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Cover letter

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Tarragona, 9th July 2016

Dear Editor,

Please find enclosed the manuscript entitled “Pressurized liquid extraction and liquid chromatography-high resolution mass spectrometry to determine high-intensity sweeteners in fish samples” by M. Núñez, F. Borrull, N. Fontanals and myself to be considered for publication in Journal of Chromatography A.
The manuscript presents for the first time a rapid and reproducible method for the quantification of ten high-intensity sweeteners in different fish samples. The developed method includes pressurized liquid extraction (PLE) with in-cell clean-up using alumina and on-cell clean-up with hexane, which were the best clean-up strategies among the different ones evaluated. The analyses were done by liquid chromatography coupled to high resolution (Orbitrap) mass spectrometry.
For the reasons above mentioned the present manuscript is new and original.
The authors work at the Universitat Rovira i Virgili in Tarragona (Spain), which agree with the submission of this paper to Journal of Chromatography A.

Looking forward to hearing from you.

Yours sincerely,

Dr. E. Pocurull

Highlights of the manuscript: “Pressurized liquid extraction and liquid chromatography-high resolution mass spectrometry to determine high-intensity sweeteners in fish samples”.

First PLE/LC-HRMS (Orbitrap) method to determine high-intensity sweeteners in fish samples.

Different clean-up strategies were evaluated to reduce the high matrix effect.

In-cell clean-up with alumina and on-cell clean-up with hexane were selected.

The method was validated in different fish species according to their % of lipid content.

PRESSURIZED LIQUID EXTRACTION AND LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY TO DETERMINE HIGH-INTENSITY SWEETENERS IN FISH SAMPLES

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ABSTRACT

An analytical method based on pressurized liquid extraction (PLE) followed by liquid chromatography-high resolution mass spectrometry (Orbitrap) was developed for the simultaneous determination of ten high-intensity sweeteners in fish samples. As the method was developed, the different PLE parameters were optimized and different clean-up strategies were evaluated, of which in-cell clean-up using alumina and on-cell clean-up with hexane were the most effective. PLE recoveries were between 43% and 94%. The limits of quantification were between 12.5 ng g^{-1} dry weight (d.w.) and 250 ng g^{-1} (d.w.) and the limits of detection between 2.5 ng g^{-1} (d.w.) and 125 ng g^{-1} (d.w.). Repeatability and reproducibility were below 16% and 25%, respectively. Fish samples from different species were analysed and, saccharin was found below its limit of quantification in the species *Scomber scombrus*.

Keywords: Sweeteners; Pressurized liquid extraction; Liquid chromatography-high resolution mass spectrometry; fish samples.

1. INTRODUCTION

Recently, high-intensity sweeteners have been included in the group of emerging organic contaminants (EOCs) as, in the last few years, their widespread occurrence in the aquatic environment has been reported. They are considered extreme persistent compounds with low degradability. Most of them are not completely eliminated in wastewater treatment plants and some of them do not display environmental degradation [1]. Different studies on the issue have developed analytical methods that allow their determination in different aquatic environments [2-4]. According to Lange *et al.* [5], the sweeteners acesulfame and sucralose have been reported in the aquatic environment at concentrations higher than other EOCs, such as most pharmaceuticals and personal care products.

High-intensity sweeteners are food additives widely used as sugar substitutes in food, beverages, cosmetics, pharmaceuticals, animal feed, tobacco and tobacco-related products [6]. They can be divided into two groups: natural sweeteners, such as stevioside and glycyrrhizic acid, which are isolated from plants; and artificial ones, such as acesulfame, alitame, aspartame, cyclamate, neohesperidine dihydrochalcone, neotame, saccharin and sucralose. They are widely used due to the fact that they do not provide calories and they do not cause blood glucose levels to rise, since the insulin level is not affected, as well as being tooth-friendly [2]. For these reasons, their consumption can help to control obesity and diabetes. However, there is controversy with respect to their usage because potential health effects have been reported. Therefore, some high-intensity sweeteners have been regulated or even banned in several countries [6].

The effects of these EOCs in the ecosystem have not yet been studied in depth and data on the environmental distribution and ecotoxicological impact is still limited [1]. So far, toxicological studies have been conducted on aquatic organisms in order to evaluate the toxicity of these contaminants, due to their occurrence into the aquatic environment [7-12]. Most of these studies have focused on sucralose and they conclude that this sweetener does not alter the survival, growth or reproduction of aquatic organisms at levels above those measured is surface waters [7,12]. They also highlight that this compound may

not cause toxicity to aquatic organisms at concentrations lower than 1000 mg L^{-1} [7], with this value being higher than the concentrations reported in the aquatic environment. Toxicity studies on *Lemna gibba* [9], *Daphnia magna*, *Pseudokirchneriella subcapitata*, *Danio renio* [10] revealed no toxic effects. However, one study [8] found that sucralose alters the behavioural response of *Daphnia magna* in terms of swimming and velocity, and also increases the time it takes *Gammarus* spp. to reach food and shelter. In another study [11], two copepod species were studied: *Calanus glacialis* and *Calanus finmarchicus*. In the case of *Calanus glacialis*, food intake increased when the concentration of sucralose also increased. Although negligible, acute and chronic toxicity have been reported. The most detailed assessments revealed behavioural changes that need to be taken into account, since they are modifications of the normal behaviour [8].

