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# Trace-level determination of sweeteners in sewage sludge using selective pressurized liquid extraction and liquid chromatography–tandem mass spectrometry

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## ABSTRACT

The occurrence of sweeteners in the environment has become a matter of concern due to the possibility of adverse effects on human health and wildlife species. One of the routes by which sweeteners enter the environment is through sewage sludge. Therefore, a method was developed with a selective-pressurized liquid extraction (S-PLE) followed by liquid chromatography–tandem mass spectrometry for the simultaneous determination of eight sweeteners in sewage sludge. The chromatographic separation was achieved in less than ten minutes using an amide polar-embedded reversed-phase column. Due to the high matrix effect present in the sample, an extensive study was conducted in order to overcome this issue, with C<sub>18</sub> in-cell and solid-phase extraction (Oasis HLB) as a clean-up method. S-PLE/SPE recoveries at two levels of concentration (50 µg/kg and 1000 µg/kg in dry weight (d.w.), *n* = 5) were higher than 61%. Repeatability and reproducibility at the same concentrations (%RSD, *n* = 5) were lower than 11% and 16%, respectively. The limits of detection were 10 µg/kg (d.w) for all compounds, except for cyclamate (5 µg/kg (d.w.)). The method was successfully applied to sewage sludge samples from three sewage treatment plants located in Catalonia (Spain). Of the eight compounds, five were determined in all of the samples analysed, with acesulfame and saccharine being recorded at the highest concentrations of up to 481 µg/kg and 591 µg/kg (d.w.), respectively.

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## 1. Introduction

Sewage sludge is the solid or semi-solid that results from the treatment of domestic and/or industry wastewater in sewage treatment plants (STPs). Sewage sludge is an important source of organic matter, nitrogen, phosphorus, potassium, calcium, sulphur and magnesium, which is suitable for use in agriculture, but it can also contain contaminants such as pathogens, heavy metals and organic contaminants [1,2]. Although the European Union regulates the use of sewage sludge to prevent harmful effects on land, vegetation, animals and humans under the directive 86/278/EEC [3], its scope is only limited to establishing the maximum limits of concentration of heavy metals in sludge for land application. Organic contaminants are not regulated and they may be adsorbed by crop plants or plants used for feed production and grazing purposes, resulting in animal and human exposure to the contaminants through feed or food [2].

Of the various groups of organic contaminants, concern about high intensity sweeteners (HIS) has grown in recent years as they have been identified as emerging organic contaminants, due to their large-scale occurrence in the aquatic environment, extreme persistence and low degradability [4]. It is still unclear to what extent HIS negatively affect the environment. While some studies suggest that HIS have low toxicity [5–7], negative effects have recently been shown with respect to the behavioural and physiological functions of certain organisms [8,9]. There is therefore a need to monitor HIS in the environment. The quest for faster, more selective and sensitive methods of analysis is one of the key objectives of researchers.

Several analytical methods have been developed for the determination of HIS in environmental matrices such as river water [10,11] and wastewater [12–14], with high concentrations having been reported. However, to date, few methods have been published for the determination of HIS in sewage sludge [15–17].

HIS have been extracted from sewage sludge by using pressurized liquid extraction (PLE) [15], ultrasonic solvent extraction (USE) [16] and liquid–solid extraction [17]. Furthermore, all of these extraction techniques have been combined with solid-phase extraction (SPE) to pre-concentrate and clean up the extract.

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For instance, Ordoñez et al. [15] extracted acesulfame, aspartame, cyclamate, NHDC, saccharine and sucralose by PLE with water 500 mM formate buffer (pH 3.5) at 80 °C for a single static cycle of 21 min, with subsequent SPE clean-up with Oasis HLB. PLE recoveries reported were between 87% and 105% [15]. Meanwhile, Subedi et al. [16] showed that USE followed by SPE is also a suitable technique for extracting HIS (sucralose, aspartame, saccharine, acesulfame and cyclamate) from sewage sludge, with good extraction recoveries being obtained when MeOH:water (5:3 v/v) was used as the extraction solvent for 30 min.

For determination purposes, liquid chromatography–(electrospray)tandem mass spectrometry (LC–(ESI)MS/MS) is the technique established as the best choice for determining HIS in either water and/or sludge. The ionization of most HIS occurs best with ESI operating in negative mode [18–20]. In fact, the two methods mentioned above [15,16] determined HIS in sludge using a triple quadrupole mass spectrometer and the authors reported higher sensitivity working in negative ionization. However, others studies have obtained more fragments under positive ionization in the case of aspartame, saccharine and sucralose [21].

The present research focuses on the determination of the most common HIS in sewage sludge, namely acesulfame, saccharin, cyclamate, aspartame, sucralose, stevioside, neohesperidinedihydrochalcone and glycyrrhizic acid [22]. For the purposes of the study, an analytical method was developed based on selective-pressurized liquid extraction (S-PLE) and SPE followed by LC–MS/MS and subsequently validated for reliable and sensitive quantification. To date, there is little information available on the prevalence of HIS in sewage sludge and some of them such as glycyrrhizic acid and stevioside have not been studied yet.

