freshwater (pharmaceutical and personal care products, hormones, flame retardants and biocides) in two microalgae is presented. Target contaminants were extracted from the microalgae employing ultrasound-assisted matrix solid-phase dispersion followed by gas chromatography-mass spectrometry analysis. The method was validated for chlorella and spirulina with recoveries ranging from 70% to 111% at concentrations of 25 and 100 ng·g^{-1}, and good linearity in the range from 5 to 400 ng·g^{-1} with limits of detection below 2.5 ng·g^{-1}, in both microalgae. The method validated was applied to a range of microalgae supplement foods and the results proved that the compounds studied were below limits of detection.

Keywords: *Arthrospira platensis*; *Chlorella* ssp.; GC-MS; MSPD; SLE; pharmaceutical; pesticides

1. Introduction

The human population is increasing and by 2050 it will probably be larger by 2 to 4 billion people [1]. As the population continues to rise, the demand for high nutritive food and healthy products will increase as well. Due to the diverse nutritional components found in microalgae, its rapid growth and environmental and health benefits [2], the demand for microalgae is on rise. Microalgae have been postulated to improve the nutritional content of conventional foods, as food or dietary supplements, prebiotic agents or in therapeutic applications with a positive effect on human health. This is mainly due to the presence of compounds such as fiber, carbohydrates, lipids, unsaturated fatty acids (with double bonds in ω-3 and ω-6), vitamins, pigments, polyphenols and minerals [3,4]. Particularly, chlorella (*Chlorella* ssp.), a unicellular green alga found in fresh and marine water, is widely sold as a healthy food and generally recognized as safe (GRAS) by the U.S. Food and Drug Administration. Studies carried out on pure extracts or isolated molecules of chlorella (mainly in vitro) have demonstrated its potential benefits to treat and prevent many diseases due to its anti-inflammatory [5] and antimicrobial

activities [6], antitumor activity [7], cholesterol-lowering properties [8], antiproliferative activity [3] or a higher antioxidant activity in comparison with other microalgae [9]. Spirulina (*Arthrospira platensis*) is a prokaryotic blue-green microalga (cyanobacteria) that is grown naturally in warm climates [10,11]. Spirulina has been used as food for centuries in Mexico, Chad and Myanmar [12]. It was the first cyanobacterium to be commercially cultivated and has been produced to be used as food supplements, due to the numerous potential benefits to human health, such as diabetes treatment [13], hypertension treatment [14], antiviral activity [15], anticancer properties [16] and to treat certain allergies and inflammatory processes [17].

Microalgae are mainly produced in countries where the legislation regarding water quality is very poor and contaminants may be introduced into the human food chain. On the other hand, because the price of biomass is high, ranging from 30 to 300 € kg^{-1} depending on the strain, the biorefinery industry of microalgae tries to minimize the inputs of the process, using wastewater as the cultivation medium [18]. However, several contaminants of emerging concern (CECs) such as pharmaceutical compounds, hormones, personal care products, biocides and flame retardants have been detected in wastewaters all over the world [19–21] and can be introduced into the food chain [22,23]. Moreover, the use of manure to substitute the nutrients input, such as poultry, pig and dairy manures [24–27], may be a path to introduce toxic compounds into the microalgae as CECs have been already reported in these matrices [28,29].

Since supplements containing microalgae are on the rise, the European Union found the necessity to harmonize and regulate the vitamin and mineral content of food supplements and fortified foods [30,31]. However, this directive misses targeting undesirable compounds such as CECs that may be present in the microalgae. Thus, Directive 37/2010 [32] on pharmacologically active substances in food of animal origin and SANTE/11813/2017 Guideline [33] on pesticide residues in food can be used as a reference. Hence, a methodology for the simultaneous determination of 31 CECs in chlorella and spirulina, based on a previous work where these analytes were determined in aquatic plants [34], was tested, validated and applied to assess the presence of these contaminants in commercialized microalgae food supplement products.