In order to study and evaluate the fate, effects and environmental risks posed by EOCs such as artificial sweeteners in aquatic ecosystems, information regarding their presence in aquatic organisms is urgently needed. For this purpose, analytical methods that allow their determination need to be developed. These methods have to deal with time-consuming sample preparation due to the complexity of these samples.

The aim of this work was the development for the first time of an analytical method for the determination of ten high-intensity sweeteners in different fish species using pressurized liquid extraction (PLE) and liquid chromatography-high resolution mass spectrometry (LC-HRMS). Finally, the method was validated and applied to the analysis of fish samples from different species.

2. MATERIALS AND METHODS

2.1 Reagents, standards and materials

Acesulfame-K (ACE), alitame (ALI), aspartame (ASP), cyclamate-Na (CYC), glycyrrhizic acid (GLY), neotame (NEO), neohesperidine dihydrochalcone (NHDC), saccharin-Na (SAC), stevioside (STV) and sucralose (SUC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All standards were of a purity higher than 96%, except for GLY (70%). Individual stock solutions of 1000 mg L^{-1} were prepared in methanol (MeOH) and stored at -20ºC. For the

preparation of the stock solution of STV and GLY, a percentage of water (water/MeOH 5:95; v:v) was needed in order to ensure the dissolution of the solid. A mix solution of all compounds at 50 mg L^{-1} in MeOH was prepared weekly and stored also at -20ºC.

The organic solvents MeOH, acetonitrile (ACN) and hexane were of HPLC grade and provided by J.K. Baker (Deventer, the Netherlands). Acetone was also of HPLC grade and purchased from Prolabo (Llinars del Vallès, Spain).

Formic acid (HCOOH), ammonium hydroxide (NH_4OH) and the sorbents tested for the in-cell clean-up (C_{18}, Florisil, silica and alumina) were supplied by Sigma-Aldrich. Diatomaceous earth was bought from Fisher Scientific (Waltham, MA, USA) and Oasis® HLB SPE cartridges (500 mg/6 cc) were obtained from Waters (Milford, MA, USA).

The ultrapure water was produced by ultrapure water system from Veolia Water (Sant Cugat del Vallès, Spain). The nitrogen gas (N_2) was obtained from Carburos Metálicos (Tarragona, Spain).

2.2 Sampling

The species *Mullus surmuletus* (striped red mullet), *Scomber scombrus* (Atlantic mackerel), *Sparus aurata* (gilt-head bream) and *Psetta maxima* (turbot) were bought in the local market, while the species *Cyprinus carpio* (common carp) and *Silurus glanis* (wels catfish) were collected from the Ebro River. Of all of the species, *Mullus surmuletus* and *Cyprinus carpio* were selected to optimize the method. For all species, the lateral fillets were separated and homogenized and frozen for 24 hours at -20ºC. Once frozen, samples were lyophilized using the Genevac miVac Duo Concentrator freeze-drying system (Ipswich, Suffolk, UK). Then, samples were ground to obtain a homogeneous powder and sieved (500 µm) to obtain particles of similar size.

To optimize the method, the matrix was covered with acetone and then the analytes were added. The sample was periodically homogenized and the acetone was left to evaporate overnight inside an extraction hood. This is a common procedure to enable good interaction between the analytes and the matrix [13,14].

2.3 Extraction and clean-up

To perform the extractions, an ASE 200 Accelerated Solvent Extraction system from Dionex (Sunnyvale, CA, USA) was used. 11 mL extraction cells were used and mounted as follows: a cellulose filter from Teknokroma (Sant Cugat del Vallès, Spain) was placed at the bottom of the extraction cell and 3 g of alumina was added, then 1 g of sample mixed with 2 g of alumina were introduced, the void volume of the cell was filled with diatomaceous earth and, finally, another filter was placed on top.

Once the extraction cell was assembled, an on-cell clean-up using hexane was performed, for the purpose of defatting the sample, followed by the extraction of the analytes. The conditions of the on-cell clean-up can be found in [14] and the main conditions were: extraction temperature of 40ºC at 1500 psi with a preheating time of 5 min with 2 cycles of 1 min each, a flush volume of 100% and a nitrogen purge of 360 s. For the extraction of the analytes, the optimal conditions were: MeOH:ultrapure water (1:1; v:v) as the extraction solvent, pressure of 1500 psi, preheating time of 5 min, 1 cycle, temperature of 60ºC, extraction time of 5 min, flush volume of 50% and purge time of 300 s. The extract obtained (~17 mL) was evaporated to dryness employing a Genevac miVac Duo Concentrator, and the dried residue was reconstituted with 5 mL of MeOH:ultrapure water (1:9; v:v). The extract was then filtered through a 0.22 µm polypropylene syringe filter obtained from Serviquimia (Constantí, Spain) before injection.

2.4 Liquid chromatography-high resolution mass spectrometry

The analyses were performed on an LC system connected to an Exactive Orbitrap mass spectrometer from Thermo Scientific. The instrument was equipped with an Acela 1250 HPLC system and the interface used was a heated electrospray ionization (HESI-II) source working in negative mode. The instrument was also equipped with a high-energy collisional dissociation cell (HCD).

