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Safety issues in nutraceutical exploitation of *Chlorella vulgaris*, *Arthrospira Platensis* and *Scenedesmus sp.* microalgae

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ABSTRACT

Microalgae contain many bioactive compounds, which may be exploited in food and nutraceutical fields. Bisphenol A (BPA) is a contaminant in microalgae that may be released from polymeric plastics. Since it is responsible for toxic effects on humans, the European legislation set the legal BPA limit within foods at 50 μg kg^{-1} of food weight. In this work, a fast ultrasounds solid-liquid extraction of BPA from commercial microalgal powders of *Chlorella vulgaris*, *Arthrospira Platensis* and *Scenedesmus sp.* was optimized. To increase selectivity, BPA was derivatized by using N,O-bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane (BSTFA-TMCS) and it was analysed by GC-MS in selected ion monitoring mode. A design of experiment (DOE) optimization study of the reaction conditions was performed. The analytical method was validated by determining selectivity, linearity (R^2= 0.99999 ± 3.3165E-07), precision, accuracy (99.92 ± 9.83E-02 %), recovery (99.65 ± 3.61E-02 %) and sensitivity (LoD= 0.547 ± 9.94E-02 μg kg^{-1}; LoQ= 1.823 ± 3.31E-1 μg kg^{-1}). The overall method proved to be fast, with high recovery and suitable to selectively and sensitively determine the content of BPA, eliminating the interferences from extraction and allowing to control the safety profile of microalgae. Dried microalgae cultivated in a polycarbonate reactor, were found to contain an amount of BPA 6 times exceeding the legal limit.

1. Introduction

Since microalgae are well-known to be rich in valuable bioactive compounds (vitamins, essential amino acids, proteins, polyunsaturated fatty acids, carotenoids and minerals), they are attracting the attention of many industries in the food, farming, cosmetic, nutraceutical, pharmaceutical and biotechnological sectors (Karpagam et al., 2021; Matos et al., 2017; Moreno-Garcia et al., 2017). Concerning the last two fields, microalgae offer interesting molecules both for designing antimicrobial, antifungal, antiviral, antibacterial and anticancer potential drugs, as well as active components with moisturizing, anti-aging, whitening and photoprotective actions (Couteau and Coiffard, 2018; Xia et al., 2021). Nonetheless, microalgae are used in the food sector, since they constitute new promising sources for preparing functional products that promote health conditions and reduce the onset risks of various diseases. Microalgae as food supplements are formulated as tablets, capsules, or they are directly integrated into common food products, such as yoghurt, snacks, pasta, sweets, and drinks (Barkia et al., 2019).

Abbreviations: ACN, acetonitrile; APCI, atmospheric pressure chemical ionization source; BPA, bisphenol A; BSTFA, N,O-bis(trimethylsilyl)trifluoroacetamide; BPA-D_{16}, bisphenol A-D_{16}; CID, collision-induced dissociation; C_std, blank microalgae samples cultivated in absence of BPA; DOE, design of experiment; EDC, endocrine disrupting compound; EI, electron ionization; EFSA, European food safety authority; EU, European union; GC-MS, gas chromatography–mass spectrometry; HPLC-DAD, high-performance liquid chromatography with diode-array detection; IS, internal standard; LC-MS, liquid chromatography-mass spectrometry; LoD, limit of detection; LoQ, limit of quantitation; Par., paragraph; PTFE, polytetrafluoroethylene; REACH, regulation on registration, evaluation, authorisation and restriction of chemicals; RSD, relative standard deviation; SIM, selected ion monitoring; SPE, solid phase extraction; *Spirulina*, *Arthrospira Platensis*; SRM, selected reaction monitoring; TDI, tolerable daily intake; TMCS, trimethylchlorosilane; TMS, trimethylsilyl; T2DM, type-2 diabetes mellitus; US, ultrasounds.

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Being microalgae at the basis of the aquatic food chain, one of the problems related to their direct or indirect intake is the possible contamination of their biomass by pollutants in the water, such as heavy metals, hexachlorobenzene, herbicides, insecticides and phenols (Baldiris-Navarro et al., 2018). Among pollutants, bisphenol A (BPA) is now considered an emerging contaminant in various environmental compartments, including water (Careghini et al., 2015).

BPA is a synthetic organic compound whose chemical structure is characterized by the presence of two phenol groups (Fig. 2, compound 1). Synthetized for the first time in 1891, it gradually attracted the attention of the plastic industry for its cross-linking property that ensures heat resistance, elasticity and durability in different materials. Thus, BPA employment and production has exponentially increased until reaching the quantity of 10 million tons per year. Currently, BPA is the primary component in polycarbonate plastic, and it is also used in epoxy and phenolic resins. Therefore, this chemical can be found in a large variety of everyday products, such as lunch boxes, bottles, kitchen utensil, food packaging, toys, dental products and many other plastic items. Especially, aquatic contamination may occur by BPA industrial manufacturing, transportation and processing, for examples by wastewater effluents or by the environmental degradation of plastics (Falcão et al., 2020; Fu et al., 2023). In this context, evidences proved that cultures of microalgae proved to absorb, accumulate and degrade BPA (Ji et al., 2014; Zhang et al., 2014). Specifically, the mechanism of degradation may be related to irradiation, which can produce hydroxyl radicals that can enhance BPA degradation (Solé and Matamoros, 2016). BPA could migrate to microalgae not only when these are in their natural habitat (fresh or salt water) but also when they are artificially cultivated in photobioreactors. Indeed, BPA may be released from bioreactor polymeric plastics (polycarbonate and epoxy resins) because of mechanical stress, thermal and UV-mediated degradation. This is a critical phenomenon in view of microalgae cultivation for both nutraceutical or food purposes, since in this way BPA contamination could cause important health risks. The most detrimental implications are related to BPA binding to estrogenic receptors mimicking or antagonizing their effects (Rubin, 2011). Nowadays, BPA is classified as an endocrine disrupting compound (EDC) that interferes with the reproductive system. An additional mechanism related to fertility disorder occurs during neonatal exposure to BPA, when it seems that epigenetic changes lead to adverse effects both on spermatogenesis and oogenesis. The BPA mechanism of action is not yet totally disclosed, and epidemiologic studies seem to correlate BPA chronic exposure to many other diseases as well, such as polycystic ovarian disease, obesity, diabetes and cardiovascular diseases (Santangeli et al., 2017). In addition, it is proved that BPA exposure is connected to insulin resistance, hyperglycemia and type-2 diabetes mellitus (T2DM). So, BPA-induced toxic effects connected to insulin resistance, as consequence intensifies the Alzheimer's disease incidence risk (Davani et al., 2022a; Engin and Engin, 2021; Montanari et al., 2021a). In light of this, BPA exposure has become a major public health concern in the last decades and its use has been restricted in Europe by regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Toys Safety Directive and Plastic Food Material regulation (Commission Regulation EU, 2018/213, 2018). In 2015, the European Food Safety Authority (EFSA) established a temporary tolerable daily intake (t-TDI) for BPA of 4 μg kg^{-1} body weight per day. Later, in 2016, EFSA was asked again by the European Commission (EC) to re-estimate the risk level to public health due to the presence of BPA in foodstuffs, and to establish a full tolerable daily intake (TDI). Finally, EU regulation established the BPA migration limit for kg of food equal to 50 μg in February 2018 (European Food Safety Authority, 2023).