2. Materials and Methods

2.1. Standards and Reagents

Standards of methyl triclosan, triclosan and pyrethroids (bifenthrin, fenpropathrin, λ- cyhalothrin, permethrin, cyfluthrin, α-cypermethrin, τ-fluvalinate, esfenvalerate and deltamethrin) (all purity >99%) were supplied by Riedel-de Haën (Seelze, Germany). Tris(2-carboxyethyl)phosphine (TCEP) and tris(2-chloroisopropyl)phosphate (TCPP), were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Standards of methyl and propyl paraben, ibuprofen, nonylphenol, gemfibrozil, fenoprofen, benzophenone-3 (BP3), naproxen, mefenamic acid, ketoprofen, carbamazepine, 2,2′,4,4′-tetra-bromodiphenyl ether (BDE-47), fenofibrate, bisphenol A (BPA), 2,2′,4,4′,6-penta-bromodiphenyl ether (BDE-100), hexestrol, diethylstilbestrol and estrone (all purity > 97%), were purchased from Sigma-Aldrich (St Louis, MO, USA). Individual stock solutions of each compound were made up at 50 μg mL^{-1} in acetonitrile (ACN) and stored in amber flasks at −18 °C. A stock mixture solution of 1 μg mL^{-1} containing all analytes was prepared by dilution with ACN. A working solution at 500 ng mL^{-1} was prepared weekly by dilution with ACN of the stock mixture solution and stored at 4 °C up to 8 weeks to ensure their stability.

Ethyl acetate (EtAc) and ACN residue analysis grade, ammonium hydroxide (NH_4OH) ≥32% and Silica Bondesil-C18 (particle diameter of 40 μm) were purchased from Varian (Palo Alto, CA, USA). Florisil 150–250 μm (60–100 mesh) was supplied by Aldrich (Steinheim, Germany) and magnesium sulfate anhydrous ($MgSO_4$) was purchased from Merck (Darmstadt, Germany). The derivatization agent N-(tert-butyldimethylsilyl)- N-methyl-trifluoroacetamide (MTBSTFA, purity

≥95%) with 1% tert-butyldimethylchlorosilane (TBDMCS) and formic acid were obtained from Sigma-Aldrich (St Louis, MO, USA).

2.2. Microalgae

Chlorella (*Chlorella sorokiniana*, strain 0002) was obtained from the Spanish Bank of Algae, Marine Biotechnology Center, University of Las Palmas de Gran Canaria, Spain and spirulina (*Arthospira platensis*) was provided by Professor Luis Mª Lubián from the Andalusian Institute of Marine Science, CSIC, Puerto Real, Cádiz, Spain. Both microalgae were cultivated aseptically in 1 L conical flasks to provide biomass to select a suitable method for the detection of CECs and validation. Both cultures were initiated with an optical density of 0.1 (measured with a spectrophotometer Pharmacia Biotech Ultrospec 2000 UV/Vis at 650 nm wavelength, pellet was centrifugated and dried during 48 h in a suitable dryer at 100 °C), corresponding to a density of 15 mg dry weight L^{-1}. Spirulina medium was kept at pH 8 with the needed nutrients [35] whereas chlorella medium used was kept at pH 7.3 [36]. Both microalgae were kept in an environmental chamber at a temperature controlled at 25 °C, illuminated with cool white fluorescent tubes (F58W/GRO) (SYLVANIA GRO-LUX F58W/GRO 5FT T8 58W, Erlangen, Germany) with an intensity of 132 µmol $s^{-1}m^{-2}$ and a light/dark cycle of 16/8 h. The culture was aerated with filtered air (0.2 µm vent filter) at a rate of 2 L min^{-1} through a mechanical air pump (KNF LABOPORT Mini Diaphragm Vacuum Pump N 86 ProfiLab24, Freiburg, Germany). The cells were harvested at exponential phase, around 14 days of cultivation, with a concentration of 1004.8 mg dry weight L^{-1} (0.1% dry matter and 99.9% moisture content) calculated from an optical density of 0.70 for spirulina and 1392.77 mg dry weight L^{-1} (0.14% dry matter and 99.86% moisture content) calculated from an optical density of 0.93 for chlorella. Then cells were centrifuged at 4000 rpm (2630× *g*) for 15 min at 25 °C. The supernatant was discarded, and the algae pellets were collected.

Seven products acquired from shops specialized in food supplements (four containing pure spirulina and the rest pure chlorella) were used as real samples to assess the presence of contaminants of emerging concern.

2.3. Sample Preparation

2.3.1. Method Extraction Selection

Two extraction methods, one classical and the other a more novel procedure, were tested to evaluate their extraction efficiency of CECs from spirulina. The best performing method was then validated with both microalgae (spirulina and chlorella) and consequently applied to microalgae food supplements. The extraction yields obtained with both methods was evaluated spiking spirulina with a mixture of 17 representative CECs.

Spirulina material pelleted was weighed (1.0 g) and spiked with 200 µL of a working mixture solution of targeted compounds (500 ng·mL^{-1}), reaching a final concentration of 100 ng·g^{-1}, allowing 24 h of rest at 4 °C to reach equilibrium before extraction with methods described below.