The column Zorbax Eclipse XDB-C_8 (150 x 4.6 mm i.d., 5 µm) from Agilent Technologies (Santa Clara, CA, USA) was used to perform the analyses. The optimal mobile phase was a mixture of solvent A (ultrapure water at pH 2.5 with

HCOOH) and solvent B (ACN). The gradient profile started with 15% B, which was raised to 45% in 13 min and then to 100% in 2 min. Afterwards, it was maintained at 100% for 3 min and, finally, it was returned to initial conditions in 2 min. The column was allowed to stabilize for 8 min between injections. The flow-rate was 0.6 mL/min, the oven temperature was set at 25ºC and the injection volume was 25 µL.

Optimized HRMS conditions were obtained in full scan mode at high resolution 50000 full width at half maximum (FWHM) over a mass range of 60 to 1000 m/z. The optimal parameters were: spray voltage of 3.5 kV; sheath gas 40 AU (arbitrary units); auxiliary gas 10 AU; tube lens voltage of -90 V; skimmer voltage of -26 V; capillary voltage of -25 V; heater temperature of 350ºC; capillary temperature 300ºC; and probe position adjustments: 0 as side-to-side position; C as vertical position and micrometer 0.5.

Four windows were used with different collision voltages in the HCD. In each window, two scan events were performed: one full scan at 50000 FWHM with 250 ms of injection time and with a scan range of 60-1000 m/z; and the other a fragmentation scan at 10000 FWHM with 50 ms of injection time with a scan range of 60-1000 m/z. In the first window (0 to 8.01 min) and in the third (12.01 to 14.01 min), a voltage of 20 eV in the HCD was selected. In the second (8.01 to 12.01 min) and fourth windows (14.01 to 18 min), a voltage of 40 eV in the HCD was selected. All of the selected ions can be found in Table 1.

3. RESULTS AND DISCUSSION

3.1 Liquid chromatography-high resolution mass spectrometry

In order to optimize the chromatographic separation, two columns were tested: Ascentis Express RP amide (100 x 2.1 mm i.d., 2.7 µm) from Supelco (Sigma-Aldrich) and Zorbax Eclipse XDB-C_8 (150 x 4.6 mm i.d., 5 µm). Both columns are suitable for the separation of high polar compounds like sweeteners. It was observed that, with the Zorbax Eclipse XDB-C_8 column, better separation was obtained with respect to the first five eluting compounds (ACE, SAC, CYC, SUC and ASP) and, consequently, further experiments were performed using this stationary phase, which enabled good separation in 15 min. The specific retention time of each analyte is detailed in Table 1.

As regards as the HRMS optimization, in accordance with the literature, the highest sensitivity was achieved when working with ESI in negative mode [1,3,15]. The HRMS conditions described by Salas *et al.* [2] were used as a starting point, although the present study includes a higher number of sweeteners. To test these conditions, standard solutions were continuously infused together with a flow of mobile phase with 50% B. The exact m/z was recorded in a full scan at 50000 FWHM for each compound in negative mode. For all of the compounds, $[M-H]^-$ was selected for quantification, with the exception of ACE, SAC and CYC, for which $[M]^-$ was selected, and STV, for which the adduct $[M+HCOO]^-$ formed due to the mobile phase was selected. Table 1 shows the selected precursor ion for each compound. The different voltages and temperatures were also optimized, and the values selected are detailed in Section 2.4.

Moreover, for confirmation purposes, fragment ions for each compound were obtained. To do so, the signal intensity was monitored when different voltages (ranging from 5 to 60 eV) in the HCD were applied. It was not possible to choose a compromise voltage for all of the compounds and, for this reason, different windows were conducted. The first window (from 0 to 8.1 min), with an HCD of 20 eV, corresponds to the compounds ACE, SAC, CYC, ASP and ALI. In this window, SUC also elutes. However, for this compound, no fragment ion was selected. Instead, due to the presence of Cl^- in the molecule, two precursor ions were selected namely 395.00858 m/z and 397.00565 m/z, with 395.00858 m/z being selected for quantification and 397.00565 m/z for confirmation. The second window (from 8.1 to 12.01 min), with an HCD voltage of 40 eV, includes the compounds NHDC and STV. The third window (from 12.01 to 14 min), with an HCD of 20 eV, includes NEO and, finally, the fourth window (from 14 to 18 min), with an HCD of 40 eV, contains GLY. All of the selected ions can be found in Table 1.

High-intensity sweeteners comprise different types of molecules and, for this reason, different fragmentation pathways were observed. Table 1 details the precursor ions and the most intense fragments selected. Some of them (i.e. the fragments for ACE, SAC, CYC and ALI) are easy to explain. Nevertheless, the fragment ion 200.07179 m/z obtained for ASP might correspond to the loss of

methoxycarbonyl (CH_3OCO), amine (NH_2) and hydroxyl (OH) groups. NHDC might break the molecule from the two hydroxyl substituted six atom rings through the carbon oxygen bond, giving the fragment ion 303.08856 m/z. As regards as STV the adduct 849.3775 m/z was selected as precursor ion, the fragment ion selected, 641.31903 m/z, might correspond to the loss of a monosaccharide. In the case of NEO, an HCD of 20 eV yielded the fragment ion of 200.07184 m/z, associated with the loss of methoxycarbonyl (CH_3OCO), 3,3-Dimethyl-1-butanamine ($C_6H_{15}N$) and hydroxyl (OH) groups. Finally, the fragment ion of 351.05847 m/z of GLY could be obtained by the loss of the aglycone group. These fragments have previously been reported in the literature [2-4,16].