## 2. Experimental

### 2.1. Reagents and standards

Acesulfame-K (ACE), saccharin-Na (SAC), cyclamate-Na (CYC), aspartame (ASP), sucralose (SUC), stevioside (STV), neohesperidinedihydrochalcone (NHDC) and glycyrrhizic acid (GLY), aspartame- $d_3$  (ASP- $d_3$ ), sucralose- $d_6$  (SUC- $d_6$ ) were purchased from Sigma-Aldrich (St. Louis, USA). Stock solutions of individual standards and deuterated standards were prepared by dissolution of pure compound in MeOH at a concentration of 1000 mg/L, and then stored at –20 °C in amber glass bottles. Mixed intermediate standard solutions were prepared every month by diluting stock solutions in MeOH at a concentration of 4 mg/L and stored at 4 °C. Mixed standard working solutions were prepared daily from intermediate standard solutions by diluting appropriately with water:MeOH (9:1 v/v). Deuterated compounds were tested as surrogates.

The following HPLC grade reagents were tested as extraction solvent: acetone, dichloromethane (DCM), isopropyl alcohol (IPA), ethyl acetate (EtOAc) and methanol (MeOH). These were purchased from Prolabo (VWR, Llinars del Vallès, Spain). Formic acid (HCOOH) for LC–MS analysis was purchased from Merck (Darmstadt, Germany), acetonitrile (ACN) from Prolabo, and ammonium hydroxide (NH<sub>4</sub>OH) from Sigma-Aldrich. Ultrapure water was obtained using an ultrapure water purification system supplied by Veolia Water (SantCugatdelVallès, Spain). Nitrogen gas was sourced from CarburrosMetálicos (Tarragona, Spain). In-cell sorbents used in PLE (Florisil, C<sub>18</sub>, alumina, and silica), as well as diatomaceous earth, were purchased from Sigma-Aldrich.

### 2.2. Sludge sampling and sample pre-treatment

For three consecutive months, sewage sludge samples were collected from three STPs located in Catalonia (Spain). These STPs

mostly receive urban sewage along with some industrial discharges. The STPs have a design flow between 18,000 m<sup>3</sup>/day and 35,000 m<sup>3</sup>/day, with a total capacity between 156,450 and 200,000 equivalent inhabitants.

Dewatered sludge samples were taken after anaerobic sludge digestion treatment by grab sampling using polyethylene bottles. They were then stored in the freezer at –20 °C until being lyophilized with the freeze dry system (Labconco, Kansas City, MO, USA). The lyophilized samples were homogenized by mortar and pestle, sieved through a 125 μm screen and stored at room temperature.

In order to optimize and validate the method, the sludge samples were spiked by adding the stock mixture of standards in acetone (~8 mL of acetone was necessary to cover 1 g of sludge sample). The solvent was slowly evaporated inside a fume hood at room temperature with frequent homogenization.

### 2.3. S-PLE and clean-up procedure

Extraction of HIS from sludge samples was carried out by PLE using an ASE 2000 (Dionex, Sunnyvale, CA, USA). The stainless steel extraction cell (11 mL capacity) was filled with 1 g of C<sub>18</sub> adsorbent in the bottom of the cell, followed by a second layer of 1 g of pre-treated sludge thoroughly mixed with 2 g of C<sub>18</sub> and, finally, a third layer of 1 g of diatomaceous earth. Whatman glass fibre filters were put at the top and bottom of the cell.

The PLE method resulted in the following optimal conditions: MeOH:water (1:1 v/v) as the extraction solvent, 5 min preheating period, extraction temperature of 40 °C, extraction pressure of 1500 psi with a static period of 5 min in one cycle, flush volume of 40% of the cell volume and nitrogen purge time of 90 s. PLE extract (~15 mL) was evaporated under a nitrogen stream approximately to 5 mL and then it was re-dissolved to 25 mL in water. Subsequently, this extract was loaded into the 500 mg Oasis HLB cartridge previously preconditioned with 5 mL of MeOH followed by 5 mL of ultrapure water. Then, the cartridges were cleaned up with 5 mL of water:MeOH (9:1 v/v) and completely dried under vacuum. The compounds were eluted with 2.5 mL of MeOH followed by 2.5 mL of a solution containing 5% of NH<sub>4</sub>OH in MeOH. The eluate was evaporated to dryness and re-dissolved to 10 mL with MeOH:water (1:9 v/v), before being filtered with a 0.22 μm polypropylene membrane filters (GHP) prior to analysis by LC–(ESI)MS/MS.

### 2.4. Determination by LC–(ESI)MS/MS

The LC–(ESI)MS/MS method was adapted from our analytical method previously developed for the determination of HIS in environmental aqueous samples [23]. The LC–(ESI)MS/MS system was an Agilent 1200 series (Waldbronn, Germany) coupled to a triple quadrupole 6410 series mass spectrometer with an ESI interface (Agilent Technologies). The LC conditions were: Ascentis Express RP-amide (100 × 2.1 mm, 2.7 μm) as the chromatographic column; the mobile phase was ultrapure water acidified with HCOOH to pH 2.5 (solvent A) and ACN (Solvent B); the elution gradient started isocratically at 5% B for 3 min and then increased to 75% B in 6 min, then increased to 100% B in 1 min, remaining constant for 1 min and finally returning to 5% B in 1 min.; the oven temperature was 25 °C; the flow rate was 0.4 mL/min and the injection volume was 50 μL.

The ESI–MS/MS conditions were as follows: nebulizer pressure of 45 psi, drying gas (N<sub>2</sub>) flow rate of 11 L/min, drying gas temperature of 350 °C and capillary voltage of 4000 V. The ionization mode was selected negative for all compounds. The acquisition mode used was selected reaction monitoring (SRM) and the most abundant SRM transition was used for quantification, while the second and third most abundant SRM transitions were used for confirmation, except in the case of SUC and CYC (one transition for confirmation). Cone

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