A classic solid–liquid extraction (SLE) was performed placing the spiked 1.0 g pelletized spirulina into a 15 mL screw-cap glass tube that contained 4.0 g of Florisil and 4.0 g of $MgSO_4$. EtAc with 3% NH_4OH (10 mL) was added and stirred intensively by magnetic agitation for one hour. Then, it was centrifuged at 4000 rpm (2630× *g*) for 4 min. The supernatant was transferred to a graduated glass tube and evaporated to dryness using a Genevac EZ-2 evaporator (NET Interlab, S.A.L., Madrid, Spain). A second extraction cycle was performed to the remaining pellet with 10.0 mL ACN containing 4% formic acid. After centrifuging, the supernatant was transferred to the same graduated tube in which the extract of the previous extraction step was collected and dried. The extract was evaporated to a final volume of 1.0 mL.

Ultrasound assisted-matrix solid-phase dispersion (UA-MSPD) was performed as described in Figure 1 carrying out three extraction cycles. In a glass mortar, the spiked 1.0 g of spirulina pellet

was mixed with 4.0 g of Florisil and 2.0 g of $MgSO_4$ and blended with a glass pestle for 5 min to reach complete homogenization. The sample is then placed in a 20 mL glass column (10 cm × 20 mm I.D., from Becton-Dickinson, Madrid, Spain) with 2 paper filters (Whatman No. 1 paper circles of 2 cm diameter, Maidstone, UK) at the end and 2.0 g of $MgSO_4$. In a first extraction cycle, EtAc with 3% NH_4OH (8.0 mL) was added to the column and 2.0 mL were used to wash the glassware and added to the column. The column was closed with a one-way stopcock and sonicated at room temperature for 15 min in an ultrasonic water bath (Branson Ultrasonics, 40 Hz, Carouge, Switzerland). Extracts were collected in tubes using a multiport vacuum manifold (Supelco, Visiprep, Madrid, Spain) and evaporated to dryness. The second extraction cycle was carried out adding 5.0 mL ACN containing 4% formic acid to the column and sonicated for 15 min before collecting the extract in the same tube where the extract from the first extraction cycle was evaporated to dryness. The extraction was done with another 5.0 mL ACN containing 4% formic acid and sonicated 15 min. The solvent was collected in the same tube combining the extracts and evaporated to 1.0 mL (same volume as SLE to be able to compare results).

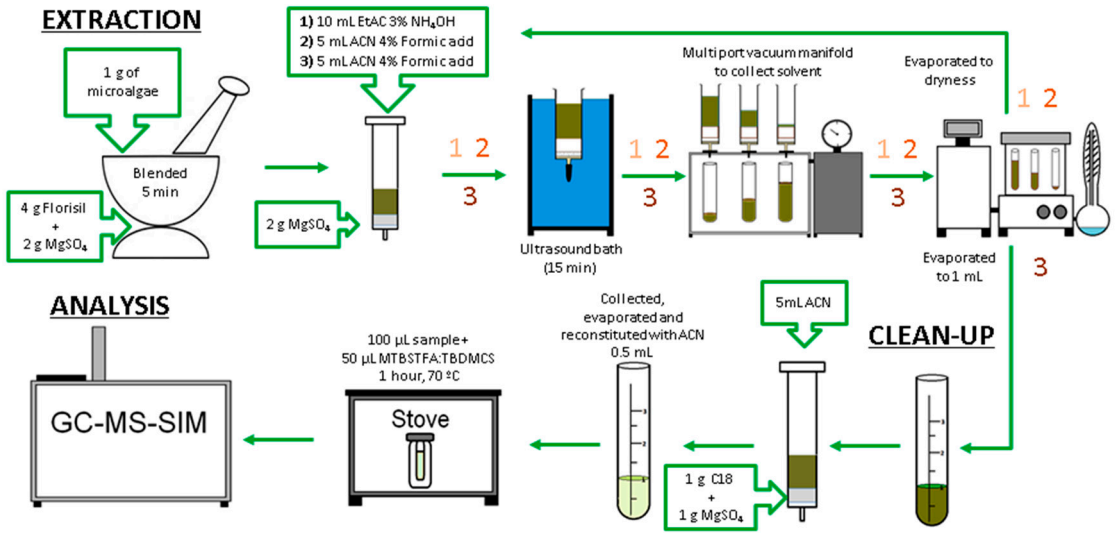

Figure 1. Workflow of the ultrasound assisted-matrix solid-phase dispersion (UA-MSPD) method used to extract contaminants of emerging concern from microalgae.