Currently, most of the researches focused on bisphenols determination are applied to beverages, water and food, such as ready-made meals. Despite the growing use of microalgae in the nutraceutical and food sector, as well as in the cosmetic and nutraceutical fields, only few studies are currently available on the development of methods able to control the level of BPA in microalgae biomass to ensure their safe intake. The analytical methods developed for determining and monitoring the content of BPA in microalgal biomass involved a preliminary sample preparation carried out with solid phase extraction (SPE) (Regueiro and Wenzl, 2015; Škufca et al., 2021). In addition, to date, most of the studies are based on the extraction and the analysis of BPA from cultivation water which is a simpler matrix than dried biomass (Giamaki et al., 2022; Salgueiro-González et al., 2012).

Hence, the present study aimed to answer the urgency of developing a selective and sensitive method for determining BPA in microalgal dried biomass samples to be used for food supplements, nutraceutical or cosmetic products. One of the major issues encountered in analysing whole microalgae cells is related to the low selectivity of solvents for BPA extraction, which gives rise to many interferences in further analytical steps. Therefore, this work validated a fast, convenient, and inexpensive solid-liquid extraction of BPA from the dried microalgae biomass endowed with high recovery, without the necessity of any further purification with SPE. Moreover, a pre-derivatization step was optimised and carried out before gas chromatography–mass spectrometry (GC-MS) analysis, in order to selectively transform BPA into a trimethylsilyl (TMS) volatile derivative (BPA-di-TMS). This selective reaction reduced the presence of non-reacting interferences in the final sample mixture and resulted strategic in lowering the number of interferences in the GC-MS analysis conducted in selected ion monitoring (SIM) mode (Montanari et al., 2022).

In the present work the optimised and validated pre-derivatization step and GC-MS analysis were applied to determine the BPA content in commercial microalgal samples, overcoming selectivity issues and reaching the limit of detection (LoD) and limit of quantitation (LoQ) values in the μg kg^{-1} range, compatible with the BPA legal limit in the microalgae dried biomass used in nutraceutical products or food.

2. Material and methods

2.1. Materials

Lyophilized microalgal samples of *Chlorella vulgaris*, *Arthrospira Platensis* were commercially available. Lyophilized *Scenedesmus sp.* was cultivated in a polycarbonate reactor. Bisphenol A ≥ 99 %, bisphenol A-D_{16} 98 atom % D, diphenylmethane 99 %, acetonitrile (ACN) suitable for high-performance liquid chromatography with diode-array detection (HPLC-DAD), gradient grade, ≥ 99.9 %, hyper-grade methanol for liquid chromatography-mass spectrometry.

(LC-MS) LiChrosolv®, N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for GC derivatization, LiChropur™ ≥ 99.0 % and trimethylchlorosilane (TMCS) for GC derivatization, LiChropur™, ≥ 99.0 % (GC), pyridine anhydrous 99.8 %, were purchased Sigma-Aldrich company (St. Luis, MO, USA).

All used materials (labware, glassware, plastics) were BPA free.

2.2. Standard solutions

Standard stock solutions in acetonitrile of bisphenol A (BPA) and bisphenol A-D_{16} (BPA-D_{16}) and of Diphenylmethane at the concentration of 1 μg mL^{-1} were prepared. The obtained solutions have been stored at −20 °C and kept out from light. The solutions were stable for more than one month after going through many freeze-thaw cycles.

2.3. Derivatization reaction

In order to prepare the volatile TMS derivatives (BPA-di-TMS and BPA-D_{16}-di-TMS), a pre-derivatization reaction, modified from described procedures (Mead and Seaton, 2011; Sigma-Aldrich, 2023), was performed after a design of experiment (DOE) optimization study of the reaction conditions described in paragraph 2.5. The optimized reaction was performed by using a thermomixer Eppendorf compact,

adding to the BPA (final concentration of 0.64–128.00 ng mL^{-1}) and BPA-D_{16} (fixed final concentration of 63.00 ng mL^{-1}) mix stock solutions in ACN (final volume= 930 μL), 10 μL of pyridine anhydrous, 69.3 μL of BSTFA and 0.7 μL of TMCS, heating at 75 ºC for 15 min. The solution was filtered with a 0.22 μm polytetrafluoroethylene (PTFE) filter and it was analysed by GC-MS.

The optimised reaction was also applied to the samples obtained after the extraction step, by dissolving the dried extracts in 930 μL of ACN and performing the described reaction.

2.4. Gas chromatography-mass spectrometry (GC-MS) method

The chromatographic method was optimized from a described method (Regueiro and Wenzl, 2015), by using a HP5MSUI (5 %-phenyl)-methylpolysiloxane (30 m × 0.25 mm × 0.25 μm, 19091S-433UI) Agilent column. An Agilent Gas-Chromatograph coupled with a single quadrupole selective mass detector (Agilent 7820 A GC System, Agilent 5977E MSD) in electron ionization (EI) mode (70 eV) under a temperature gradient elution was applied.

The gas carrier was helium with a flow rate of 1 mL min^{-1}. An aliquot of 1 μL of the pre-derivatized sample was injected in splitless mode. The MS source temperature was set at 230 °C, the MS quad temperature was adjusted to 150 °C, the AUX-1 temperature was fixed at 280 °C and the Front Inlet temperature at 250 °C. The GC oven temperature program started at 70 °C with hold time of 1 min. The temperature was increased to 250 °C by a linear gradient rate of 10 °C min^{-1}, then to 280 °C by a rate of 5 °C min^{-1} and hold for 3 min. The analyses were carried out in SIM mode. According to the signal intensity, the 357 *m/Q*, 368 *m/Q* and 168 *m/Q* ions were selected for BPA-di-TMS, BPA-D_{16}-di-TMS and diphenylmethane monitoring, respectively. The total run time was of 28 min. Data were acquired with MassHunter GC-MS Acquisition B.07.00, 2013 and processed with MassHunter Workstation Software Qualitative Analysis B.06.00, 2012.

Calibration curves were then obtained by linear least-squares regression analysis by plotting the ratio BPA-di-TMS/BPA-D_{16}-di-TMS peak areas versus analyte concentrations.

2.5. DOE optimization of reaction conditions

The GC-MS analyses were performed after the analytes derivatization. The derivatization process was optimized starting from a described procedure using BSTFA and TMSC as silylating agents (Mead and Seaton, 2011). BPA-D_{16} was used as internal standard (IS) and diphenylmethane was used as additional internal standard unable to react with reagents and other analytes.

To investigate the best conditions, a design of experiment (DOE) was conducted. For the DOE study it was selected an optimization design, type full factorial (three levels). Two responses were selected to be maximized: a) yield of BPA derivatization, b) yield of BPA-D_{16} derivatization.

Three main multilevel factors were considered: temperature, incubation time and BPA concentrations. Each factor was evaluated at three levels: temperatures= 55, 65, 75 ºC; time= 15, 30, 60 min; concentration of a mix of BPA, BPA-D_{16}, diphenylmethane= 25, 50, 75 ng mL^{-1}. These conditions were studied to evaluate whether the yield of reactions was not influenced by different concentrations in this experimental domain. Furthermore, the worksheet describing the experiments to be performed was generated (table 1SI), producing 27 experiments and 3 centre points. Then, reactions were carried out in Eppendorf secure lock, by using different mixtures of the three standards in ACN, at the defined concentrations. Subsequently, 10 μL of anhydrous pyridine, 69.3 μL of BSTFA and 0.7 μL TMCS were added together with ACN as solvent to reach the final volume of 1 mL. Then, the solutions were transferred in a thermomixer Eppendorf compact and the reactions were performed at different temperatures and over different reaction times. Before the GC-MS analyses, the obtained solutions were filtered with a 0.22 μm PTFE filter. Reactions were repeated twice and each sample was injected twice. The yields of the reactions products obtained at different conditions were compared considering the results obtained by applying the following formulas:

Yield BPA reaction= peak area BPA-di-TMS / peak area diphenylmethane(1)

Yield BPA-D_{16}= peak area BPA-D_{16}-di-TMS / peak area diphenylmethane(2)

Then, the worksheet was filled with the obtained data. Regarding the yield of BPA derivatization, since considered factors were temperature, time and BPA concentrations, in the coefficients plot the principal effects, the interaction effects and squares effects were evaluated. To obtain the best regression (R^2) and prediction (Q^2) models, the most significant effects were selected: time, temperature, temperature*temperature and time*temperature.