It should be pointed out that several compounds displayed poor fragmentation. This is the case of CYC, for which the fragment ions described in the literature [17] when a QqQ analyser was used were 80 m/z $[M\text{-}H\text{-}C_6H_{12}N]^-$ and 96 m/z $[M\text{-}H\text{-}C_6H_{10}]^-$, with the highest response being recorded for 80 m/z, which was selected as the fragment ion in the present study. Moreover, the most intense fragment reported in the literature [3] for SAC is 42 m/z, which corresponds to the $[NCO]^-$ fragment. This ion could not be monitored with an Exactive Orbitrap mass spectrometer since the scan range starts at 50 m/z. As for GLY, it should be mentioned that the fragments from GLY had a very low response, although the fragment ion 351.05847 m/z had the highest intensity.

Instrumental limits of detection (LODs) and quantification (LOQs) were calculated. The LOD for each compound was attributable to the concentration giving a peak signal of the precursor ion with intensity higher than $1x10^3$, in line with [18]. For most of the compounds, the LODs were between 0.1 and 1 µg L^{-1}, with the exception of SUC, which had an LOD of 2.5 µg L^{-1}. The LOQ was considered to be the first point of the calibration curve. For most of the compounds, the linear range started between 0.25 µg L^{-1} and 1 µg L^{-1} up to 500 µg L^{-1}, with the exception of GLY, SAC, ASP and NEO which had a linear range between 2.5 and 500 µg L^{-1}, and SUC ranging between 5 and 500 µg L^{-1}.

3.2 Extraction

To optimize the extraction process, the species *Mullus surmuletus* (striped red mullet) was selected. In order to obtain efficient extractions, several parameters of PLE were optimized. Based on previous experience [13], initial PLE conditions were fixed as: 1 g of sample, 1500 psi, extraction temperature of 80ºC, preheating time of 5 min, static time of 10 min, flush volume of 100%, 1 cycle and a purge time of 300 s.

The first parameter to be optimized was the extraction solvent. The solvents tested were: ACN, MeOH, ultrapure water, ultrapure water adjusted to pH 2.5 with HCl and a mixture of MeOH:ultrapure water (1:1; v:v).

PLE recoveries (PLE REs) were calculated to evaluate the solvents. To do so, the ratio between the signal of the analytes obtained in a fish sample spiked before PLE at 2500 ng g^{-1} (d.w.) and the signal of the analytes obtained in an extract spiked at the same concentration after PLE extraction was conducted. In order to obtain good peak shape, the final solution (25 mL) was a composition of ultrapure water:MeOH (9:1; v:v), similar to the initial mobile phase composition. Thus, in the case of the organic solvents, such as ACN and MeOH, the extracts were evaporated to dryness under a stream of nitrogen and the dried residue was re-dissolved to the final solution. In the case of the mixture of MeOH:ultrapure water (1:1; v:v), the PLE extract was half evaporated, assuming that all MeOH was evaporated, and then diluted to the desired composition. In the case of water as the extraction solvent, the extracts were also diluted. Figure 1 shows the PLE REs when the abovementioned solvents were tested.

As can be seen in Figure 1, the highest PLE REs were obtained when using ultrapure water for ACE, SAC, STV and GLY. However, with this solvent, NHDC and NEO could not be extracted. In fact, NHDC was hardly extracted with any of the solvents. With MeOH, all of the compounds were extracted with values ranging from 42% to 107%, with the exception of NHDC (10%) and ACE, which could not be extracted. With the mixture of MeOH:ultrapure water (1:1; v:v), all of the compounds were extracted with values higher than 75%, with the exception of NHDC, NEO and GLY, which had PLE REs of 20%, 26% and 47%, respectively. As in the case of ultrapure water, with ACN and water at pH 2.5, some compounds could not be extracted. ACN could not extract ACE, STV and

GLY and, in the case of water at pH 2.5, NHDC and NEO were also not extracted. The mixture MeOH:ultrapure water (1:1; v:v) was chosen as the extraction solvent as it allowed all of the compounds to be extracted. The same solvent was used in our research group for extracting a group of sweeteners from sludge [17].

The second optimized parameter was the extraction temperature, which was tested at 40ºC, 60ºC and 80ºC (data not shown). It was observed that the compound most affected by the variation of temperature was NEO. At 80ºC, NEO showed a PLE RE of 26%, while at 60ºC, it displayed a PLE RE of 86%. NHDC increased from 20% at 80ºC to 39% at 60ºC. Meanwhile, the PLE REs of GLY slightly increased (around 8%) when the temperature increased from 40ºC to 60ºC. For this reason, 60ºC was selected as the extraction temperature as a compromise.

The third parameter optimized was the extraction time and 5, 10 and 20 min were evaluated. It was observed that, with an extraction time of 5 min, there was no decrease in the PLE REs compared to 10 min and, with 20 min, there was no improvement (data not shown). For this reason, the extraction time was set at 5 min in order to make the extraction process shorter.

Finally, in order to obtain a smaller extraction volume, a reduction in the flush volume (50% and 75%) was evaluated. Similar PLE REs were obtained and a flush volume of 50% was selected and incorporated into the method. So, the final volume obtained was approximately 17 mL, which is faster to evaporate.

Other PLE parameters, such as purge time, preheating time and pressure, are considered of minor importance and they were kept at initial conditions [14,19].