2.3.2. Clean-Up

All algae extracts (1.0 mL), using the above described extraction techniques, were purified through a 5 mL glass column (Normax, Lisbon, Portugal) with 2 paper filters (Whatman No. 1, Maidstone, UK) containing 1.0 g of $MgSO_4$ and 1.0 g of C18. Analytes were eluted with 5.0 mL of ACN and extracts were collected in tubes using a multiport vacuum manifold, evaporated to dryness and reconstituted to 0.5 mL with ACN before their derivatization.

2.3.3. Derivatization

Prior to the gas chromatographic analysis, some of the studied analytes need to be derivatized to increase their volatility and stability. The derivation agent MTBSTFA: TBDMCS (99:1, v/v) was selected following previous experience with the target contaminants [37,38]. Thus, an aliquot (100 µL) of the microalgae extract was transferred to a 250 µL micro insert placed within a 2 mL glass vial and 50 µL of derivatization agent were added. Then, vials were closed, vortexed and the mixture left to react for 1 h at 70 °C before analysis.

2.4. Chromatographic Analysis

Gas chromatography-mass spectrometry (GC-MS) analysis was performed on an Agilent 7890A (Waldbronn, Germany) gas chromatograph coupled to a mass spectrometer (HP 5977A) equipped with an automatic injector. Separations were carried out using a ZB-5MS column, (30 m × 0.25 mm i.d. and 0.25 μm film thickness) from Phenomenex (Torrance, CA, USA). Helium (purity 99.995%) was used as carrier gas at a flow-rate of 1.2 mL/min. Solvent-vent mode operating conditions were as follows: 2 μL of plant extracts were injected in a simple-taper glass liner with glass wool. The injection port temperature was programmed to start at 50 °C (held 0.1 min) and reach 300 °C at 600 °C min^{-1} (held 5 min). The split vent was open for 0.1 min with an inlet pressure of 5 psi and a flow rate of 100 mL min^{-1} and then closed for analyte transfer into the column. After 2.6 min, the purge value was activated at a 60 mL min^{-1} flow rate. The column temperature was maintained at 50 °C for 2.6 min, then programmed at 20 °C min^{-1} to 300 °C and held for 5 min. The total analysis time was 20.1 min.

The mass spectrometric detector was operated in electron impact ionization mode with an ionizing energy of 70 eV. Ion source and transfer line temperatures were 230 and 280 °C, respectively. Retention time and mass spectra of all analytes were acquired in the full scan mode (mass range from 50 to 600 m/z). Selected Ion Monitoring (SIM) mode was employed for quantitative analysis, using one target and two qualifier ions to identify each analyte. Table 1 lists the compounds with their retention times and ions selected for the analysis. The compounds were confirmed by their retention times, the identification of target and qualifier ions and the determination of qualifier to target ratio. Retention times must be within ±0.1 min of the expected time and qualifier-to-target ratio within a 20% range for positive confirmation. The quantification was accomplished by matrix-matched calibration to overcome the matrix effect produced in GC-MS by complex matrices [39].

2.5. Method Validation

Several quality parameters were assessed through the process of method validation: recovery, precision, limits of detection (LOD), limits of quantification (LOQ), linearity and matrix effect [33]. Both microalgae (chlorella and spirulina) were spiked with the target contaminants at two levels (25 and 100 $ng{\cdot}g^{-1}$; $n = 4$), to study the extraction efficiency. The precision of the analytical procedure was evaluated as the relative standard deviation (RSD) of the recovery test. The limits of detection (LODs) and limit of quantification (LOQs) of the selected method were determined analyzing ten replicates of blank extracts of both microalgae, spiked at 5 $ng{\cdot}g^{-1}$. The equation to calculate the LOD was as follows (1):

$$LOD = t_{99} \times SD \quad (1)$$

where t_{99} is the Students' value for a 99% confidence level with n-1 degrees of freedom and SD is the standard deviation of the replicate analyses. The LOQ was calculated as 10 times the SD of the results of the replicate analysis used to determine LOD. The linearity and matrix effect were studied by analyzing two sets of seven calibration points each, one set was prepared in neat solvent (ACN) and the other was prepared spiking blank microalgae extracts (400 μL) with 100 μL of the corresponding standard solution in ACN to reach the same concentration range (5 to 400 $ng{\cdot}g^{-1}$).

Table 1. Use, retention times and mass spectrometric parameters of the studied analytes.