As reported above, the same considerations were applied when considering the yield of BPA-D_{16} derivatization. In the coefficients plot the principal effects, the interaction effects and squares effects were analysed. As above, the most significant effects were selected: time, temperature, temperature*temperature and time*temperature. Then, the response contour of BPA and of BPA-D_{16} were generated by plotting time (x axis) and temperature (y axis). The colour scale indicated that best reaction conditions for both BPA and of BPA-D_{16} were in the area of the experimental domain characterized by the following conditions: 75 °C, 15 min (Fig. 5, par. 3.5). Therefore, these conditions were applied for this work.

2.6. Sample preparation

The sample preparation consisted in an ultrasound (US) assisted solid-liquid extraction. An accurately weighted amount (approximately 0.3 g) of lyophilized microalgal powder was weighted in a 15 mL polypropylene centrifuge tube. The internal standard BPA-D_{16} was added to reach the fixed final concentration of 63.00 ng mL^{-1}. Then, aliquots of 2 mL of ACN and 1 mL of MeOH were added, in order to reach BPA solubility in ACN and microalgae wall cell degradation by MeOH (S R et al., 2022; Sun et al., 2020). The sample mixture was vortexed for 30 s and sonicated for 20 min. Then, it was centrifuged (Thermo Scientific CL10 centrifuge) for 5 min at 4000 rpm and the supernatant was collected in a flask. The procedure was repeated three times, by adding 2 mL of ACN. The collected solvent was evaporated by rotavapor, re-suspended with 1 mL of ACN, sonicated for 2 min, transferred in an Eppendorf secure lock and evaporated under nitrogen stream. Finally, the derivatization reaction was performed by using an Eppendorf compact thermomixer, adding to the dried extract 930 μL of ACN, 10 μL of anhydrous pyridine, 69.3 μL of BSTFA and 0.7 μL of TMCS and heating at 75 ºC for 15 min. Then, the solution was filtered with a 0.22 μm PTFE filter and an aliquot of 1 μL was injected twice into the GC-MS. Three independent solid-liquid extraction and further derivatization were performed for each sample (n = 3).

The amount of BPA in the microalgae sample was determined by interpolating the ratio of BPA-di-TMS peak area and BPA-D_{16}-di-TMS peak area into the BPA calibration graph.

BPA concentration (μg kg^{-1}) in the dried powder was determined by using the following formula:

BPA (μg kg^{-1})= (μg of BPA in microalgal sample * 1000) / weighted g of dried microalgal powder (3)

Four commercial samples of *Chlorella* (**C_std, C1-C3**) five commercial samples of *Spirulina* (**S1-S5**) and one sample of *Scenedesmus* (**Sc1**) grown in a polycarbonate reactor were analysed.

2.7. GC-MS method validation

The proposed method consisting in BPA US extraction from

microalgae powder, pre-derivatization and GC-MS analysis was validated considering specificity, linearity, sensitivity, precision, accuracy and recovery (European Medicines Agency, 1995).

Specificity. The specificity of the method was determined by using three blank *Chlorella* (**C_std**) microalgae samples and by comparing the chromatograms obtained after injecting the pre-derivatized BPA-di-TMS and BPA-D_{16}-di-TMS non-spiked and spiked samples respectively. After each sample analysis, two solvent injections were performed in order to demonstrate the absence of any carry-over effect.

Linearity. Three standard calibration curves were determined by analyzing eight BPA standard solutions diluted in ACN in the concentration range comprised between 0.64 and 128.00 ng mL^{-1}, each containing BPA-D_{16} at a fixed concentration of 63.00 ng mL^{-1}. Each solution was subjected to the pre-derivatization reaction (par. 2.3). Further on, linearity was determined by preparing three calibration curves by analysing, for each curve, five samples of blank *Chlorella* (**C_std**) microalgae spiked with BPA (concentrations range 1.28 – 128.00 ng mL^{-1}) and BPA-D_{16} (63.00 ng mL) standard solutions $^{-1}$. The enriched samples have then been subjected to the US solid-liquid extraction procedure reported in par. 2.6, pre-derivatization (par. 2.3) and subsequently analysed by GC-MS.

Sensitivity. The limit of detection (LoD= 3 *SE/m) and limit of quantitation (LoQ= 10 *SE/m) values, were obtained by a statistical evaluation, considering the standard signal deviations (Wu et al., 2011).

Precision. The intra- and inter-day precisions were evaluated by analysing standard samples at low (6.40 ng mL^{-1}) medium (32.00 ng mL^{-1}) and high (96.00 ng mL^{-1}) BPA concentrations, each containing BPA-D_{16} at a fixed concentration of 63.00 ng mL^{-1}, after the pre-derivatization step (par. 2.3). In addition, the intra- and inter-day precisions were evaluated by analysing spiked *Chlorella* (**C_std**) microalgae samples at low (12.80 ng mL^{-1}) medium (32.00 ng mL^{-1}) and high (64.00 ng mL^{-1}) BPA concentrations, each containing BPA-D_{16} at a fixed concentration of 63.00 ng mL^{-1}, after the pre-derivatization step (par. 2.3). Spiked *Chlorella* (**C_std**) microalgae samples were extracted daily. After the pre-derivatization step (par. 2.3), each final solution was injected twice into the GC-MS.

Intra-day analyses were performed three times in a single day (n = 3) at three different concentrations of BPA both as standard solutions and for the in spiked *Chlorella* (**C_std**) microalgae solutions. Inter-day analyses were performed twice a day for three days during a week on standard solutions (n = 6) and twice a day for four days during a week (n = 8) on the spiked *Chlorella* microalgae solutions.

Accuracy. Accuracy (n = 3) was determined by calculating the percentage of the deviation between the experimental concentrations of BPA-di-TMS of spiked blank *Chlorella* (**C_std**) microalgae and the nominal ones considering three concentrations: low (12.80 ng mL^{-1}) medium (32.00 ng mL^{-1}) and high (64.00 ng mL^{-1}) each containing BPA-D_{16} at a fixed concentration of 63.00 ng mL^{-1}.

Recovery. Recovery (n = 3) determination was carried out on blank *Chlorella* (**C_std**) samples spiked with three incremental concentrations of BPA (12.80, 32.00, 64.00 ng mL^{-1}) and a fixed concentration of BPA-D_{16} (63.00 ng mL^{-1}). The recovery values were obtained by the following formula:

Recovery= [(peak Area BPA-di-TMS spiked blank *Chlorella* (**C_std**) microalgae sample / peak BPA-D_{16}-di-TMS spiked blank *Chlorella* (**C_std**) microalgae sample) / (peak Area BPA-di-TMS standard solution / peak area BPA-D_{16}-di-TMS standard solution)] * 100 (4)

2.8. Statistical analysis

Regarding the BPA content in microalgae, US solid-liquid extraction and further derivatization were exhausted in triplicate for each sample. (Steel et al., 1997).

Statistical data regarding the GC-MS method validation and the BPA content in microalgae were obtained by using Microsoft Office Excel (Microsoft Office LTSC Professional Plus 2021). Data regarding DOE optimization of reaction conditions were processed with MODDE® Design of Experiments Solution, MODDE® Pro 13, Sartorius Stedim Data Analytics AB software.