The final extraction conditions were therefore 1 g of sample, MeOH:ultrapure water (1:1; v:v), 5 min extraction time, 60ºC, 1500 psi, 50% flush volume, 1 cycle, 5 min preheating and 300 s purge time. Under these optimal conditions, the PLE REs were evaluated for *Mullus surmuletus* and *Cyprinus carpio* (Table 2). According to [20], a different % of lipid content can lead to changes in the figures of merit, and the matrix effect (ME) is expected to be higher when the percentage of lipids increases. These two species were selected since they have different % of lipid content: 23% in the case of *Mullus surmuletus* and 15% in the case of *Cyprinus carpio* [19]. Moreover, apparent recoveries (App REs)

and the ME were also evaluated for both species. App REs were calculated by interpolation of the signal of the analytes obtained from a fish sample spiked at 2500 ng g^{-1} (d.w.) before PLE with an external calibration curve. The ME was calculated with the following formula:

ME (%) = -[100- (B/A*100)]

where (A) is the instrumental response for standards injected directly to the LC-HRMS and (B) is the analytes' response in a fish extract spiked just before being injected into the LC-HRMS. App REs (Table 2) were higher for *Cyprinus carpio* (between 118% and 45%) than for *Mullus surmuletus* (between 95% and 8%). As expected, the ME was higher for *Mullus surmuletus* and some compounds had values higher than 50% in terms of ion suppression. This was the case of ASP (-68%), SUC (-68%), NHDC (-87%), STV (-79%), NEO (-61%) and GLY (-56%). A lower ME was observed for *Cyprinus carpio*, with a maximum value of ion suppression of -39% for NHDC. These results are in line with the values of % lipid content, since *Mullus surmuletus* has a higher lipid content than *Cyprinus carpio*.

Due to the high ME observed, particularly for the species *Mullus surmuletus*, different strategies were evaluated in order to reduce this ME and all the tests were performed with this species.

3.3 Clean-up

One strategy often used to clean the matrix is SPE. In the present study, SPE using the copolymer sorbent Oasis® HLB (lipophilic divinylbenzene-hydrophilic N-vinylpyrrolidone copolymer) was evaluated. The protocol recommended by suppliers was followed, which is summarized as follows: the cartridges were conditioned with 5 mL of MeOH and 5 mL of ultrapure water at pH 3. The PLE extract of fish sample was half evaporated, assuming that all of the MeOH was evaporated, and then the remaining aqueous extract was diluted to 25 mL with water and adjusted to pH 3 with HCOOH, before being loaded into the cartridge. A clean-up step was performed with 5 mL of a mixture of ultrapure water:MeOH (9:1; v:v) and then the cartridge was vacuum dried. The analytes were eluted with 5 mL of MeOH and the eluate was evaporated to dryness using a Genevac miVac Duo Concentrator. The dried residue was re-dissolved in 25 mL of

363 ultrapure water:MeOH (9:1; v:v) and filtered before being injected into the LC-
364 HRMS. SPE REs were calculated as the signal ratio of the analytes of a sample
365 spiked before SPE and after SPE at the same concentration. With this
366 parameter, only the SPE was evaluated and no losses in other steps were
367 taken into account. Some compounds showed low SPE REs, as is the case of
368 ACE, SAC and GLY, which presented SPE REs lower than 50%. For the rest of
369 the compounds, the SPE REs were higher than 78%. Nevertheless, it was
370 observed that the SPE did not improve the App REs.
371 In order to improve these low SPE REs, the SPE procedure using the Oasis®
372 HLB sorbent was tested, as described by Arbeláez *et al.* [3] to evaluate eight of
373 the ten sweeteners from the present study in sewage sludge. The protocol was
374 the same as the one described by the suppliers with the exception that, in the
375 elution step, instead of eluting with 5 mL of MeOH, the analytes were eluted
376 with 2.5 mL of MeOH and 2.5 mL of a mixture of MeOH:NH_4OH (95:5; v:v), and
377 the eluate was also evaporated to dryness and the dried residue was re-
378 dissolved in 25 mL of ultrapure water: MeOH (9:1; v:v) before being injected into
379 the LC-HRMS. In this case, the SPE REs increased (>70% for all of the
380 compounds), but no improvement of the App REs was observed. As this
381 strategy did not entail any improvement and actually lengthened the analysis
382 time considerably, it was rejected.
383 Another strategy used to reduce the ME is an in-cell clean-up that was also
384 evaluated. This step consists of the use of a sorbent inside the extraction cell in
385 order to retain interfering compounds and obtain a cleaner extract. To do so,
386 instead of adding diatomaceous earth at the bottom of the extraction cell, it was
387 filled with a sorbent, and the sample was also mixed with the sorbent. Finally,
388 the void volume of the extraction cell was filled with diatomaceous earth. The
389 sorbents evaluated in the present study were Florisil, C_{18}, silica and alumina, all
390 of which are often used to clean complex matrices and recommended by
391 Dionex. Although similar results were obtained from the different sorbents
392 tested (Figure 2), alumina was selected as the sorbent as it slightly improved
393 the App REs of some compounds (SAC, ALI) and it is an inexpensive material
394 often used in biota studies [20,21].

Another strategy that was evaluated was on-cell clean-up, which consists of performing a defatting step prior to the extraction, once the extraction cell was assembled. Hexane was selected as the on-cell solvent based on the good results obtained in previous studies [14,22]. Although this step did not significantly improve the App RE of the method, an extract with a cleaner appearance was obtained, which helps to prevent the deterioration of the chromatographic column. In addition, it is a step that does not require any sample manipulation and does not involve a significant increase in the analysis time. Therefore, with the two clean-up strategies adopted, the App REs improved by 5% to 10%, with respect to those shown in Figure 2.