Name	Use	t_R	SIM Parameters		
			T	Q_1	Q_2
Methyl Paraben (tBDMS)	Preservative	11.27	209	210	266
TCEP	Plasticizer	11.38	249	250	63
TCPP	Plasticizer	11.57	125	99	277
Ibuprofen (tBDMS)	NSAID	11.85	263	264	117
Propyl Paraben (tBDMS)	Preservative	12.15	237	238	294
Methyl Triclosan	Antifungal	13.38	302	304	252
Nonylphenol (tBDMS)	Surfactant	13.38	334	277	278
Gemfibrozil (tBDMS)	Lipid regulator	13.45	243	179	307
Fenoprofen (tBDMS)	NSAID	13.68	299	197	206
Benzophenone-3 (BP3) (tBDMS)	Sunscreen	14.11	285	242	286
Naproxen (tBDMS)	NSAID	14.13	287	185	288
Triclosan (tBDMS)	Antifungal	14.34	347	345	200
Mefenamic acid (tBDMS)	NSAID	14.64	298	224	355
Ketoprofen (tBDMS)	NSAID	14.65	311	295	105
Bifenthrin	Pesticide	14.80	181	165	166
Fenpropathrin	Pesticide	14.87	125	181	265
Carbamazepine (tBDMS)	Antiepileptic	14.95	193	194	293
BDE-47	Flame retardant	15.16	486	326	488
Fenofibrate	Lipid regulator	15.19	121	273	139
λ-Cyhalothrin	Pesticide	15.24	197	181	208
Permethrin	Pesticide	15.74	183	163	165
BPA (tBDMS)	Plasticizer	15.70	441	442	456
BDE-100	Flame retardant	15.92	404	406	566
Cyfluthrin	Pesticide	16.06	163	206	226
Hexestrol (tBDMS)	Hormone	16.35	249	250	337
α-Cypermethrin	Pesticide	16.41	163	165	181
Diethylstilbestrol (tBDMS)	Hormone	16.50	496	497	498
τ-Fluvalinate	Pesticide	17.09	250	252	181
Estrone (tBDMS)	Hormone	17.10	327	384	328
Esfenvalerate	Pesticide	17.20	125	167	181
Deltamethrin	Pesticide	17.74	181	253	251

tBDMS, tert-butyldimethylsilyl ethers group formed after derivatization of -OH groups; NSAID, nonsteroidal anti-inflammatory drug; t_R = retention time, min; T = target ion, m/z; Q_1 and Q_2 = qualifier ions, m/z.

2.6. Statistical Analysis

The overall datasets are expressed as mean values of four replicates. ANOVA test and Tukey–Kramer's HSD test were used to determine significant differences (>95%) among treatments using the XLSTAT 2016 software (Addinsoft, Paris, France).

3. Results and Discussion

3.1. Method Selection

An UA-MSPD extraction method developed in our laboratory for the determination of CEC in four different aquatic plants was selected to assay its applicability to other aquatic organisms such as microalgae [34]. In addition, a conventional extraction procedure, SLE was tested employing the same extraction solvents, salts and sorbents as in the UA-MSPD method. In this assay, spirulina was spiked with a mixture containing 17 compounds representative of all the families of targeted compounds, reaching a final concentration of 100 $ng \cdot g^{-1}$, allowing 24 h of rest at 4 °C to reach equilibrium before performing the extraction. The results of this assay are shown in Figure 2.