3. Results and discussion

Since microalgae are naturally rich in lipids, proteins, polysaccharides, pigments and many bioactive compounds such as carotenoids and vitamins, they have recently gained a prominence for their potential health benefits. In particular, these nutrients can be exploited for human nutrition, farming, supplements for athletes, cosmetic industry, food industry and food dye (Matos et al., 2017). BPA is considered a contaminant in microalgae since it can be released from manufacturing sites and it can severely contaminate the environment, in particular the aquatic systems. In addition, microalgae can grow in polycarbonate photobioreactors (Baldiris-Navarro et al., 2018; Narala et al., 2016). In 2018, the European commission set the specific migration limit of 50 μg of BPA per kg of food (European Food Safety Authority, 2023).

This work was aimed at developing and validating an accurate, fast and reproducible method in order to control the safety profile of lyophilized powders of microalgae. A first attempt was made by HPLC-DAD method. The HPLC-DAD analysis was performed by Agilent HPLC, in reverse phase and isocratic mode (par. 2, SI). The sample preparation consisted in a liquid-liquid extraction. The algal lipid extract (about 0.6 – 0.8 g) obtained with an already-described procedure (Davani et al., 2022b) was solubilized in chloroform and three sequential extractions were performed with NaOH solution, pH = 12.5. Subsequently, the aqueous fractions were combined and acidified with HCl 0.6 M (pH = 3). Three extractions with chloroform were carried out from the acidic aqueous fraction. The obtained extract in chloroform from **S2** was dried and then, it was solubilized in methanol and analyzed. The calculated amount of BPA in sample **S2** was found to be 1682.23 ± 4.12E01 μg kg^{-1}, higher than the legal limit. In Fig. 1-a, the overlapped chromatograms of BPA standard (at the bottom) and of sample **S2** (at the top) shows the presence of many peaks and a disturbing background noise that probably increase the peak area of BPA. Despite some parameters changes in the HPLC-DAD analysis, such as the mobile phase composition (90:10; 80:20; 75:25), consisting in triethanolammonium phosphate buffer, pH= 3, 0.05 M and ACN, 70/30 (v/v) (Montanari et al., 2021b), and the flow rates (from 0.8 to 1.5 mL min^{-1}), the chromatographic separation of BPA from interferents was not achieved. Furthermore, the content of BPA in sample **S2** was investigated with LC-MS analysis, by using a reverse phase column and an atmospheric pressure chemical ionization (APCI) negative ion source (par. 3, SI). The sample was subjected to the liquid-liquid extraction to obtain BPA, as already described, and then the extract was injected into the chromatographic system. In Fig. 1-b1, the selected reaction monitoring (SRM) chromatogram of sample **S2** is reported, in which BPA peak is still not well resolved from other coeluting compounds. In Fig. 1-b2 the LC-MS full MS2 mass spectrum of the selected ion peak of BPA (MW= 228.29 g mol^{-1}) with the product ion scan m/z = 212.1, after collision-induced dissociation (CID) fragmentation in an ion trap of the molecular ion m/z = 227.1 is shown.

The calculated amount of BPA in sample **S2** was found to be 741.69 ± 5.24E01 μg kg^{-1}. Although a selective scanning in SRM mode was used in the LC-MS analysis, the complexity of the matrix generated high interference, which led to an overestimated result, likewise in the HPLC-DAD method.

Supposing that these results were likely influenced by interferents, a sample preparation consisting in a direct US liquid-microalgae extraction step was combined with the GC-MS analysis with a pre-derivatization step. This was conceived as a more selective approach,

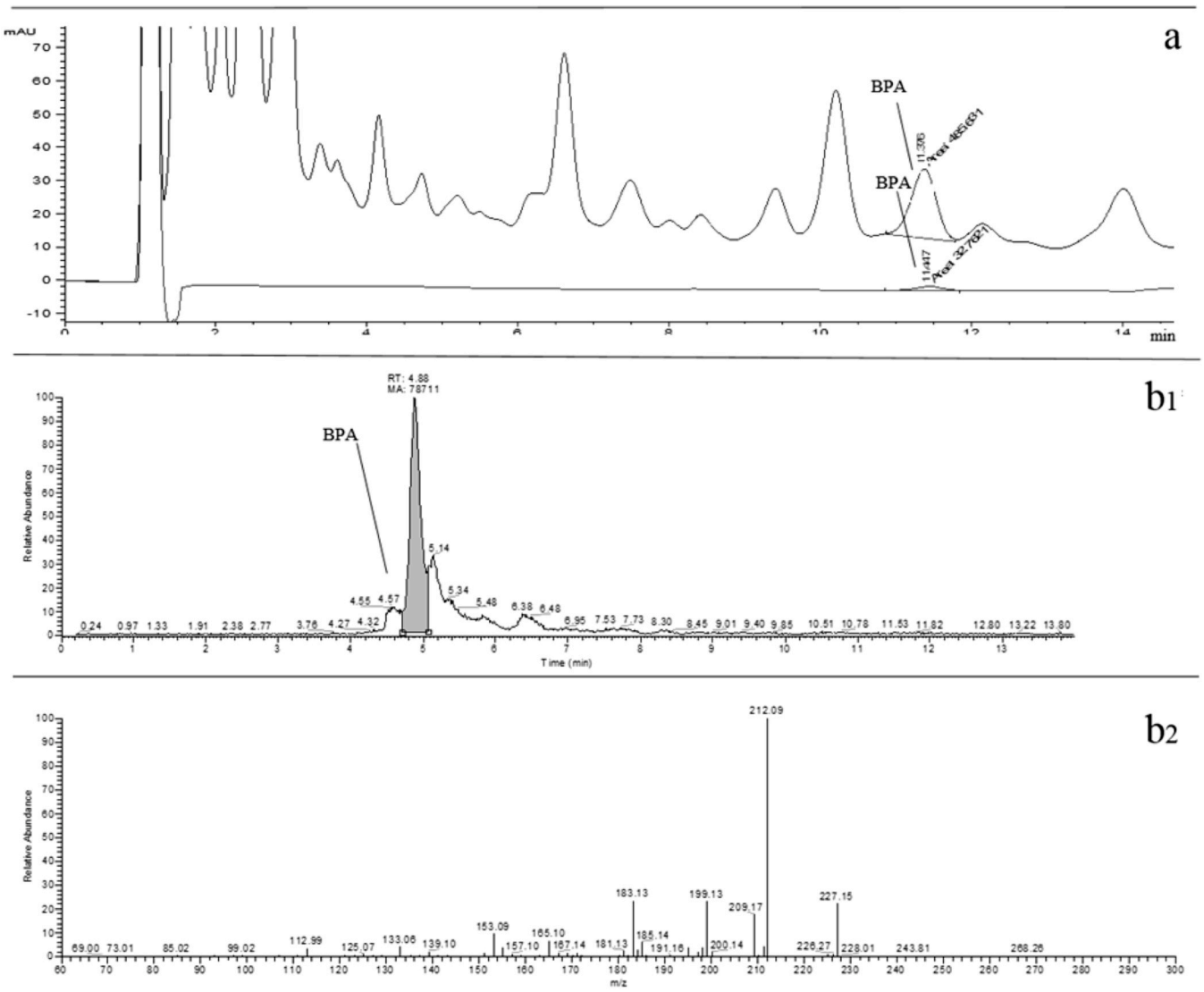

Fig. 1. a) overlapped HPLC-DAD chromatogram: sample S2 at the top, BPA standard in methanol at the bottom; b1) LC-MS SRM chromatogram of sample S2; b2) LC-MS full MS2 of the selected peak (r.t.= 4.88) of sample S2.

aimed at extracting and transforming BPA into a volatile TMS derivative. A selectivity step was therefore introduced by the BPA pre-derivatization reaction (Fig. 2) aimed to forming the trialkylsilyl derivatives.