All of the strategies above were evaluated when the final volume was 25 mL. However, in order to achieve lower LODs and LOQs, a reduction of the final volume was assayed; thus, instead of 25 mL, volumes of 10 mL and 5 mL were evaluated. Between 25 mL and 10 mL, a reduction of the App RE was observed, with SAC, CYC being the compounds that showed a higher reduction (~20%). However, between 10 mL and 5 mL, there was almost no reduction. For this reason, 5 mL was chosen as the final reconstitution volume.

3.4 Method validation

The method validation was performed for the species *Mullus surmuletus* and involved the evaluation of the linear range, LODs, LOQs, repeatability and reproducibility, App REs and ME. Blank samples were evaluated in order to subtract the signal if any compound was present. However, none of the studied sweeteners was present. Moreover, some of these parameters were also evaluated for *Cyprinus carpio*. All of the validation parameters can be found in Table 3.

For the species *Mullus surmuletus*, the App REs were evaluated at two concentration levels 125 ng g^{-1} (d.w.) and 500 ng g^{-1} (d.w.), with the exception of NHDC, which was only evaluated at 500 ng g^{-1} (d.w.) as this compound had a low PLE RE and high ME, as well as the fact that, in the 125 ng g^{-1} (d.w.) concentration, it was below its LOQ (LOQ = 250 ng g^{-1} (d.w.)). At both levels, the App REs were very similar for all of the compounds, with values ranging from 11% to 91%. The ME was also evaluated for the highest level, with most of

428 the compounds being subject to ion suppression, with the exception of ACE,
429 which displayed ion enhancement. The most affected compounds in terms of
430 the ME were NHDC and ASP, with MEs of -93% and -89%, respectively. The
431 option of using internal standards to correct the high ME observed was ruled out
432 since the selected compounds showed different responses in the LC-HRMS. In
433 addition, they also belong to different chemical classes (sulfamates, peptides
434 and carbohydrate derivatives) and cover a wide range of polarities that might
435 result in different behaviour. These features mean that most likely ten
436 isotopically labelled compounds would be needed, increasing the costs of the
437 study.
438 In order to quantify the analytes, matrix-matched calibration curves were
439 plotted. Linear range, LODs and LOQs were obtained experimentally by spiking
440 fish samples at different concentrations before PLE. All of the compounds
441 showed good linearity (in the ranges shown in Table 3) with R^2 above 0.9913.
442 LOQs and LODs were calculated as explained in Section 3.1. LOQs, which
443 were the lowest point of the calibration curve, for most of the compounds, were
444 between 25 ng g^{-1} (d.w.) and 50 ng g^{-1} (d.w.), with the exception of ALI and
445 ACE, with a lower LOQ (12.5 ng g^{-1} d.w.), and NHDC, with a high LOQ of 250
446 ng g^{-1} (d.w.). As for LODs, they ranged between 12.5 ng g^{-1} and 25 ng g^{-1} for
447 most of the compounds, with the exception of ALI and ACE (2.5 ng g^{-1} d.w.) and
448 NHDC (125 ng g^{-1} d.w.), as can be seen in Table 3.
449 Repeatability and reproducibility (expressed as the % relative standard
450 deviation) were obtained with five replicated samples performed on the same
451 and different days, respectively, spiked at 500 ng g^{-1} (d.w.). As can be seen, the
452 values of repeatability were always below 16% and reproducibility lower than
453 25%.
454 In the case of *Cyprinus carpio*, App REs were evaluated at the highest
455 concentration (500 ng g^{-1}) and the values ranged between 20% and 122%. In
456 general, they were higher than for the species *Mullus surmuletus*, as mentioned
457 previously. The lipid content of these two species is different, with it being
458 higher in the case of *Mullus surmuletus* (23%) than for *Cyprinus carpio* (15%).
459 This fact means that a higher ME is observed and a lower App RE is obtained

for *Mullus surmuletus*. Repeatability was also evaluated for *Cyprinus carpio*, ranging from 4% to 17%.

3.5 Method applicability

As mentioned, the method was developed for the two species with different lipid content: *Mullus surmuletus* with a high lipid content and *Cyprinus carpio* with a lower lipid content. The optimized method was applied to evaluate the occurrence of the selected compounds in different fish species that had similar % of lipid content (which is indicated in brackets) to the above mentioned species. The evaluated species considered to have a high lipid content [19] were: *Mullus surmuletus* (striped red mullet, 23%), *Scomber scombrus* (Atlantic mackerel, 21%), *Sparus aurata* (gilt-head bream, 35%) and *Psetta maxima* (turbot, 31%). The species with a lower lipid content were: *Cyprinus carpio* (common carp, 15%) and *Silurus glanis* (wels catfish, 12%) [19].

The criteria to evaluate the presence of the selected compounds were the retention time, the exact mass of the precursor ion with a mass error of 5 ppm and the fragment ion and corresponding ion ratio [18,23,24]. However, in the case of CYC, GLY and SAC, the presence of the fragment ion was not considered, as these compounds displayed poor fragmentation (Section 3.1). Moreover, the signals of the fragment ions were highly affected by the noise. In any case, the high confirmation capabilities of high-resolution techniques should be noted.