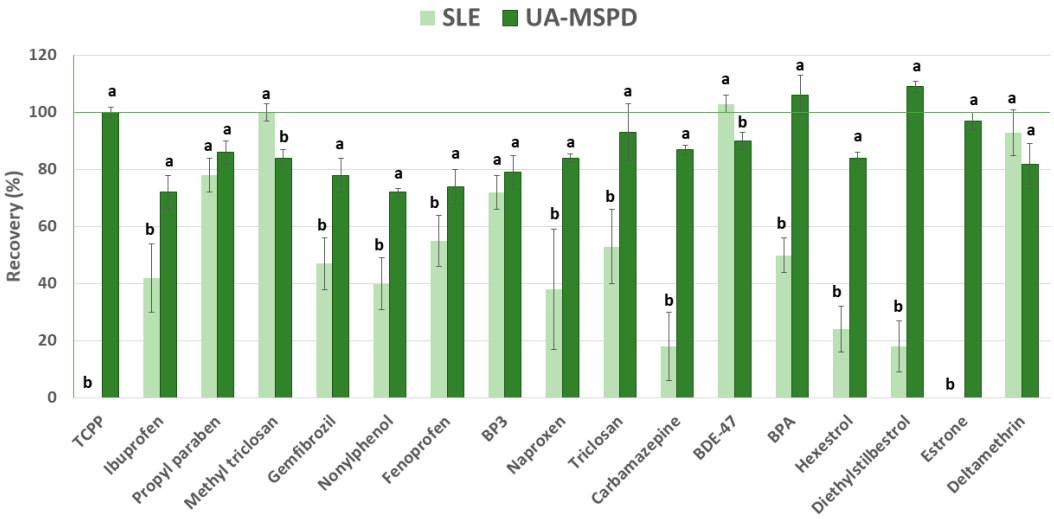

Figure 2. Recovery in percentage of the studied compounds in spirulina spiked at 100 ng·g^{-1} employing two extraction procedures, solid–liquid extraction (SLE) and UA-MSPD. Different letters indicate significant differences ($p \leq 0.05$).

Compounds such as methyl triclosan, BDE-47 and deltamethrin were easily extracted with recoveries close to 100% using SLE. However, other compounds, such as TCPP and estrone were missing or very poor recoveries were obtained for carbamazepine or diethylstilbestrol, which may be explained due to the lack of penetration into the cell as SLE is a non-disrupting cell technique.

Ultrasound-assisted extraction is a mild technique that uses the cavitation effect that produces the ultrasound bath to penetrate into the cell [40], helping the transfer of the target contaminants to the extraction solvent. In addition, the sorbent used in MSPD acts as an abrasive that promotes the disruption of the physical structure of the sample matrix facilitating the extraction of the analytes. Thus, UA-MSPD performed the best results, having recoveries that ranged from 70% to 111%. Hence, UA-MSPD, depicted in Figure 1, was selected to perform method validation with chlorella and spirulina and apply it to food supplements.

3.2. Method Validation

The method was validated using UA-MSPD extraction for each matrix (chlorella and spirulina) in terms of recovery, precision, LODs, LOQs, linearity and matrix effect.

The accuracy of the method was assessed by determining the recovery of 31 CECs from spirulina and chlorella samples spiked with standard solutions at two concentration levels (100 and 25 ng·g^{-1}). Satisfactory recoveries were achieved ranging from 70% to 103% and from 70% to 111% for chlorella and spirulina, respectively (Table 2). The precision was determined by analyzing four spiked samples, where the RSDs were lower than 11% in both cases (Table 2).

Table 2. Recoveries (%) and relative standard deviations (RSD (n = 4), % in parenthesis), limits of detection (LOD) and limits of quantification (LOQ) (n = 10) obtained for target compounds in chlorella and spirulina.