After this reaction, the resulting solution containing BPA-di-TMS and BPA-D_{16}-di-TMS derivatives was injected into the GC-MS. The GC-MS analysis was carried out in SIM mode on BPA-di-TMS derivatives, confirming their production in the specific reaction. Thus, by performing a previous derivatization of BPA (using BSTFA and TMSC as silylating agents) the obtained BPA-di-TMS derivative was found to be more volatile and the sensitivity of the analysis was increased when compared to the HPLC analysis (GC-MS LoD and LoQ values of 0.164 ± 2.98E-02 ng $mL^{-1}$and 0.547 ± 9.94E-2 ng mL^{-1} respectively, versus HPLC LoD and LoQ values of 0.067 ± 4.34E-02 μg mL^{-1} and 0.222 ± 1.45E-01 μg mL^{-1} respectively).

Thus, as a final result, the combination of US extraction of BPA from the microalgal matrix, together with the selective pre-derivatization step (only molecules with nucleophilic groups such as phenols can react) and the GC-MS analysis in SIM mode, avoided the wasting time with further expensive purifications of the extract performed with SPE (Regueiro and Wenzl, 2015) as previously described and allowed to increase the selectivity and the sensitivity of the analysis (Škufca et al., 2021). In these conditions, the BPA determination resulted straightforward and selective, not affected by significant interferences and the recovery almost quantitative (99.65 ± 3.61E-02).

Then, a DOE study was performed regarding the yield of the pre-derivatization reaction (par. 2.5 and par. 3.5). Furthermore, the GC-MS method was validated concerning specificity, linearity, sensitivity, precision, accuracy and recovery. The optimised and validated GC-MS method with ultrasound extraction and derivatization was applied to the analysis of BPA in four samples of *Chlorella* (**C_std, C1-C3**), five samples of *Spirulina* (**S1-S5**) and one sample of *Scenedesmus* (**Sc1**).

3.1. GC-MS analysis

3.1.1. Pre-derivatization step

The GC-MS analyses were performed after the derivatization of the analytes using BSTFA and TMSC as silylating agents by modifying a previously described procedure (Sigma-Aldrich, 2023).

Introducing the use of acetonitrile as solvent since it was proved that it can facilitate the reaction. In addition, the reaction temperature was modified (from 70 °C to 75 °C), since an improvement of the yield was demonstrated by the DOE study (Mead and Seaton, 2011). BPA-D_{16} was used as IS and diphenylmethane was used as an additional IS unable to

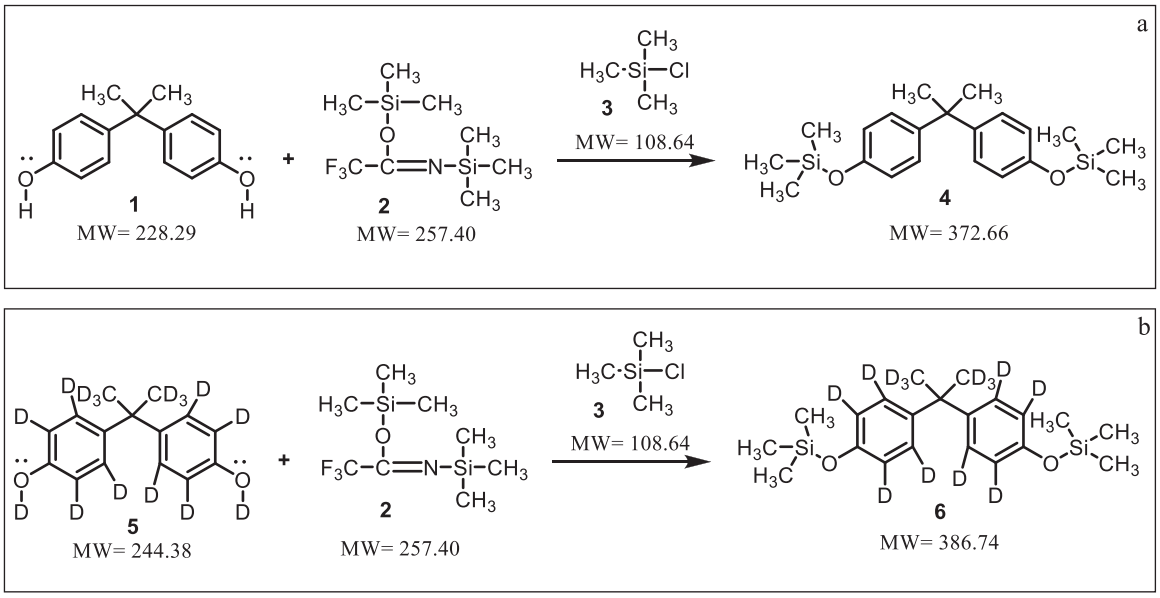

Fig. 2. a- silylation reaction of BPA (1) with BSTFA (2) and TMCS (3) to obtain BPA-di-TMS (4); b- silylation reaction of BPA-D_{16} (5) with BSTFA (2) and TMCS (3) to obtain BPA-D_{16} -di-TMS (6).

react with reagents and other analytes, and useful to verify the BPA-di-TMS yield of the reaction. The reaction consisted in a silylation, a nucleophilic attack of the oxygen of the phenol functional group onto the Si atom of the silyl donor. As a result, the two active hydrogens of the phenolic groups of the BPA and BPA-D_{16} were replaced by two TMS moieties (Fig. 2).

3.1.2. *Chromatographic analysis*

The chromatographic conditions were optimized on Agilent GC-MS System with a HP5MSUI Agilent column by using a temperature gradient elution. The analyses were conducted in SIM mode, using BPA-D_{16} as IS. Diphenylmethane was introduced as additional IS to control the BPA-di-TMS yield of the reaction. The quantitative analysis was performed in the positive mode. Signals at 357 m/Q, 368 m/Q and 168 m/Q ions showed the highest intensity in the fragmentation mass spectrum. Therefore, they were selected for the single ion monitoring of BPA-di-TMS, BPA-D_{16}-di-TMS and diphenylmethane, respectively, in the GC-MS SIM analysis (Fig. 3).

Under the described single ion selected chromatographic conditions, the GC elution of BPA-di-TMS and BPA-D_{16}-di-TMS peaks were found to be comprised in the narrow range of 17.85–18.05 min. The respective chromatographic peaks were baseline resolved and not affected by interferences. Five BPA-di-TMS standard incremental dilutions comprised in the range 0.64 – 128 ng mL^{-1} with the addition of BPA-D_{16}-di-TMS (at the fixed concentration of 63.00 ng mL^{-1}) as internal standard, were injected under the chromatographic conditions reported in par. 2.4.

By plotting the five incremental concentrations of BPA-di-TMS standard versus the ratio BPA-di-TMS/BPA-D_{16}-di-TMS peak areas, a calibration curve (n = 3), y = (2.164E-2 ± 5.460E-06x) + (5.023E-4 ± 4.605E-04) was obtained by linear least-squares regression analysis, with a good correlation coefficient (R^2 = 0.99999 ± 5.908E-08, p-value= 0.18). Compared to previous results, LoD and LoQ values were found improved, [0.126 ± 5.83E-03 ng mL^{-1}, (p-value= 0.46) and 0.419 ± 1.94E-02 ng mL^{-1}, (p-value= 0.46) respectively] (Owczarek et al., 2022; Yu and Wu, 2012). Then, the method was validated on blank microalgae samples cultivated in absence of BPA (**C_std)** in terms of specificity, linearity, sensitivity, precision, accuracy and recovery (par. 3.3) (European Medicines Agency, 1995).