Among the studied compounds, SAC was found in one of the analysed samples, in the species *Scomber scombrus* at a concentration below its LOQ. Figure 3 shows the accurate mass extracted ion chromatogram of the molecular ion for SAC in a fish sample. To the best of our knowledge, this is the first time that these compounds have been studied in aquatic organisms and, thus, it is not possible to compare the results obtained.

4 CONCLUSIONS

A PLE method followed by LC-HRMS to determine simultaneously ten high-intensity sweeteners was successfully developed and validated.

PLE REs ranged from 43% to 94%. Several clean-up strategies were tested to reduce the high ME encountered and in-cell clean-up using alumina and on-cell clean-up employing hexane were selected as the best options.

The repeatability (n=5) and reproducibility (n=5) of the method were less than 16% and 25%, respectively.

The method was applied to determine the occurrence of the selected sweeteners in different fish species with different % of lipid content. Of these sweeteners, SAC was found in one of the samples analysed, below its LOQ.

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601
602 **Figure captions:**
603
604 **Figure 1.** PLE RE (%) using different extraction solvents when the fish sample
605 was spiked at 2500 ng g^{-1} (d.w.). See the text for the rest of the
606 conditions.
607
608 **Figure 2.** App REs (%) of fish samples spiked at 2500 ng g^{-1} (d.w.) when
609 different in-cell sorbents and when no in-cell (diatomaceous earth)
610 were used.
611
612 **Figure 3.** Accurate mass extracted ion chromatogram of the molecular ion for
613 SAC in a sample of the fish *Scomber scombrus*.
614
615
616

Figure

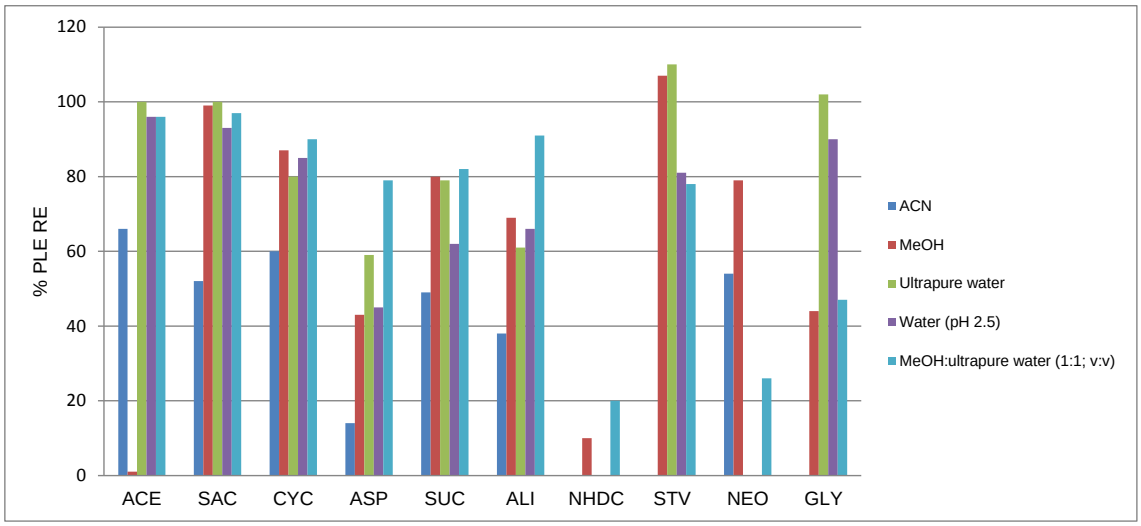

Figure 1.

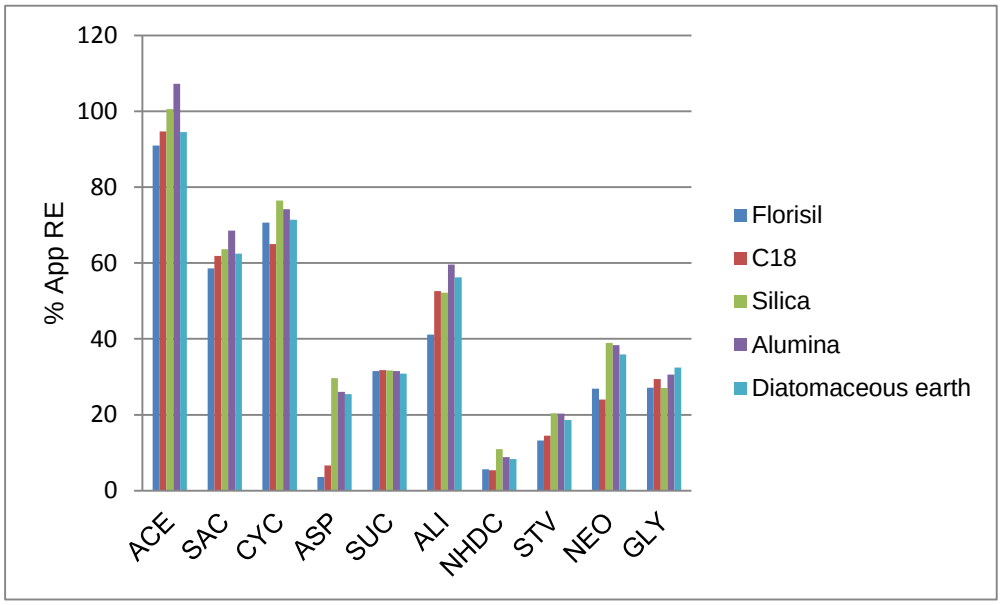
120
100
80
60
40
20
0
% App RE
ACE
SAC
CYC
ASP
SUC
ALI
NHDC
STV
NEO
GLY
Florisil
C18
Silica
Alumina
Diatomaceous earth

Figure 2.