	Chlorella spp.				*Arthrospira platensis*			
	100 ng·g^{-1}	**25 ng·g^{-1}**	**LOD ng·g^{-1}**	**LOQ ng·g^{-1}**	**100 ng·g^{-1}**	**25 ng·g^{-1}**	**LOD ng·g^{-1}**	**LOQ ng·g^{-1}**
Methyl Paraben	70 (8)	82(8)	0.4	1.2	76 (5)	72 (9)	1.8	3.6
TCEP	84 (4)	80 (9)	1.5	3.2	74 (3)	94 (6)	1.5	3.2
TCPP	94 (9)	81 (8)	0.8	2.4	100 (3)	96 (9)	0.9	1.7
Ibuprofen	100 (3)	71 (3)	0.8	2.7	70 (6)	72 (5)	1.3	2.6
Propyl Paraben	89 (3)	80 (6)	0.3	1.0	86 (2)	80 (5)	1.5	2.9
Methyl Triclosan	88 (3)	74 (7)	1.3	4.0	87 (4)	83 (8)	2.3	4.6
Gemfibrozil	86 (6)	81 (5)	0.5	1.5	77 (7)	79 (10)	0.8	2.6
Nonylphenol	87 (2)	83 (8)	0.4	1.5	70 (2)	77 (5)	1.3	2.6
Fenoprofen	78 (9)	71 (3)	0.9	2.6	72 (8)	78 (2)	2.1	4.1
BP3	97 (4)	98 (6)	0.8	2.5	78 (7)	80 (4)	0.8	1.7
Naproxen	82 (8)	98 (2)	1.0	3.1	86 (3)	79 (5)	0.8	1.8
Triclosan	89 (4)	84 (2)	0.3	1.0	94 (11)	80 (7)	2.1	4.0
Mefenamic acid	91 (8)	94 (8)	0.4	1.4	70 (9)	70 (3)	1.3	2.8
Ketoprofen	73 (2)	72 (2)	0.3	1.0	75 (10)	78 (8)	0.7	1.9
Bifenthrin	86 (3)	89 (7)	0.4	1.3	94 (5)	82 (5)	0.3	1.2
Fenpropathrin	84 (3)	89 (7)	1.2	3.6	90 (6)	73 (5)	2.1	3.9
Carbamazepine	90 (2)	83 (2)	0.5	1.5	88 (3)	75 (9)	2.4	4.9
BDE-47	76 (8)	84 (9)	0.7	2.5	89 (3)	82 (6)	1.3	4.5
Fenofibrate	83 (3)	82 (8)	1.0	2.7	89 (5)	83 (5)	1.8	3.9
λ-Cyhalothrin	83 (11)	80 (5)	0.9	3.0	85 (6)	90 (11)	1.9	3.9
Permethrin	83 (2)	83 (8)	1.1	3.6	91 (5)	79 (6)	1.6	4.8
BPA	77 (7)	77 (4)	1.5	3.2	107 (10)	105 (3)	1.0	1.9
BDE-100	86 (2)	80 (9)	1.1	3.6	88 (4)	79 (9)	1.8	5.4
Cyfluthrin	80 (4)	84 (7)	1.0	3.1	87 (5)	82 (8)	1.2	3.6
Hexestrol	90 (0)	80 (7)	0.5	1.8	85 (3)	78 (6)	1.9	3.7
α-Cypermethrin	81 (3)	79 (8)	1.1	3.5	83 (4)	87 (10)	1.1	3.7
Diethylstilbestrol	97 (6)	101 (2)	0.3	1.0	111 (4)	106 (6)	0.7	2.1
τ-Fluvalinate	74 (3)	85 (8)	0.9	2.8	82 (6)	86 (6)	1.2	2.6
Estrone	103 (11)	97 (9)	0.5	1.8	95 (5)	74 (10)	1.2	3.1
Esfenvalerate	82 (11)	74 (6)	0.6	2.0	95 (6)	89 (2)	1.5	4.8
Deltamethrin	80 (5)	73 (11)	1.3	3.3	83 (7)	95 (7)	1.4	3.8

Microalgae are a complex matrix and the different physicochemical properties of the target analytes make the development of a method for the simultaneous determination of 31 CECs difficult. To the best of our knowledge, there are no published papers determining levels of a high number of CECs in microalgae so the results included in this study could not be compared with other obtained using other methods. In a very recent work, BDE-47 was extracted from *Chlorella spp.* after a 24 h Sohxlet extraction, a time-consuming technique, but no information on the performance of the method was provided [41]. Research has focused mainly on the ecotoxicological effects of these compounds in algae [42] or on the application of microalgae on the bioremediation of contaminated water [43]. Regarding the uptake of CECs by microalgae, most of the studies have determined what remains in the aqueous phase rather than the amount taken up by the algae [44,45].

Low limits were obtained due to the high selectivity and sensitivity of mass spectrometry, allowing the determination of these compounds at trace levels in microalgae food supplement. As shown in Table 2, LODs and LOQs for chlorella ranged from 0.3 to 1.5 ng·g^{-1} and from 1.0 to 4.0 ng·g^{-1}, respectively. Similar results were obtained for spirulina matrix, ranging from 0.3 to 2.4 ng·g^{-1} and from 1.2 to 5.4 ng·g^{-1} for LODs and LOQs, respectively.

The linearity of the method was evaluated by comparing the curves obtained by injecting standards in a neat solvent (ACN) and spiked microalgae extracts ranging from 5 to 400 ng·g^{-1} for all studied

compounds. Good linearity was obtained for the 31 CECs studied with correlation coefficients equal to or higher than 0.992. The chromatographic response of target analytes may be affected by the presence of co-extracted components that produce an enhancement transferring analytes from the inlet to the column, having a negative impact on the correct quantification. Figure 3 shows how diethylstilbestrol does not present matrix effect as both calibration curves match perfectly (ACN and spiked matrix) on the opposite, as expected several compounds such as methyl triclosan, BDE-47 and BP3, presented an enhancement of the chromatographic response when injected in matrix extract.