3.2. *Sample preparation*

Many methods are reported for the extraction of BPA from liquids (water, beverages, wastewater culture medium, and seawater) by SPE, but there are just few studies regarding the quantification of bisphenol on food, in particular on microalgae lyophilized powders (Ji et al., 2014; Karalius et al., 2014; Śkufca et al., 2021). In this work, considering the feasibility of the procedure in terms of a fast, easy and inexpensive preparation, an accurate US solid-liquid extraction of BPA from

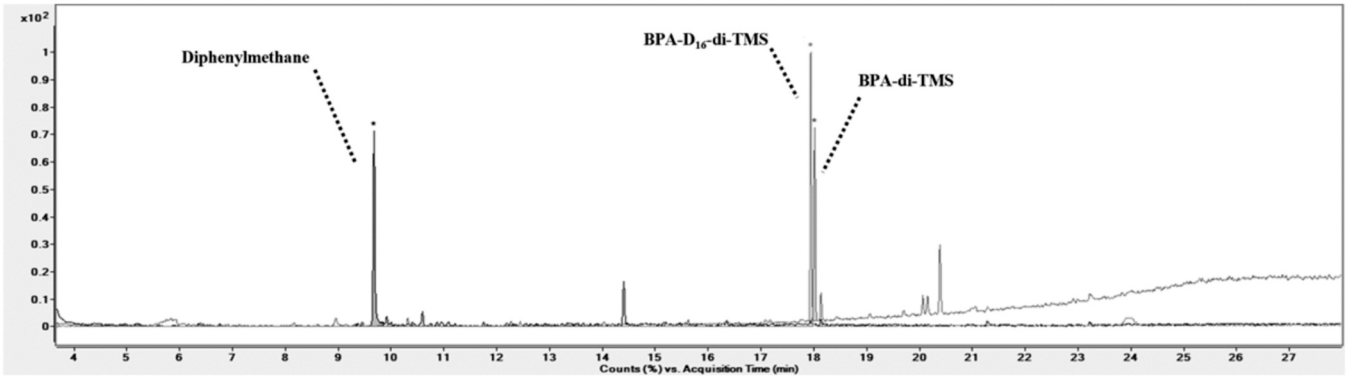

Fig. 3. overlapped SIM (357 m/Q, 368 m/Q and 168 m/Q ions) chromatograms showing the peaks of diphenylmethane, BPA-di-TMS and BPA-D_{16}-di-TMS.

lyophilized microalgal powder was validated. Since BPA is soluble in methanol and acetonitrile, these solvents were used for its extraction (Sun et al., 2020). In particular, in order to facilitate the extraction of BPA, present not only on the surfaces but also inside the cells, the first extraction was carried out with methanol, since it induces cell wall breakage and better extraction yield (S R et al., 2022). In addition, through the emitted shock waves, ultrasounds bath contributed to further disrupting the microalgae cell walls (Grabski, 2009). In comparison to data reported in literature (Škufca et al., 2021) and also to the less selective extraction previously adopted for the HPLC-MS and DAD analyses, a higher recovery was achieved by this extraction approach (99.65 ± 3.61E-02 %). Moreover, another advantage regards the opportunity to inject the obtained sample extract into the GC-MS without any further purification after the pre-derivatization step. The final sample featured a lower number of interferences (Fig. 4).

3.3. Method validation

The method specificity was determined by using three blank microalgae samples cultivated in absence of BPA (**C_std**) and comparing the chromatograms obtained after injecting the non-spiked and spiked samples respectively (chromatogram of **C_std,** that verify the absence of BPA-di-TMS after the derivatisation, is reported in SI: par. 4, figure 2SI). Moreover, each sample analysis was followed by a double solvent injection. The absence of any signal at BPA retention time demonstrated the lack of the carry-over effect confirming that analyses were not affected by sample matrix residues. Due to the absence of any interferences in the pre-derivatization step, after the solid-liquid extraction of both blank microalgae and BPA and BPA-D_{16} spiked microalgae (**C_std**), a good selectivity proved by GC-MS analysis was achieved.

Then, a calibration curve (n = 3) was obtained by analysing five microalgae samples **C_std** spiked with BPA (concentration range 1.28 – 128 ng mL^{-1}) and BPA-D_{16} (fixed concentration of 63.00 ng mL^{-1}) after the solid-liquid extraction and pre-derivatization step. The calibration curve of **C_std**, obtained by plotting analyte concentrations added to the samples versus the corresponding BPA-di-TMS/BPA-D_{16}-di-TMS peak area ratio, demonstrated a good correlation coefficient [y = (2.158E-2 ± 2.969E-5x) + (2.363E-4 ± 3.9343E-4), R^2 = 0.99999 ± 3.3165E-07]. LoD and LoQ, lower than those reported in literature (Škufca et al., 2021) were found to be 0.547 ± 9.94E-02 μg kg^{-1} (0.164 ± 2.98E-02 9 ng mL^{-1}, p-value= 0.25) and 1.823 ± 3.31E-1 μg kg^{-1} (0.547 ± 9.94E-2 ng mL^{-1}, p-value= 0.25) respectively. Therefore, the validated GC-MS method with a pre-derivatization step showed a satisfactory and suitable selectivity and very high sensitivity for evaluating the legal limit of BPA (50 μg kg^{-1}).

The determination of the intra-day and inter-day precision of the method were carried out both on the standard solutions and on the spiked samples. The variation coefficient for intra-and inter-day of standard solutions, tested at three different BPA concentration levels, demonstrated an average value of 0.39 ± 3.10E-01 % and 0.35 ± 2.38E-01 % respectively, p-value> 0.05 (table 4SI, 5SI). The relative standard deviation (RSD) for intra-and inter-day of spiked **C_std** proved an average value of 0.57 ± 5.87E-01 % and 0.58 ± 4.30E-01 % respectively (table 6SI, 7SI), p-value> 0.05.

Accuracy, that was determined at three different BPA concentration levels by calculating the percentage of the deviation between the experimental concentrations of BPA obtained from spiked **C_std** analysis and the nominal ones, was 99.92 ± 9.83E-02 %, p-value= 0.42 (table 8SI), thus confirming the closeness between experimental and true value. In order to determine recovery, three microalgal samples of **C_std**