Figure

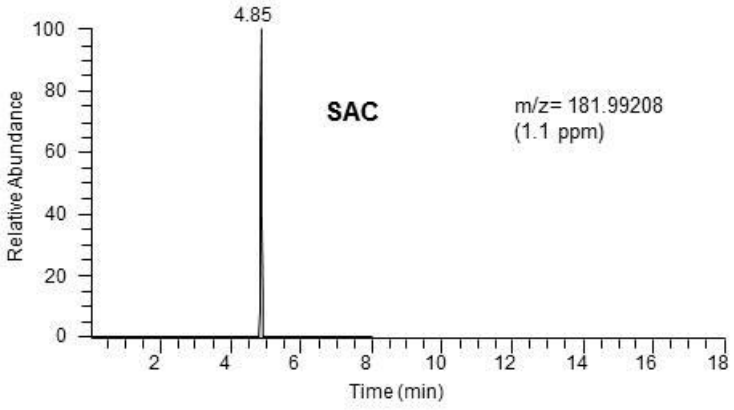

Figure 3.

Tables

Table 1. Chemical formula, retention time and accurate masses of the studied sweeteners.

Compound	t_R (min)	Formula	Precursor ion (m/z)	Fragment ion (m/z)
ACE	4.07	$C_4H_4NO_4S$	161.98621 $[M]^-$	82.02899 $[M-SO_3]^-$
SAC	4.86	$C_7H_4NO_3S$	181.99188 $[M]^-$	105.95982 $[M-C_6H_4]^-$
CYC	4.90	$C_6H_{12}NO_3S$	178.05428 $[M]^-$	79.95647 $[M-C_6H_{12}N]^-$
SUC	6.27	$C_{12}H_{19}Cl_3O_8$	395.00858 $[M-H]^-$	397.00565$[(M+2)-H]^-$
ASP	6.42	$C_{14}H_{18}N_2O_5$	293.11542 $[M-H]^-$	200.07179 $[M-C_2H_7NO_3]^-$
ALI	7.47	$C_{14}H_{24}N_3O_4S$	330.15048 $[M-H]^-$	312.13998 $[M-H_2O]^-$
NHDC	10.30	$C_{28}H_{36}O_{15}$	611.19934 $[M-H]^-$	303.08856 $[M-C_{12}H_{20}O_9]^-$
STV	11.35	$C_{38}H_{60}O_{18}$	849.3775 $[M+HCOO]^-$	641.31903 $[M-C_6H_{10}O_5]^-$
NEO	13.06	$C_{20}H_{30}N_2O_5$	377.20935 $[M-H]^-$	200.07184 $[M-C_8H_{19}NO_3]^-$
GLY	15.06	$C_{42}H_{62}O_{16}$	821.39838$[M-H]^-$	351.05847 $[M-C_{30}H_{46}O_4]^-$

Tables

Table 2. PLE REs (%), App REs (%) and ME (%) for *Mullus surmuletus* and *Cyprinus carpio* when the fish samples were spiked at 2500 ng g^{-1} (d.w.). See the text for the rest of conditions.

Compounds	*Mullus surmuletus*			*Cyprinus carpio*		
	PLE RE	App RE	ME	PLE RE	App RE	ME
ACE	94	95	-2	93	118	27
SAC	93	62	-37	96	106	11
CYC	92	71	-29	86	93	8
ASP	77	25	-68	69	61	-11
SUC	84	31	-68	96	76	-21
ALI	79	56	-37	78	75	-4
NHDC	46	8	-87	74	45	-39
STV	77	19	-79	88	75	-15
NEO	82	36	-61	82	74	-10
GLY	43	32	-56	69	54	-21

RSD (n=3) ≤14

Tables

Table 3. Method validation data when the samples were analysed by PLE and LC-HRMS.

Compounds	*Mullus surmuletus*							*Cyprinus carpio*		
			125 ng/g	500 ng/g						
	Linear range ng/g (d.w.)	LOD ng/g (d.w.)	App RE (%)	App RE (%)	ME (%)	Repeatability (%RSD; n=5)	Reproducibility (%RSD; n=5)	App RE (%)	ME (%)	Repeatability %RSD
ACE	12.5-1000	2.5	66	91	9	4	13	122	50	7
SAC	25-1000	12.5	26	35	-54	2	14	88	19	6
CYC	25-1000	12.5	30	44	-56	4	12	72	-3	10
ASP	50-1000	25	20	11	-89	5	25	23	-59	7
SUC	50-1000	25	25	37	-58	11	16	57	-34	4
ALI	12.5-1000	2.5	40	53	-27	4	24	168	112	8
NHDC	250-1000	125	-	<10	-93	15	20	20	-70	17
STV	25-1000	12.5	13	16	-78	16	22	39	-51	10
NEO	50-1000	25	19	17	-76	11	13	38	-54	5
GLY	25-1000	12.5	26	21	-70	3	9	29	-46	7