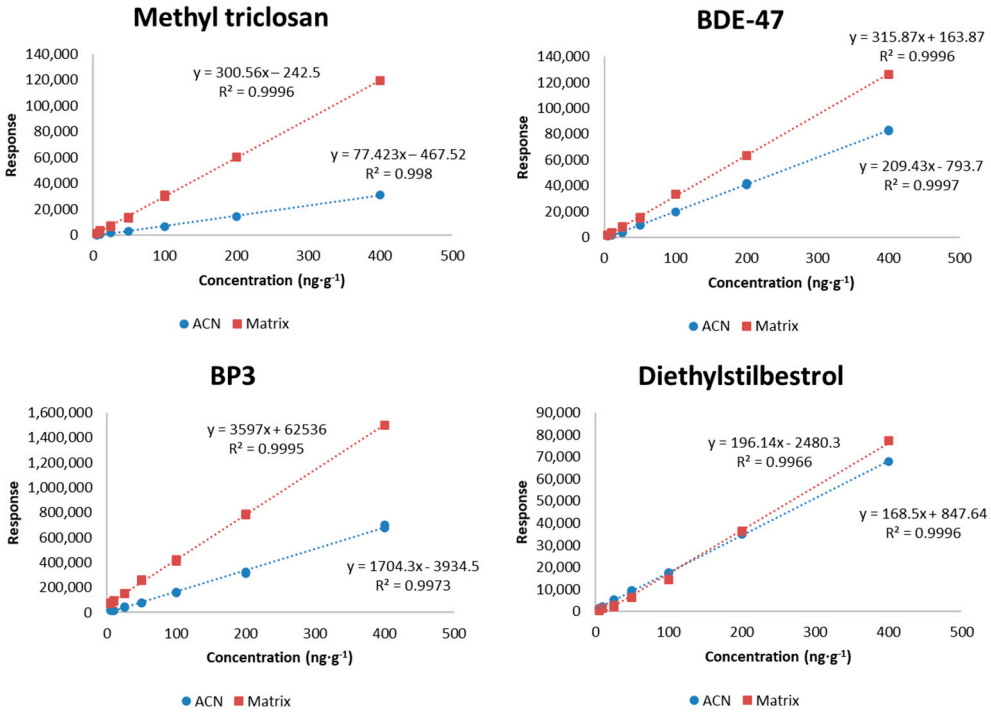

Figure 3. Comparison of seven-point calibration curves of methyl triclosan, BDE-47, BP3 and diethylstilbestrol, obtained by injection of standards in neat solvent ACN and spiked spirulina extracts, ranging from 5 to 400 ng·g^{-1}.

Hence, to overcome matrix effect there are several approaches, but due to the high price and the nonexistence of isotope-labeled standards for all targeted contaminant studied, matrix-matched calibration was selected [37].

3.3. Food Supplements

The UA-MSPD described above was applied to different food supplements containing pure chlorella and spirulina. Samples were hydrated and centrifuged as it was described in Section 2.1 to reproduce the same conditions assayed. The levels in the food supplements analyzed were found to be below the LODs (presented in Table 2). The supplements assessed have shown no risk for human consumption however the use of regained waters or the use of manure during microalgae production will increase over the years as this industry expands so it is necessary to keep screening CECs in these food supplements.

4. Conclusions

A method, based on UA-MSPD, was successfully validated for the determination of 31 CECs in supplements of two different microalgae. The method showed satisfactory recovery results for all the studied compounds and low LODs (<2.5 ng·g^{-1}). After the validation of the recovery, precision, LOD, LOQ, linearity and matrix effect parameters, the method was applied to commercial supplements of

chlorella and spirulina, showing that all the compounds were below the LODs of the proposed method and consequently are safe to be taken. With the expanding market of microalgae, the current method could be used to assess the safety of microalgae supplements due to the increasing use of reclaimed waters and manure that can lead to the introduction of CECs into the food chain. Although this work has provided a sensitive method to detect and quantify CECs in chlorella and spirulina, more studies involving new species that will be accepted for human consumption will be necessary to ensure food safety in the near future.

Author Contributions: Conceptualization, I.M.-G., R.A. and B.A.; methodology, I.M.-G., R.A. and B.A.; validation, E.M., R.A. and B.A.; data analysis, E.M., R.A. and B.A.; writing—original draft preparation, I.M.-G. and R.A.; writing—review and editing, R.A.; B.A. and B.K.T.; project administration, R.A. All authors have read and agreed to the published version of the manuscript.

Funding: Authors wish to thank EU INTERREG Atlantic Area European programme Enhance microalgae project (EAPA_338/2016) for financial support.

Acknowledgments: Authors wish to thank Consuelo Sanchez-Brunete and José Luis Tadeo for their advice in this project.

Conflicts of Interest: The authors declare no conflict of interest.

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