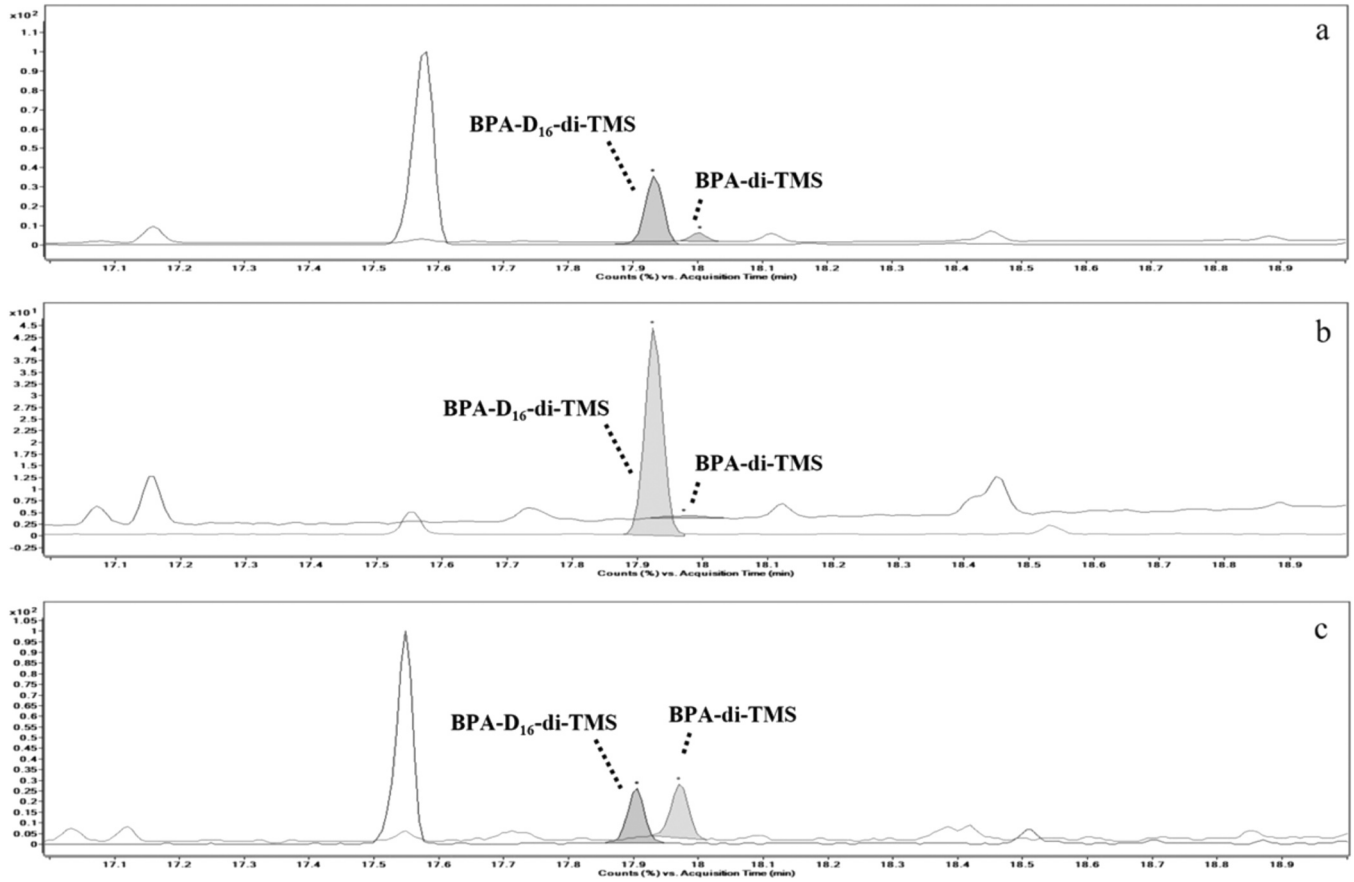

Fig. 4. a) overlapped SIM chromatograms showing the peaks of BPA-di-TMS and BPA-D_{16}-di-TMS in sample **C2**; b) overlapped SIM chromatograms showing the peaks of BPA-di-TMS and BPA-D_{16} -di-TMS in sample **S2**; c) overlapped SIM chromatograms showing the peaks of BPA-di-TMS and BPA-D_{16}-di-TMS in sample **Sc1**.

spiked final concentrations level (12.00, 32.00, 64.00 ng mL^{-1} of BPA) and of a fixed final concentration (63.00 ng mL^{-1} of BPA-D_{16}) before the extraction were added to the blank microalgae sample, cultivated in absence of BPA **C_std**. Given the good mean recovery value (99.65 ± 3.61E-02 %, table 9SI, p-value= 0.25), the results confirmed the high efficiency of the reported GC-MS method, including extraction and derivatization steps.

3.4. Sample analysis

In literature, some publications report the analysis of BPA in microalgae, but these methods are focused on the BPA determination only in the microalgae liquid medium, without investigating the whole cells content (Ben Ouada et al., 2018; Ji et al., 2014).

This newly optimised and validated method was applied in particular to the determination of BPA in lyophilized powders of microalgal samples (Fig. 4) in order to quantify the amount of the contaminant, below the legal requirements. Each microalgae sample was subjected three times to the US assisted solid-liquid methanol-acetonitrile extraction and pre-derivatization steps (par. 2.4, par. 2.6). The resulting solution was injected twice in the GC-MS. To exclude any other interfering co-eluting compound and in order to confirm the chromatographic identification of BPA-di-TMS, a third GC-MS injection was performed on a spiked solution. An external addition to the analysed sample of BPA-di-TMS obtained by a parallel derivatization reaction on a separate scale was carried out. The solution of BPA-di-TMS for the external addition was obtained by using the same derivatization procedure reported in par. 2.6. Then, an aliquot of 50 μL of the BPA -di-TMS solution was mixed with 50 μL of the already derivatized microalgal extract sample. Finally, the resulting spiked sample was analysed by GC-MS.

The estimated amount in terms of μg kg^{-1} of BPA in each algal dried powder sample (n = 3), calculated with the formula reported in par 2.6., is reported in Table 1 (p-value> 0.05). *Chlorella* (**C1**-**C3**) and *Spirulina* (**S1**-**S5**) samples demonstrated a level of BPA in the dried powders lower than the legal limit (50 μg kg^{-1}). In particular, sample **S2** showed a BPA content of 10.86 ± 4.54E-01 μg kg^{-1} (RSD= 4.18), confirming that the HPLC-DAD and the LC-MS methods were affected by the interferences of other analytes and repositioning **S2** to be safe within the legal limit of BPA 50 μg kg^{-1} of food weight. An amount of BPA 301.59 ± 4.15E+ 00 μg kg^{-1} (RSD= 1.37), was found in **Sc1**, exceeding the legal limit. Supposedly, this amount in excess was due to a migration of the contaminant from the polycarbonate photobioreactor where the microalgae **Sc1** was cultivated.

3.5. DOE optimization of reaction conditions

In this work, a described procedure of BPA pre-derivatization, obtained with BSTFA and TMSC reagents, was improved by a DOE optimization of reaction conditions (Mead and Seaton, 2011) with the aim to maximize and increase the selectivity and the sensitivity of the analysis. In order to verify the best conditions to increase the reaction yield, an additional internal standard (IS) unable to react with reagents and other extract components, was added before GC-MS analysis. Many compounds were examined: 1–3 dimethoxybenzene, 3-acetylpyridine, biphenyl, 2-acetonaphthone, anethol, benzyl benzoate, triacetin and diphenylmethane. The best one, resulted to be the diphenylmethane, chosen as the internal standard because well resolved from BPA-di-TMS and BPA-D_{16}-di-TMS. The yields were calculated considering the ratio between the BPA-di-TMS and the diphenylmethane peak areas (table 2SI, 3SI). A higher ratio corresponded to higher yield.

With the effort of MODDE® Pro 1, the design in the optimization DOE study was set in full factorial in three levels. Despite it necessitated a high number of runs and it was low customizable, it allowed a high tolerance against unplanned events and a low prediction error.

The yield of BPA derivatization and the yield of BPA-D_{16} derivatization were selected as responses to be maximized since it was necessary to find the best reaction conditions for increasing the sensitivity of both analytes.

Temperature, time, and concentration of analytes were the selected factors. The latter was studied in order to evaluate that the yield of reactions was not conditioned by different concentrations in this experimental domain. The performed 3 centre points of BPA and BPA-D_{16} demonstrated an acceptable experimental error, lower than the 30 % of the response variability. Since the response distribution showed a *bell shaped* normal distribution for both analytes, it was not necessary to be transformed according to the skewness test. The analysis of the coefficient plot (Fig. 5-a) evaluated the primary effects of time, temperature and concentration of BPA and BPA-D_{16}, the interaction effects (time*-temperature, time*concentration and temperature*concentration) and squares effects (time*time, temperature*temperature and concentration*concentration) of each factor. To improve the regression (R^2) and prediction (Q^2) models, the primary effect of concentration was eliminated since in the coefficient plot it demonstrated to be not significant. Consequently, its interaction and squares effect were excluded too. Thus, according to the purpose of the study, the yield of the reaction was not influenced by the concentration in experimental domain. Therefore, time and temperature showed primary significant effects and interaction effects on both responses (time*temperature). In addition, temperature demonstrated a square effect (temperature*temperature) improving the model. Regression coefficient of the model was R^2= 0.849, assessing a high value of significance. Prediction coefficient, Q^2= 0.789, estimated that it was a good model for future prediction. Then, the 2D response contour of BPA and of BPA-D_{16} (Fig. 5-b), plotting time (x axis) and temperature (y axis), showed the predicted responses value determined by the two selected factors. The colour scale, similar for both BPA and BPA-D_{16}, highlighted the best reaction conditions in the red areas. Finally, the optimal reaction conditions in the studied experimental domain to perform all the following experiments in this work were defined to be 75 °C for 15 min.

Table 1
Average BPA concentration (μg kg^{-1}), n = 3, determined in microalgal samples; *lower than LoQ value; p-value was calculated by considering the three independent solid-liquid extraction and further derivatization performed for each microalgal sample.

Sample	BPA (μg kg^{-1}) n = 3	SD	RSD	p-value
C_std	0.00	0.00E+00	0.00	/
C1	15.72	1.48E+00	9.42	0.19
C2	4.59	4.08E-01	8.89	0.07
C3	6.29	1.36E-01	2.16	0.30
S1	2.61	3.99E-01	15.32	0.45
S2	10.86	4.54E-01	4.18	0.11
S3	1.20*	3.28E-02	2.74	0.50
S4	3.04	1.24E-01	4.06	0.46
S5	5.56	1.67E-01	3.00	0.26
Sc1	301.59	4.15E+00	1.37	0.46

4. Conclusions

BPA can be present in microalgae as contaminant and it is classified as an EDC, responsible for toxic effects on humans. In this work, a selective, accurate (99.92 ± 9.83E-02 %), sensitive (LoD and LoQ values of 0.547 ± 9.94E-02 μg kg^{-1} and 1.823 ± 3.31E-1 μg kg^{-1} respectively), reproducible and endowing a good recovery (99.65 ± 3.61E-02 %) GC-MS method in SIM mode was validated, aimed to determine the content of BPA in microalgae. The fast and inexpensive US liquid-solid extraction, that provided a quantitative recovery of BPA, was combined with pre-derivatization step with BSTFA and TMSC as silylating agents to enhance the volatility of the analyte. This sample preparation allowed to increase the selectivity of the analysis by reducing the interferences generated by the other microalgal components in the extract.

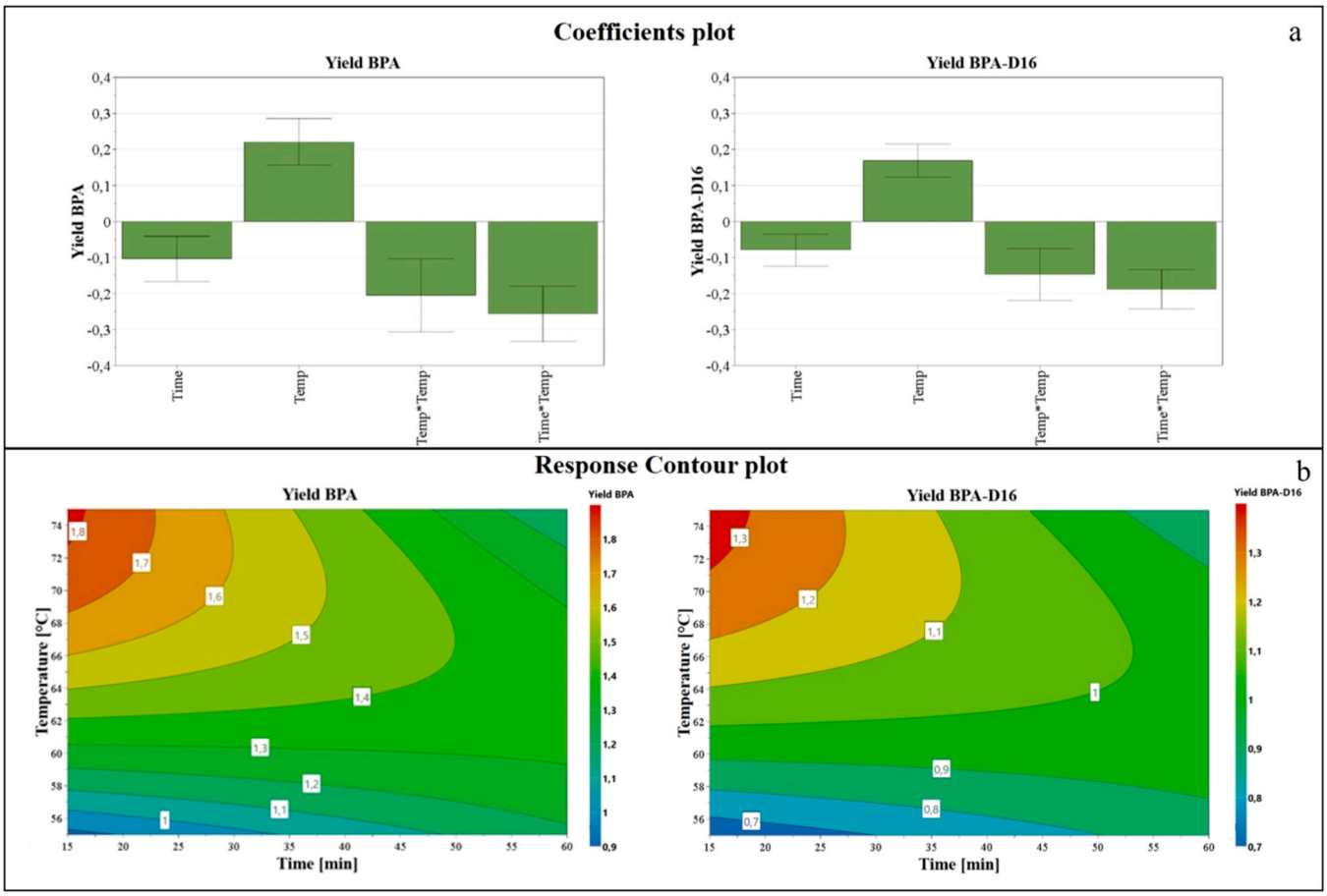

Fig. 5. a) coefficient plot reports the primary effects of time and temperature, the interaction effect of time*temperature and squares effect of temperature*temperature for yield of BPA and BPA-D_{16} respectively; b) the 2D response contours of BPA and of BPA-D_{16}, time in the x axis and temperature in the y axis, reported the predicted responses value.

DOE optimization of reaction conditions (75 °C for 15 min regardless of concentration) was performed to maximize the yield of BPA-di-TMS, increasing the sensitivity of the analysis. All the analysed commercial microalgae powders of *Chlorella* and *Spirulina* samples were found to contain BPA below the legal limit. However, in the *Scenedesmus* sample exposed to polycarbonate, BPA was found to be 301.59 $\pm$ 4.15E+ 00 μg kg^{-1}, six times higher than the threshold value. Considering the increasingly consistent use of microalgae in food, nutraceutical, and pharmaceutical sectors, this method can be successfully applied to the quality control of microalgal samples to verify the BPA legal limit compliance.

Author statement

I hereby certify that all authors have seen and approved the final version of the manuscript being submitted. The article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2023.105568.

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